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ROLE OF PI3K-AKT PATHWAY IN THE AGE ASSOCIATED DECLINE IN TLR MEDIATED ACTIVATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Immunosenescence results in reduced immune response to infections with *Streptococcus pneumoniae* as well as to pneumococcal polysaccharide vaccines. The antibody response to the capsular polysaccharide (CPS) provides protection against *S. pneumoniae* infection. CPS immunoresponse is T cell independent and needs the macrophage-derived cytokines such as IL-12, IL-6 and IL-1β to elicit an antibody response. We showed a cytokine dysregulation, i.e. a decrease in IL-12, IL-6 and TNF-α but an increase in IL-10, in the aged (18-24 months old comparable to >65 years in human) compared to young adult mouse (8-12 weeks less than 65 years old) splenic macrophages (SM) or bone marrow derived macrophages (BMDM) activated via TLR4, TLR2 or TLR9 as well as heat killed *Streptococcus pneumoniae* (HKSP). There is also an age-associated defect in splenic B cells in the production of IgG3 upon stimulation with these ligands. A microarray analysis in SM followed by validation by both qt-RTPCR and western blots indicated that this age-associated defect in aged SM, BMDM and B cells was due to a heightened activity of the PI3K-Akt signaling pathway. We hypothesized that the senescence of immune responses in macrophages and B cells is due to an increase in activity of PI3K/Akt and decrease in the activity of GSK-3, the downstream kinase. Inhibition of the PI3-kinase with either LY294002 or Wortmannin restored the TLR2, 4, 9 and HKSP induced cytokine phenotype of the aged to that of the young adult in both the SM and BMDM and an enhanced IgG3 production in aged mice.

We also showed that inhibition of glycogen synthase kinase-3 (GSK-3) the downstream target of the PI3K-Akt signaling pathway with SB216763 in SM, BMDM and B cells resulted in an enhancement in production of IL-10, IL-6 and IL-1β by macrophages and in B cell activation. Treatment of B cells with SB216763 in the presence of ligands for TLR-1/2, 4 or 9 as well as HKSP under in vitro conditions led to enhanced production of IgG3 and IgA, plasma cell formation and a slight increase in the proliferation of the B-cells with no adverse effects on the viability of the cells. Therefore, targeting the PI3K-AKT-GKS-3 signaling pathway could rescue the intrinsic signaling defect in the aged macrophages, increase IL-12 and IL-6, and enhance anti-CPS antibody responses.

KEYWORDS: Toll-like receptor, PI3 kinase, Glycogen synthase kinase-3, macrophages, aging,

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DEDICATION

I dedicate this dissertation to my DEAR FATHER, MR. KAMANO FALLAH, who taught me and my siblings that education was a sure escape route from poverty. A few months ago he was struck with a massive stroke and became paralyzed and speechless back home in Liberia. He passed away on Father's day, June 19, 2011. The memories of his desire to give us an education forever remains fresh in my heart.

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Chapter 1: LITERATURE REVIEW

Introduction

Immunosenescence: In humans and rodents it is known that the potency and vitality of life takes a downward trend with age resulting in increased neurological, metabolic and infectious diseases, as well as higher incidence of cancer as a result of defects in their immune system (15, 187, 288). This population of people becomes a candidate for vaccines to help them mount effective defenses against common pathogens that have the propensity to cause them to have higher hospitalization, morbidities and mortalities. Frustratingly, the vaccine response in this more vulnerable population is less efficacious than in their young counterpart. But an additional challenge is the fact that with the advent of modern technology and an improved health system, we see an ever increasing number of elderly people who are susceptible to these debilitating age-associated conditions. Mathematical modeling and other studies predict that 40% of the population in both in the USA and Europe will be above 65 years of age by 2050 (214). In spite of scientific efforts to improve the vitality and health conditions of this population, an increase in incidence of infectious diseases is expected (376).

The elderly are more susceptible to diseases and mount impaired vaccine response to infections, such as *Streptococcus pneumoniae,* because there are defects in both their innate and adaptive immune systems. This generalized decline in innate and adaptive immunity is referred to as immunosenescence (15, 197). Aging affects the immune functions of innate cells like macrophages and dendritic cells which are critical for the early detection and clearance of infectious agents through early non-specific immune

response like phagocytosis and inflammatory mediated clearance of pathogens (5, 63, 120). The age-associated defects in the innate cells, which are crucial for initiating the delayed but more specific adaptive immune system, result in defects in both the B cell and T cell mediated responses in the elderly (40, 92, 182, 234).

T-cells and aging

It is generally accepted that the T-cell arm of the adaptive immunity is seriously affected by aging due to thymic involution, which begins around late teens and, thus, negatively affects both humoral and cell mediated responses (236, 327).There is an age-related decrease in the naïve T cells but an increase in the memory T cells (92, 190). The memory T cells in the aged have impaired proliferation and IL-2 secretion, which associates with a defect in the MAP kinase pathway. These memory T cells have limited repertoire and thus are not able to repond to new pathogen (58, 370). Another mechanism for the age-related defect in T cells, especially the CD4 T compartment, is the formation of a weakened immunological synapse resulting in reduced proliferation, differentiation and cytokine production (136-138).

In the frail elderly, when peripheral blood monocytes (PBM) are stimulated with *Staphylococcus aureus Cowan* (SAC) there is a predilection for the development of Th2 cells (138) . Defects in Th_1 development results in impaired delayed type hypersensitivity (DTH) reaction and higher incidence of intracellular infections, such as tuberculosis (TB) in the elderly (138). More recent studies show that the age-related defective polarization results in a reduction in the proliferation of both Th_1 and Th_2 subsets, with an enhancement of Th17 cells (138, 155). The age-associated increase in Th_{17} cells is due to

rapid production of these cells by memory T cells. The increase in Th_{17} cells is not due to any influence from dendritic cells as demonstrated by co-culture experiments. The most important finding is that Th_{17} cells ($CD4^{+}$ $CD45Rb^{hi}$) from the aged upon transfer into Rag knockout mice induce more severe colitis than those from young mice (274). This could contribute to the increase in autoimmunity observed in the elderly. Another common feature in aged T cells is the decrease in the expression of CD28 costimulatory molecules with aging (57, 58). Aging is also associated with an overproduction of regulatory T cells (Treg) and a change in the T cell receptor (TCR) repertoire usage. The age-associated increase in Treg is considered another factor responsible for the suppression of immune response to pathogens and tumors, and for reactivation of chronic infections (138, 193, 364). Because of these defects in the helper T cells population in the aged, the T cells have a reduced ability to help B cells, which then negatively impinges upon antibody responses, both quantitatively and qualitatively, and the eventual poor vaccine response (50, 138, 251). This may partly explain why the current conjugate vaccines or the improved pneumococcal conjugate vaccines may only have partial efficacy in the elderly as both vaccines function under the T-cell dependent mode to support B-cell mediated humoral responses (92).

Aging also affects the immune potency of CD8 T cells. There is a reduction in CD8 TCR repertoire diversity and a shift in recognition of immunodominant viral epitopes. Because the naïve T cells in the aged have a limited T cell diversity there is a lack of robust response to infections and vaccines. This may be due to the lack of fresh T cell emigrant pool from the thymus, causing the T cell population in the periphery to increase by

homeostatic expansion. Thus, there will be an increase in T cells that have a limited repertoire (11, 138).

B-cells and aging

The two major changes that affect B cell immune function in the aged are changes in the B cell compartment and the impaired responsiveness of the peripheral B cells. While the total numbers of B cells remain constant, the follicular B cells are reduced which is compensated for by an increase in antigen experienced B cells. As a result, there is a restriction in naïve B cells with receptor repertoire diversity to respond to new and different antigens irrespective of the strain of mice (117, 165, 228).

The alteration in the peripheral B cell compartment which leads to impaired humoral response associates with increased B cell longevity and decreased emigration of B cells from the bone marrow hematopoietic stem cell (HSC) compartment (182). The peripheral B cell pool can exhibit homeostatic expansion and, thus, increase their population without migration of newly generated B cells to the periphery. In addition to these factors, the restricted B cell pool in the periphery can undergo clonal expansion leading to a restricted B cell repertoire (164, 234). Plausible mechanisms include defects in the pre B-cell compartment or inability of new B cells that are normally produced in the bone marrow to migrate to the periphery due to the presence of long-lived mature B-cells in the periphery (165). During aging the B-cell repertoire changes from BCR with specificities for foreign antigens to autologous antigen as a result of shift in B-cell population from B-2 to B-1 cells. There is also a reduction in the quality of the antibody response as measured by affinity and avidity (40, 394) (163, 327, 377). The B1 cells are

innate cells that constitutively produce natural antibodies in the absence of antigen. They are located in specialized areas like the spleen and peritoneal cavity unlike the B2 cells that circulate and initiate antibody response upon activation by antigens (91). These changes lead to such humoral defects as a reduction in the duration and protective abilities of the antibodies produced in the aged compared to the young. The anti-pneumococcal polysaccharide response upon vaccination of the elderly humans is found to be reduced in the elderly compared to the young. A study of both the healthy old and elderly nursing home residents compared to young adults shows that there is an age-associated decrease in the memory B cell compartment compared to the young (40). Not only that, but there is a persistently higher IgM concentration in the aged that is attributed to impaired class-switching to other isotypes with specific effector functions (103, 220, 256).

Another consequence of this shift in peripheral B cell pool in the aged from young naive follicular B cells to antigen experienced B cells is the increase in auto-antibodies in the sera from aged mice and humans. However, the levels of auto-antibodies do not always correlate with autoimmunity probably due to a decrease in their binding affinity (164, 183). This is validated in classic experiments in which lupus prone, autoimmune NZB and MRL/lpr mice are compared with normal Balb/c over a period of different ages (183). At eight months of age, the autoimmune mice have a repertoire biased towards autoantibodies production and indeed produce autoantibodies, while the nomal Balb/c mice do not exhibit any of these phenotypes. However, by 20 months, the NZB autoimmune mice have an accelerated autoimmune repertoire and autoantibody production and this is comparable to the normal, aged Balb/c mice.

Studies from the Cancro laboratory highlight an exciting factor that may influence the B cell homeostatic regulation in response to the low emigration of B cells from the bone marrow. They show that the competition among various B cells for BAFF/BLYS, which is normally secreted by activated macrophages, dendritic cells or some subtypes of B cells greatly, influences the homeostatic process (232, 235). The ability of BAFF to influence this process is dependent on its specific binding to the BAFF receptor (BAFFR) expressed by transitional B cells. This specific interaction has dual effects of guiding the B cell developmental process and influencing the longevity of both marginal zone and mature B cells (133). The negative selection process of autoreactive B cells is efficient in young rodents and humans. As a result of this elimination process, there are adequate amounts of BAFF available for normal B cell development. With aging there is a reduction in the emigration of B cells from the bone marrow leading to a reduction in the competition for BAFF. This creates favorable conditions for the production and maintenance of autoreactive B cells in the peripheral B cell pool (235). The aged B cells become hyper responsive to the limited amounts of BAFF. Thus, they are able to bind more potently to BAFF which increases their survival, further enhancing these B cells with limited repertoire over naïve B cells (234).

Age-associated bone marrow mediated defects affect B cells in aging

The bone marrow is the site for antigen-independent development of B cells, including pro- and pre-B cells. If the pro-B cell has a productive heavy chain rearrangement then it can proceed to the pre-B cell stage. During aging, the low production of pro-B cells and the impaired transition of Pro-B cells to pre-B cells is one of the factors responsible for the low emigration of B cells from the bone marrow (182, 233). The decrease in the

transition of the pro to pre- B cell is due to an age-related defect in productive V-DJ heavy chain rearrangements as a result of a defect in the E2A-encoded E12 and E47 transcription factors. These transcription factors are known to regulate this process (104, 345). Another mechanism for the impaired pro- to pre- B cell transition is attributed to the decreased production of IL-7 by the aged bone marrow stromal cells, as well as an impaired IL-7 receptor signal transduction (164, 233).

Signaling mechanism governing the impaired humoral response in the elderly

A critical hallmark of the humoral responses to pathogens is the ability to class switch to non IgM isotypes with functions more suitable to eliminate the pathogen. However, studies show that this ability to undergo class-switching is impaired in aged humans and mice. The Bloomberg laboratory showed that there is impaired class-switching to the typical Th2 –cell dependent isotypes IgE and IgG1. They further show a defect in class switching to the typical T-cell independent isotypes, IgG2a and IgG3 in senescent mice (105). Further they show that the impaired class switching in the elderly mice can be correlated with a decrease in activation-induced cytidine deaminase (AID), and E47, one of its transcriptional regulators. The enzyme AID is critical for both class switch recombination and somatic hypermutation which are necessary for enhancing both the effector function and increased affinity of antibodies produced by B cells (248). When the study is extended to human peripheral blood, there is a similar decrease in both AID and E47 in B cells recovered from aged compared to the young. Hence, they conclude that the impaired ability of the B cells to class-switch in response to antigen and cytokines or CD-40 mediated signaling is due to an intrinsic signaling defect involving an age-associated decrease in both AID and E47 (103, 105). Other intrinsic signaling defects

in B cell signaling include lower expression of costimulatory molecules like CD86 (396). Another study in human B cells showed that the enzymatic activity of protein tyrosine kinases and protein kinase C, major upstream signaling molecules of B cells, are impaired in the aged compared to the young (379).

The microenvironmental and intrinsic signaling defects in bone marrow hematopoietic stem cells (HSC) affect B lymphopoiesis

Aging also affects HSC which are the progenitors of lymphoid and myeloid cells. As a result of the age-related defects in the HSC, there is a decrease in lypmphopoiesis resulting in an increase in myelopoiesis (179, 305). Studies to date, point to a dual regulation of HSC by factors intrinsic to individual HSC and cues from the bone marrow microenvironment or stromal niches. For example, age-related defects in both types of signals affect generation of B cells and macrophage lineage cells (33, 54, 170, 372, 386). Age-associated intrinsic signaling defects can affect HSC such as loss of the integrity of the genome. This is due to accumulation of DNA damage and downregulation of genes involved in maintaining the integrity of the genome as a result of epigenetic dysregulation at the chromatin level (59, 304, 372). The alteration in the expression of crucial genes in the long-term HSC sets the stage for the downstream defects observed in lymphoid and myeloid cells (305). Aging leads to a remodeling of the bone marrow, with a decrease in osteoblasts, which constitutes the stromal niche, but increase in adipocytes and osteoclasts (303). The age-related alterations in bone marrow microenvironemnt result in a decrease in B cell production and immunoglobulin diversity, due in part to an impaired expression of Rag2 at the level of the pro-B cells (192).

Aging and Dendritic cells (DC)

DCs are crucial sentinel innate cells that bridge the innate and adaptive immunity via antigen presentation to T cells and expression of costimulatory molecues that are needed for T cell activation and cytokine production. Aging-related defects in DC function contribute to age-related impairment in T cells. Aged DCs exhibit reduced antigen processing, costimulatory molecules and IL-12 production leading to reduced capacity for antigen presentation (6). This is even more prominent in the frail elderly humans (354). However, studies in human blood monocyte derived DC via GM-CSF and IL-4 show that the DCs from the aged and young are equally efficient in antigen presentation and inducing T cell activation (212)**.** The migration of DCs in the aged is impaired as a result of their failure to upregulate chemokine receptors, such as CCR7, and due to reduced production of chemokines such as CCL19 and CCL21 in their microenviroment upon immunization. As a result there is decreased accumulation of DCs in draining lymph nodes in aged mice (187, 209). Aged plasmacytoid dendritic cells (pDC) show a dysregulation (lower) in the secretion of type I interferon in response to CpG and HSV-2, which signal via TLR-9 and 7 respectively, and thus accounts for the higher incidence of viral and bacterial infections in the elderly (187, 211) .

Aging and Macrophages:

Resident tissue macrophages are affected in multiple ways in the aged which could account for age-related increases in viral and bacterial infections, as well as increase in cancers incidence. Aged peritoneal macrophages exhibit a decrease in adherence, opsonization, chemotaxis, phagocytosis and antibody-dependent cytoxicity involved in

the killing of tumors when compared to similar macrophages recovered from the young (5, 77, 187, 264, 288). Liver macrophages (Kupffer cells) also exhibit age-associated reductions in respiratory burst and endocytotic capacity (288, 363). A body of literature intimates that cytokine secretion is dysregulatated in both splenic and peritoneal macrophages activated through engagements of different TLRs. There is a decrease in pro-inflammatory cytokines like IL-6, IL-12, TNF-α, and an enhancement in the anti-inflammatory cytokine, IL-10 by aged macrophages (34, 63, 65, 187). Both humans and rodent macrophages exhibit reduced expression of MHC-II (143, 288). While studies in human monocytes demonstrate similar number of cells between the aged and young, some studies indicate a reduction in the precursor cells in the bone marrow of the aged (264, 288).

Several studies have investigated the signaling mechanisms governing the age-associated defect in macrophages. In this regards, studies in rodents and humans show age-associated decreases in TLR expression (187)**.** In contrast others including our laboratory, show no differences in TLR-2 and TLR4 expression by macrophages recovered from aged and young (63, 187, 298, 359). Another possible mechanism for the cytokine dysregulation and other aging defects is enhanced production of cycloxyenase-2 (COX-2) due to an increase in prostaglandin E2 (PGE). The enhanced COX-2 suppresses the pro-inflammatory cytokines and enhances IL-10 secretion; and it is also known to suppress MHC-II (120, 289, 384). Studies which investigate the TLR-mediated signaling mechanism by aged phagocytic cells have come to different conclusions, and this may be due to the type of macrophages, purity, and experimental conditions. We show that increases in phosphorylated and total p38 MAP kinase levels have a role in cytokine

dysregulation which could be rescued with p38 MAP kinase inhibitors; however, others show an age-associated reduction in the activation of p38 MAP kinase (34, 65). We and others show a decrease in the NF-κB signaling pathway in the aged as a possible mechanism (34, 35, 65). In this thesis we tested the importance of PI3K pathway, since our microarray analysis showed an increased expression of PI3 kinase subunits in aged macrophages and since the pathway is known to have negative regulatory effects on macrophage-derived cytokines (107, 194). In summary, aging affects macrophages and may be a major player in the increased susceptibility of these individuals to infections and impaired immune response to vaccines.

Streptococcus pneumoniae **as a model pathogen to study the effect of aging on infection with gram positive bacteria**

History of S. pneumonia infection and pneumococcal vaccine

The gram positive bacterium *S. pneumoniae* was independently isolated by Louis Pasteur and George Miller Sternberg in 1881 (373). Not long after that, the Klemperers made the startling discovery that serum from patients infected with *S. pneumoniae* could confer protection against homologous organisms (19, 369, 373). The first attempt to use the existing knowledge about *S. pneumoniae* in vaccine development was done by Sir Almroth E. Wright in 1911, when he showed that the use of killed whole pneumococci could confer some protection (369). However, the protection was suboptimal due to limited knowledge on serotype specific responses and the dosage. The evolution of the current pneumococcal polysaccharides vaccine is a result of the work of Felton and Bailey in 1926, who isolated pure pneumococcal polysaccharide for the first time (369).

As a result the first pneumococcal polysaccharide vaccine was developed from purified polysaccharides in 1931 and was shown to avert the spread of pneumococcal infections (329). However, upon the development of antibiotics and the realization that they can serve as effective treatments for pneumococcal infections, the polysaccharide vaccine fell out of favor for almost forty years (20, 37). Renewed interest in the use of polysaccharide vaccine came from the seminal work of Robert Austrian and colleagues in the 1960s and 1970s, which resulted in their creation of the 14-valent pneumococcal polysaccharide vaccine in 1977. Less than 10 years later, the vaccine was expanded to the current 23-valent polysaccharide vaccine that contains the most common 23 serotypes that account for greater than 80% of pneumococcal infections (19, 21, 37).

Ecology, Epidemiology and pathogenesis of S. pneumoniae

Humans are the only natural reservoir for *S. pneumoniae* and the bacteria is found in the nasopharyngeal cavities (352). The carriage rate is between 30 to 40 percent in normal humans. There is a horizontal transfer from person to person by close contact, and this transfer is limited by competition with other microbial occupants of the nasopharyngeal flora, as well as the innate host defense mechanisms. The carriage and colonization rates has been associated with the extremes in age, that is higher in children less than two years and elderly above 65 years (352, 357). There are other factors in addition to age that contribute to increases in colonization and carriage, which are considered precipitating factors for spread of the infection and invasive pneumococcal disease (IPD). Some of these factors among children less than 5 years include having young siblings, attendance at day care centers and for adolescent and young adults, preexisting respiratory tract infections, primary or secondary smoke and being asthmatic (124, 148,

217, 260). If the natural commensal relationship between the pneumococcal bacteria and the host is compromised due to breach in the host natural defense, then disease ensues and the bacteria migrate from the nasopharynx to normally sterile sites in the upper or lower respiratory tracts. This results in infections, such as otitis media (if migration is to the middle ear), sinusitis (to the sinuses) and pneumonia (if migration is to the lungs). When the bacteria migrate from the middle ear or sinuses or through the blood stream to the meninges of the brain it results in meningitis (281). This makes S. *pneumoniae* an etiological agent for meningitis, and bacteremia in adults and children, especially in people with comorbidities or impaired immune systems (180, 250, 312).

Epidemiology

S. pneumoniae is the most common cause of bacterial pneumonia and other associated diseases like pleural effusion and emphysema (pus in the pleural space) (281). It affects people of all ages and all geographical regions, as well as those with chronic and immunocompromising diseases (156, 263). *S. pneumoniae*-related infections are the leading cause of death in the world (WHO, 2000). *S. pneumoniae* is a pathogen that associates with community acquired pneumonias both in the United States and the world. Pneumoccocal infections account for the most upper respiratory tract infections and otitis media in children (218). Pneumococcal infections account for 20-70% of hospitalizations per year in the United States, 915,000 cases of community acquired pneumonia in the elderly and 4,600 deaths per year in adults greater than 50 years old. Other consequences include neurologic sequelae common among survivors (96, 218, 273).

Invasive pneumococcal disease (IPD)

Invasive pneumococcal disease (IPD) is defined as the presence of *S. pneumoniae* in normally sterile sites like the blood, cerebrospinal fluid, surgical sites, and lungs. It is a major cause of morbidity and mortality both in the United States and globally. In the United States, pneumococcal IPD is responsible for increased incidence of hospitalization due to bacteremia with 15-40 per 100,000 overall hospitalizations (16, 300, 380).

*Risk factors for IPD***:** The most common risk factors are genetic defects, such as defects in the classical complement pathway, the extremes of age, patients with comorbidities, defective immune defenses, living in crowded places like prisons or long term care facilities; abuse of alcohol and cigarettes and asthma (148, 149, 217, 300, 310, 357).

Cell Surface and virulence Factors and host defense:

Some of the virulence factors are polysaccharide capsule (CPS), cell wall and pneumolysin which are depicted in Figure 1.1.

Polysaccharide capsule (CPS): The most important virulence factor of *S. pneumoniae* is the polysaccharide capsule, which encircles the cell wall and resists phagocytosis conferring greater survival of the bacteria (129, 218, 253, 352). The polysaccharide capsule is found in serotypes that are mostly pathogenic, resulting in colonization, invasion and causing IPD. It is used to determine the sereotypes of the various strains. There are about 91 different serotypes, each of which elicits a specific anti-capsular polysaccharide (CPS) antibody response. The global IPD are caused by 20% of these serotypes, which is the basis for their inclusion in the 23-valent polysaccharide vaccine.

These IPD causing serotypes include serotypes: 14, 4, 1, 6A, 6B, 3, 8, 7F, 23F, 18C, 19F and 9V (142, 273).

The Cell Wall: The cell wall of *S. pneumoniae* is also a strong virulence factor as it recruits polymorphonuclear leukocytes to the lungs and enhances permeability of the alveolar epithelial cells. During progression of the infection, the cell wall is degraded releasing inflammatory materials leading to massive inflammation and this aggravates the disease condition, as well as the mortality (272). Lipoteichoic acid (LTA) is a component of the cell wall and plays a major role in inducing inflammation (281). The cell wall also contains a polysaccharide called C-polysaccharide that is distinct from capsular polysaccharide. Phosphocholine is a major component of the C-polysaccharide. Naturally present antibodies expressing the T15 idiotype in many species react with C-polysaccharide. Passive administration of the natural antibodies that express the T15 idiotype protects against pneumococcal infection in animal models (43)

Pneumolysin: Pneumolysin exhibits endotoxin-like properties and is found in all pathogenic strains. It is released during autolysis and becomes a pore forming cytotoxin. It is thought to stimulate macrophages via TLR4 and induce secretion of pro-inflammatory cytokines. Once released by autolysis it lyses the host epithelial cells, inhibits mucociliary action, and impairs phagocytosis resulting in massive inflammation leading to morbidity and mortality associated with infection of *S. pneumoniae* (147, 357).

Innate Immune response and Streptococcus pneumonia: TLRs and cytokines

Host Response to S. pneumoniae: The lungs and the spleens are two of the most important organs in the clearance of infection by this normally commensal bacterial

organism, but deadly under disease conditions (37, 48). The first line of defense in a normal host is the mucociliary movement, cough, sneeze and epiglottis reflexes which help to prevent the pathogen from penetrating beyond the superficial epithelium in the respiratory tract (281). The second layer of defense is by the host phagocytes, especially alveolar macrophages in the lungs and the neutrophils recruited by the inflammatory response. The alveolar macrophages have a critical role in the removal of bacteria via phagocytosis and intracellular killing. The phagocytic response is promoted by serotype specific antibodies, like IgA, IgM, and complement that bind the bacteria and allow for efficient clearance by a mechanism known as opsonophagocytosis.

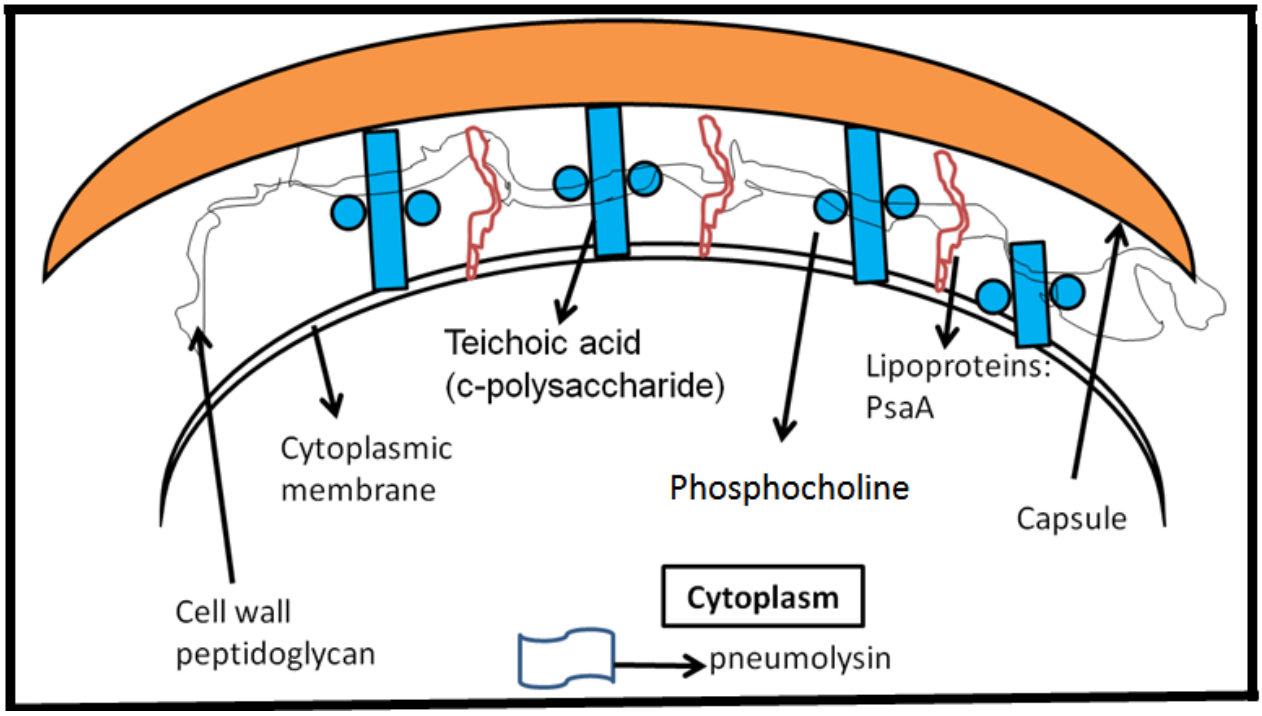
Toll like receptors (TLR)

Toll like receptors (TLR) are the major class of receptors by which innate cells recognize microbial pathogens. The basis for recognition of microbial pathogens is recognition of specialized structures that are unique to the invading pathogens. These are known as pathogen associated molecular patterns (PAMP) and are recognized by the pattern recognition receptors (PRR) on the innate immune cells like macrophages, dendritic cells and other type of cells like epithelial cells (162, 174). These PRRs help in the recognition of germline encoded PAMPs that are associated with viruses, bacteria, yeast, fungi and even damaged tissues (288).

Figure 1.1: *S. pnenmoniae* morphology displaying virulence factors

Streptococcus pneumoniae has a cell wall comprising of teichoic acid and is capsulated by by a polysaccharide. The capsular polysaccharide and other component of the cell wall constitute the virulence factors that are associated with the pathogen.

Figure 1.1


Teichoic acid
(c-polysaccharide)
Lipoproteins:
PsaA
Cytoplasmic
membrane
Phosphocholine
Capsule
Cell wall
peptidoglycan
Cytoplasm
pneumolysin

Once the PRR recognizes PAMP it initiates an intracellular signaling pathway that culminates in induction of cytokines, chemokines and interferons. This same intracellular signaling drives the maturation of dendritic cells which ushers in the adaptive immunity (174).

Though Drosophilia lack an adaptive immunity, they elicit responses to fungal infections via toll like receptors. This supports the concept that toll like receptors are evolutionarily conserved across simple to more complex species (152).

The TLRs have amino terminal leucine-rich repeats which are used to recognize PAMPs. The C-terminal domain which contains the Toll/interleukin-1 (IL-1) receptor homology (TIR) domain induces the intracellular signaling (348). As of now 11 TLRs have been discovered and each has specificity for distinct PAMPS, as well as self molecules under different conditions (174). The TLR-1/ 2 and TLR-2/ 6 heterodimers are activated by cell wall component of Gram positive bacteria while TLR4 is recognized by lipopolysaccharide (LPS), a component of Gram negative bacteria, and endogenous heat shock proteins (31, 288). TLR-3, 5 and 9 receptors interact with double stranded RNA (PolyI:C), bacterial flagellin and CpG DNA motifs respectively (288). Murine TLR-7 and human TLR-8 recognize uracil-rich single stranded RNA present in many viruses (141). TLR-11 can be ligated by ligands from uropathogenic bacteria in mice but is nonfunctional in humans. TLR-10 is present in humans but has no known ligand. Currently, there are no TLR-10 homologs in mouse (347, 392). TLR-1, 2 and 4 are surface receptors while TLR-3,7, 8 and 9 are intracellular, residing on endosomes (347).

TLR signaling

Ligand-mediated activation of TLRs results in intracellular signaling by means of their TIR domains which associate with the TIR domains of adaptor molecules such as MyD88, TIRAP–(MAL), TRIF–(TICAM1), and TRAM–(TICAM2). All TLRs signal via MyD88 with the exception of TLR3. This interaction further induces other adaptor molecules, such as TIRAP-MAL, TRIF-TICAM, depending on the specific type of TLR. The TLR-MyD88 interaction sequentially recruits IRAK-4 and IRAK1. IRAK-4 undergoes autophosphorylation and then phosphorylates IRAK-1 leading to its disengagement from the MyD88 complex and association with TRAF-6 (162). This complex then activates TAK, which in turn activates intermediate molecules, like IKK. Upon activation IKK phosphorylates IκB, which inhibits the translocation of NF-κB. The phosphorylated IκB is degraded leading to an eventual nuclear translocation of NF-κB. This pathway also works via the MAP kinase pathway to activate both NF-κB and AP-1 trancription factors (162, 174, 348). The activation of these transcription factors results in induction of pro-inflammatory cytokines like TNF-α, IL-12 and IL-6. If TLR 2 and 4 are activated then the TIRAP-Mal adaptor molecules will associate with MyD88 (174, 387)(Figure 1.2). While MyD88 may be dispensable to TLR4 signaling , TLR9 signaling totally depends on it (162). TLR4 has a MyD88-dependent and independent pathway. The MyD88 dependent pathway is dependent on an interaction of MyD88 with TIRAP-MAL. The MyD88-independent pathway signals via TRIF-TRAM heterodimers and is activated via both the TLR4 and 3 pathways. The latter pathway activates IRF3 leading to production of type-1 interferon (162, 347).

Role of TLR in mediating Immune response to S. pneumonia

TLRs are some of the major PRRs necessary to sense S. *pneumoniae* and activate phagocytes to clear the pathogen via phagocytosis (185). TLR-2 is critical in the recognition of *S. pneumoniae* since its cell wall components contain peptidoglycan, lipoteichoic acid and other lipopeptides that are ligands for TLR2.

TLR-2 knockout mice have impaired responses to infection with *S. pneumoniae* though the lethality is not greater; this is thought to be due to the role of other TLRs in immune response to *S. pneumoniae* (177, 184, 240). When the TLR-2 knockout mice are challenged with polysaccharide from serotype 3 and 14 bacteria and the 23-valent pneumococcal polysaccharide (PPS23) vaccine or the pneumococcal conjugate vaccine the anti-PPS IgG response disappears while the IgM response is much lower compared to the wild type. These studies show that both the PPS23 and the conjugate vaccine contain a TLR-2 ligand (317). Similarly, PPS23 vaccine is also reported to contains a ligand for TLR-4 (317). TLR-4 is important in the clearance of *S. pneumoniae* because of its ability to recognize pneumolysin (222, 240). Because of the existence of CpG motifs in the bacterial DNA, it is possible that TLR-9 is also critical for the recognition and clearance of *S. pneumoniae*. Thus, TLR-9 knockout mice exhibit an enhancement in susceptibility to *S. pneumoniae* (14).

Pro-inflammatory cytokines are critical for the clearance of S. pneumoniae.

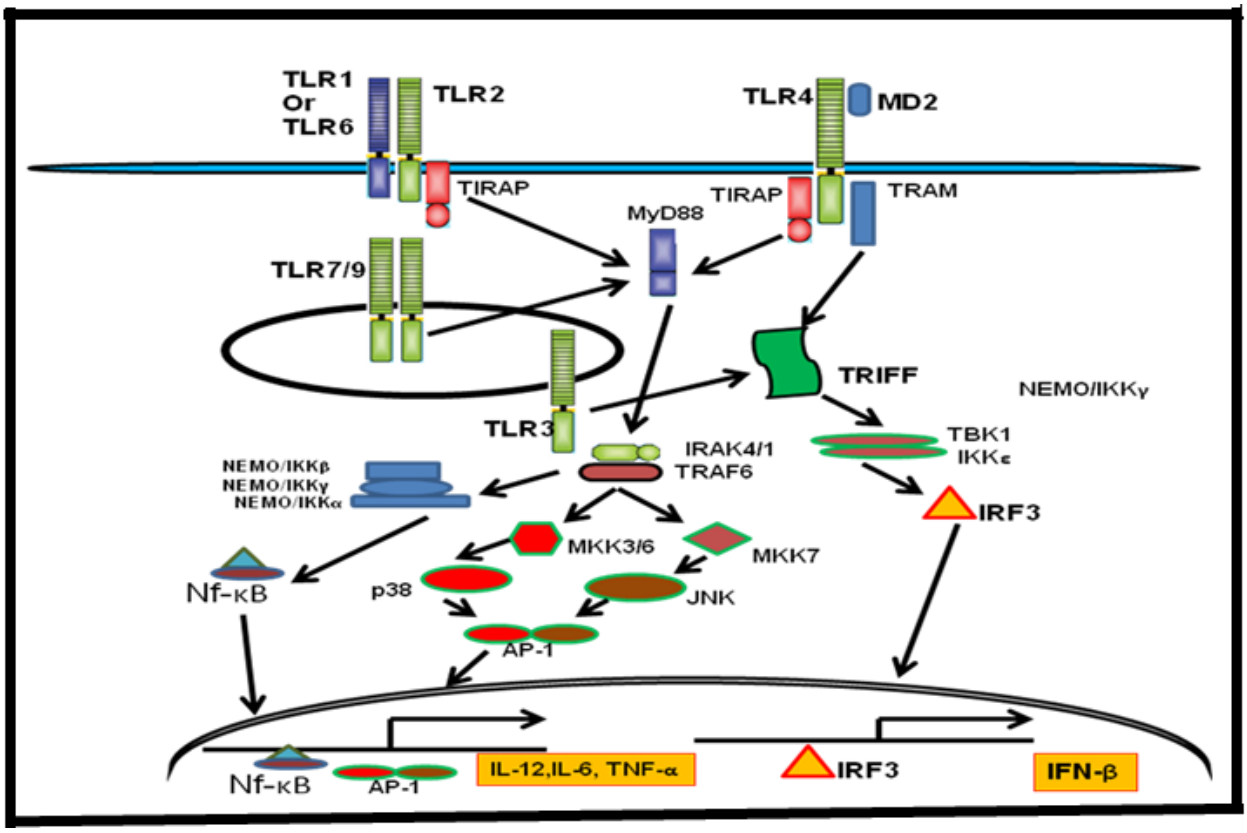
The critical role of cytokines in protecting the human host from *S. pneumoniae*-mediated pathogenesis is highlighted in human genetic defects involving both the pro-inflammatory cytokines and the inflammatory signaling pathways. A recurrence of

pneumococcal disease occurs in humans who are deficient in IL-12 (132). In animal studies a critical role of cytokines such as IL-1, TNF-α and IL-6 in the clearance of *S. pneumoniae* is seen (262, 398). Mutations in IRAK4 and NF-κB result in recurrent pneumococcal infections in the affected individuals emphasizing an important role for pro-inflammatory cytokines (286).

Figure 1.2: The TLR signaling pathway.

The toll-like receptors are pattern recognization receptors that serve as sensors of both endogenous and external danger. The TLR mediated signaling culminates in the activation of crucial transcription factors like NF-κB and AP1 which regulate cytokine production.

Figure 1.2


TLR1
Or
TLR6
TLR2
TLR4
MD2
TIRAP
TIRAP
TRAM
MyD88
TLR7/9
TRIFF
NEMO/IKKγ
TLR3
IRAK4/1
TRAF6
TBK1
IKKε
NEMO/IKKβ
NEMO/IKKγ
NEMO/IKKα
IRF3
MKK3/6
MKK7
Nf-κB
p38
JNK
AP-1
Nf-κB
AP-1
IL-12,IL-6, TNF-α
IRF3
IFN-β

Some studies show that the anti-inflammatory cytokine, IL-10, either precipitates or attenuates the infections with *S. pneumoniae* depending on the stage of the infection and the level of the host immune response (358). IL-10 is an immunomoduatory cytokine that is known to suppress LPS-induced pro-inflammatory cytokines. If given exogenously, it suppresses the pro-inflammatory cytokines and increases the lethality of *S. pneumoniae* infection by inhibiting NF-κB activation and secretion of IL-12 and TNF-α by macrophages (358). Low levels of IL-10 induce resting and activated B-cells to switch from IgM to IgG3 (325). IL-10 can also inhibit B cell proliferation induced by TLR ligands (328). The ability to stimulate macrophage response when pathogens are encountered is partly dependent upon differential expression of IL-10 and pro-inflammatory cytokines like IL-12. Interleukin-12 (IL-12) is produced mainly by macrophages and DCs and directly or indirectly (via IFN- γ) acts on B-cells and induces increased production of IgG2a and IgG3 (42). IL-12 is tightly regulated by several cytokines, including IFN-γ which enhances its production and IL-10 which is inhibitory (17, 203, 231).

The adaptive immune response to S. pneumoniae: the role of B cells:

The adaptive immune response to *S. pneumoniae* involves both humoral and cell mediated responses. There is a delicate interplay among the monocytes/macrophages, T cells, PMN and anticapusular polysaccharide antibodies produced by B-cells (352). The humoral response is directed against the capsular polysaccharide on the whole *S. pneumononiae* and is serotype specific (232, 352). The adaptive immune response is often initiated in situations where the pneumococcal bacteria are more virulent or the immune clearance mechanism is breached due to comorbidities (48). As a result, the

pathogen can escape this protective mechanism, penetrate the lung interstitium and become systemic via the blood or lymphatic system and, thus, result in the often-fatal condition known as bacteremia (48, 374). The spleen plays an important role in mediating adaptive responses to this latter stage of the pneumococcal infection. The indispensability of the spleen to the clearance of *Streptococcus pneumoniae* is clearly demonstrated in splenectomized patients from surgery or auto-infarction. In these patients there is an increase in bacteremia and sepsis due to *S. pneumoniae* (70, 366). Patients who have functional and anatomical aspleenia also show increased incidence of invasive pneumococcal disease (IPD) (142, 202). The spleen is basically divided into the red pulp, the white pulp and the marginal zone (MZ) (which is the specialized area between the red pulp and the white pulp and contains the marginal zone B cells and the marginal zone macrophages). TI-2 antigens localized exclusively within the marginal zone where they associate with the MZ macrophages and MZ B-cells (127, 360). When MZ macrophages engage *S. pneumoniae* via receptors such as SIGNR1, both the MZ macrophages and B-cells migrate to the red pulp where they interact leading to rapid differentiation of the pre-primed B-cells to antigen forming cells (AFC) and subsequent rapid production of anti-pneumococcal antibodies by a mechanism that is still not delineated (169, 171, 224).

T-cell independent B- cell mediated response to capsular polysaccharides

B-cell responses to capsular polysaccharide can be elicited in nude mice that lack mature T cells, suggesting that these antigens did not need T-cell help to elicit an antibody response. This conclusion is supported more rigorously by Nemazee et al. using TCR $\beta^{-/-}$ and $\alpha^{-/-}$ mice (241, 254, 336, 368). Hence, the capsular polysaccharide is classified as a T cell independent (TI) antigen. These antigens can cross-link the B cell receptor and

induce B cell proliferation, but are incapable of inducing immunoglobulin (Ig) secretion or isotype switching. When the proliferating B cells are exposed to exogenous or macrophage derived cytokines such as IL-1, IL-6 or IL-12, or T cell derived cytokines such as IL-4 or IL-5, they could now differentiate into antibody producing B cells (38, 63, 317, 331). However, there are studies that showed that TI antigen responses may be modulated by T-cells either directly or indirectly, but this is not an absolute requirement (73, 336).

Studies of TI antigens identified a subset of Ti antigens which included polysaccharides and haptenated-Ficoll that do not elicit antibody responses in mice with an X-linked imunodeficiency (XID), neonatal mice and human infants. These are referred to as TI-2 antigens and are high molecular weight polymers with repeating antigenic determinants for which immune responses appeared after a year or so of age in humans. These are distinguished from the TI-1 antigens that can elicit responses in both the XID and neonatal mouse model and are characterized by their ability to activate B cells polyclonally. TI-antigens are often components of bacterial products with lipids attached and included groups like LPS, TNP-LPS and TNP coupled to inactivated bacteria like *Brucella abortus* (241, 244, 331, 336).

T cell dependent antibody response: B-cells can bind soluble antigens or whole bacteria via their membrane immunoglobulin receptor a resulting in the internalization processing and presentation of antigenic determinant to T-cells in the context of MHC-II. The resultant cognant interaction between B-cells and T-cells activates B-cell expansion and differentiation into plasma cells. Hence, the B-cell interaction with the antigen via its B cell receptor (BCR) is considered as signals 1 while the cognate T-cell interaction is

considered signal 2. Therefore, the activation and effector functions of the B cells are dependent on T-cells and such antigens are designated T-cell dependent (TD) (279, 336, 368).

Concept of signals 1 and 2 in the context of immune response to PS from S. pneumoniae

In a typical TD response the antigen binding by the BCR elicits the first signal for activation of B cell, but the second signal is derived from the cognate B cell: T cell interaction. However, in the context of the B cell mediated response to PS from *S. pneumoniae*, the T-cell signal 2 is either absent or not very critical. The capsular polysaccharide (CPS) of *S. pneumoniae* contains repetitive epitopes that provide maximum cross-linking with the B-cell receptor (BCR) and activate B-cells, without the participation of T cells help, and thus engenders low isotype switching, low affinity antibody and no memory (38, 79, 207, 241, 336). But where does the second signal come from (49, 65) ? In this case the signal 2 is provided either by (1) stimuli that directly target the B-cell or (2) stimuli that indirectly target B-cells via induction of stimulatory cytokines like IL-1β, IL-6, IL-4 or IL-5 (Fig. 1.3). Such cytokines can be produced by phagocytes like splenic or alveolar macrophages, when the *S. pneumoniae* or some of its cell wall components like LTA, the bacterial DNA or peptidoglycan, engage the toll like receptors (Figure 1.2) (65, 100, 325). Activation of T cells by bacterial proteins may also provide such cytokines.

Antibiotics, success and failures in treating S. pneumoniae:

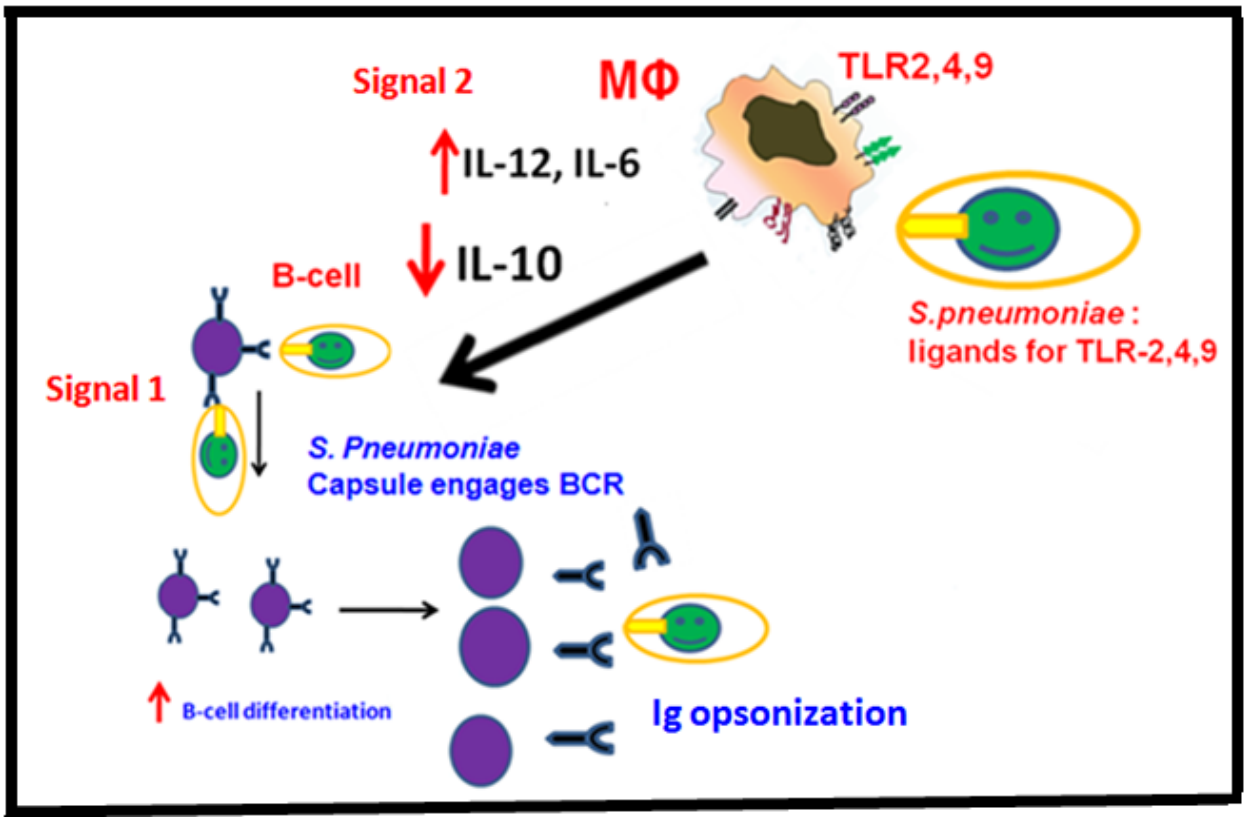
The first recorded incidence of penicillin resistance by *S. pneumoniae* was reported in Australia in 1967 with additional episodes reported in North America in 1974. In 1992

there was about 69% resistance to penicillin in Hungary. Between 1988 and 1990, there was a report of 15-20% resistance in the United States. In 1977, the first reports of multi-antibiotic resistant strains were reported in South Africa which were followed by reports of rapid global spread of antimicrobial resistance (216, 217). Some strains of *S. pneumoniae* have resistance to tetracycline, erythromycin, chloremphenicol, clindamycin, streptomycin and the beta-lactam antibiotics (281).

Figure 1.3: Concept of Signal 1 and signal 2 in the immune response to *Streptococcus pneumoniae*.

Upon engagement of the BCR by the repetitive epitopes of the capsular PS of *S. pneumoniae* (signal 1) there is B-cell proliferation, but the proliferating B cells require pro-inflammatory cytokines (signal 2) derived from macrophages, activated via *S. pneumoniae* engagement of TLRs, to differentiate into cells that secrete anti-capsular Ig.

Figure 1.3


Signal 2
MΦ
TLR2,4,9
IL-12, IL-6
IL-10
B-cell
S.pneumoniae :
ligands for TLR-2,4,9
Signal 1
S. Pneumoniae
Capsule engages BCR
B-cell differentiation
Ig opsonization

The mechanism of resistance includes point mutations in genes encoding key bacterial proteins, such as penicillin binding protein (PBP) (281) (216, 217). As a result, it is crucial to develop effective vaccine adjuvants to confer greater efficacy of the current pneumococcal polysaccharide and conjugate vaccines in the elderly. Thus, this thesis attempted to explore potential signaling pathways that would enhance immune response to the current vaccines.

Evolution of polysaccharide vaccine as a viable and cost efficient alternative to antibiotics

The 23-valent capsular polysaccharide vaccine (PPSV23)

A combination of increased antibiotic resistance and the differences in the degree of severity of the pneumococcal infections in different hospitalized patients led to the evolution of the polysaccharide vaccine as an efficacious alternative (21, 46) (20, 21). Hence, there is an advocacy for the reintroduction of the polysaccharide vaccines resulting in the approval of the 23-valent pneumococcal polysaccharide vaccine. The United States Food and Drug Administration (FDA) approved the 23-valent pneumococcal polysaccharide vaccine (PPSV23) in 1983 to be used in the elderly, children greater than two years who are at risk of the infection due to chronic diseases (like sickle cell anemia), and the immunocompromised (due to lymphoma, aspleenia and HIV/AIDS) and all adults at risk for chronic diseases. The PPSV23 vaccine is composed of purified capsular polysaccharides from 23 of the most common clinical serotypes. These serotypes account for 89% of all IPD (19, 46, 281).

Following the introduction of the (PPSV23) vaccine, post vaccine efficacy studies conducted in South Africa and other countries discovered that the vaccine elicits a robust response in the young, but only has a 60% efficacy in the elderly. The functional activity of the antibodies for the vaccine, and the ability to promote opsonophagocytosis is impaired in the elderly (>65) compared to the young (<45) (200). Another finding is that the vaccine can not elicit potent protective antibody response in children less than 24 months old (46, 74, 321). A meta-analysis and systematic review of studies of 19 trials comprising 82,665 participants from diverse settings in Western Europe, North America, Russia, Papua New Guinea and the Caribbean concludes that PPS23 offers little protection in the elderly over 65 years old and people in the ages from 2 to 64 years old with chronic respiratory illnesses (156). The vaccine elicits no memory and the serum antibodies did not last long (39, 69, 218, 272, 321, 357). Since the vaccine confers protection in a serotype specific antibody response, it can not offer protection against serotypes not covered in the vaccine, i.e., serotype restriction which is dependent on the geographical region of the world (4, 18). Finally, revaccination with PPS23 is not recommended as it results in reduced antibody production (159).

Immunological basis for the poor response of children less than 2 years to the polysaccharide vaccine

A commonly held theory is that there is a selective and developmental disadvantage in eliciting an anti-polysaccharide response in young children (46, 93). A second concept is that in children and neonates there are tissue polysialylated glycoproteins that are capable of eliciting cross-reacting antibody responses in a similar manner as the bacterial polysaccharides and thus could be deleterious to the infants. Thus, there is a form of

tolerance to the polysaccharide antigens. These polysialated glycoproteins were found in human embryonic brains, new born rat kidney, heart and muscle, but not in the similar tissues in adults (98, 99). Another plausible explanation for the impaired response in children is the fact that the polysaccharides bind the complement factor C3d generated via the alternate pathway and form complexes which bind the complement receptor CD21 on marginal zone B cells, and this is known to enhance BCR signaling. However, in children less than two years old, there is reduced expression of CD21 which may have a role in the impaired response to the polysaccharide vaccine due to a lack of CD21 mediated amplification of the BCR signal. But with the conjugate vaccine this need for CD21 is overcome at least in the rat model (41). The defective antibody responses of the human infants to model TI-2 antigens and pnemococcal polysaccharides is closely mimicked in the murine model (195). Our laboratory has shown that neonatal unresponsiveness to PS antigens is due to an inability of neonatal macrophages to produce adequate levels of IL-1, IL-12 and IL-6 (195).

Immunological basis for the impaired antibody response of the elderly to PPS23 vaccine

A major factor that predisposes the elderly to increased infection with *S. pneumoniae* or impaired antipolysaccharide vaccine response is attributed to a compromised immune function as a result of the normal aging process (75). For example the naturally acquired IgG and IgM to six of the of the most common IPD causing serotypes (3, 4, 6B, 9V, 14 and 23F) are impaired in the elderly compared to the young (327). Not only that, but the natural IgG and IgM antibodies to some common pneumococcal surface proteins including PspA are impaired in the elderly compared to the young (327). Hence, this age-related defect can also account for the impaired PPS vaccine response in the elderly.

There are also studies that indicate that aging affects B cell immune functions and specificities, such as class switching to Ig Isotypes to IgG3 in mice and IgG2 in humans (43, 105, 227, 278). Since, the immune responses to *S. pneumoniae and* PPS vaccine are mainly B cell mediated, such age-associated defects in B cells can account for the lower vaccine efficacy in this population. Aging is also associated with a decrease in cytokines such as IL-12, IL-6 which are needed for the induction of antibody response to the TI antigens, like the PPS vaccine (38, 63).

The shift to conjugate vaccine:

The seven valent conjugate vaccine (PCV-7)

Because the immune response to the PPS23 was late onset and thus could not protect children less than two years from pneumococcal pneumonia or acute otitis media (AOM), the conjugate vaccine was developed by coupling some of the PS to a protein carrier to make the PS, a T dependent antigen. It was approved for use in the USA in 2000 by the FDA (218, 319). It is recommended for children within the first 6 months of their lives and children greater than 2 years who are at higher risk for IPD due to HIV/AIDS and sickle cell anemia (261, 357). The conjugate vaccine, designated PCV-7, is made up of the purified capsular polysaccharides of seven of the most common clinical isolate serotypes, which are 4, 6B, 9v, 14, 18C, 19F and 23F and they are conjugated to a protein carrier, such as tetanus-toxoid. As a result of this composition the antibody response is now shifted to a T-cell dependent response. The carrier protein is able to activate T-cells that are specific for the protein and thus provide help for the polysaccharide specific B-cells. As a result B-cells can undergo affinity maturation, Ig class-switching and

induction of memory B-cells. The 7 serotypes contained in the conjugate vaccine account for more than 80% of all IPD in North America in children less than two years of age and the elderly (69, 261). PCV-7 vaccine protects children less than two years old from IPD and offers some protection from otitis media, and also indirectly protects adults and elderly as a result of herd immunity (154, 218, 319).

Upon the introduction of the conjugate vaccine it became obvious that non-clinical serotypes that are not included in the vaccine have emerged and are leading to increased incidence of IPD. For example, serotype 19A that is not included in the 7-valent conjugate vaccine began to emerge, thus reversing the initial gain in the reduction of otitis media in children (26, 218, 319). The heptavalent conjugate vaccine lacks a global appeal as the seven serotypes selected for inclusion are geographically restricted to North America. For instance, it will not work in sub-Saharan Africa because the predominant IPD causing *S. pneumoniae* consists of serotypes 1 and 5. The cost of producing the conjugate vaccine is too high and can not be afforded by poorer countries (2, 76, 79, 276). It also requires multiple immunizations to elicit long lasting protective antibody responses (69, 297). Five of the serotypes included in the PCV-7 vaccine are known to contribute to 80% penicillin resistance and thus with the introduction of the PCV-7 vaccine there is an overall reduction in the IPD caused by the erythromycin and multiple drug resistant strain through herd immunity (191, 283).

The 13-valent conjugate vaccine

The 13-valent conjugate vaccine after some field testing is finally approved by the FDA on February 24, 2010. This new vaccine has broader serotype coverage and is expected to

prevent IPD among infants and children, and to help in the reduction of otitis media (32, 261). The new 13-valent vaccine is made of serotypes from the initial PCV-7 conjugate vaccine along with six new serotypes (1, 3, 5, 6A, 7E, and 19A). Note that there is an inclusion of the newest nonclinical serotype that has become prominent, serotype 19A and the target population is for infants 2 – 59 months old and children between the ages 60 to 71 months old who present with underlying medical condition or children who received only a single dose of the PCV-7 vaccine (242, 261, 287).

Prime boost effect of PCV-7 and 23-PPSV

Studies show that an enhanced response can be elicited in a prime-boost immunization with the conjugate vaccine and with the 23-valent polysaccharide vaccine. If the priming is done with the PCV-7 vaccine followed by a boost with the PPSV23 vaccine there is an enhanced anti-capsular response (263). This prime-boost response is further potentiated with highly active antiretroviral therapy (HAART) treatment in HIV/AIDS children and infants (3). This latter regimen shows some promise in the elderly and the immunocompromised young adults (1, 160, 261). However, if the priming is done with the PPSV23 vaccine it results in hyporesponsiveness, even if the conjugate vaccine is used as a boost.

Pneumococcal proteins as vaccine candidates

Due to the current disadvantages of both the polysaccharide and the conjugate vaccines, there have been efforts made to utilize the pneumococcal proteins that confer cross-protection and, thus, are independent of any particular serotypes. There are many surface proteins that are being evaluated but the three most promising ones are the

pneumococcal surface protein A (PspA), pneumococcal surface adhesion protein A (PsaA), and pneumolysin (37, 45). PspA is a choline binding protein on the surface of the *S. pneumoniae* that blocks the alternative complement pathway (252). Despite its antigenic variability among different strains, studies in mice show that it has a broad protective effect against invasive infections and nasopharyngeal carriage (44). In a Phase I clinical trial it is shown that recombinant *PspA* from a single strain along with alum as an adjuvant can elicit broadly cross reactive antibodies to different antigenic forms and that passive immunization with antibodies from the participants can confer protection to mice (44). PsaA is a member of the metal binding lipoproteins. Immunization with this protein has a greater effect on colonization and nasopharyngeal carriage with little or no effect on the invasive disease in mice (37, 42, 45). Pneumolysin is another choline binding protein shown to have the best effect in protecting against invasive diseases but not colonization in mice (265, 280). As a result of the unique action of each of these proteins, the studies show that combining them exploits the synergistic effects and induces protection. A combination of PspA and PSaA protects against both colonization and otitis media in a mouse model, while a combination of PspA and pneumolysin is additive in protecting against invasive disease (42, 265). Some of the advantages of the PS protein vaccines using a mixture of different pneumococcal proteins: (1) able to target diverse pathogenic mechanisms; (2) useful to both children less than two years and adults as the children elicit responses to conjugate vaccines but not polysaccharides; (3) less expensive as there is no need for conjugation or polysaccharide purification; and (4) totally avoid the problem of serotype replacement (44, 344). However, there are several

challenges to develop a protein based vaccine, not the least of which is finding a suitable adjuvant.

Cytokines as potential adjuvant for PS vaccines

There are many vaccine adjuvants that have been evaluated in murine models and proven to potently enhance the immunogenicity of vaccines response. However, most of these adjuvants cannot be used in humans due to their severe side effects. For instance complete and incomplete Freund's adjuvant are potent immunostimulatory adjuvants but are not approved for human use due to side effects (27). The approved adjuvant, alum, is of very low potency necessitating multiple immunizations (389). As a result of these short-comings, there are many attempts to find a potent adjuvant that can still be very tolerable in humans. MF-29 is a new vaccine adjuvant approved for use in Europe but not in the USA. Monophosphoryl lipid A (MPL) is an adjuvant recently approved for use in USA and is thought to work via ligation of TLRs (72). Many researchers have also started looking into cytokines as a potential answer due to some of their unique properties. Cytokines are endogenous potentiators of immune response; hence enhancing the appropriate types of cytokines with antigen can be more highly immunogenic (67) (Table 1.1). Luo et al. showed that supplementing Pnu-immune vaccine with IL-10 elicits a better immune response than the vaccine alone (213). In a mouse model injecting IL-12 followed by PPS23 immunization resulted in an enhanced production of both IgG2a and IgG3 anti-vaccine antibodies. The IL-12 cytokine activates cell mediated immunity as well (49, 271). Physically fusing an antigenic protein with cytokines can elicit potent antibody responses consisting of various isotypes like IgG3and IgG1 (67, 383). Systemic immunization with PspA protein genetically fused to IL-2 produces higher anti-PspA

IgG1 and IgG2a responses compared to PspA adsorbed to alum (383). Another study demonstrates that immunizing mice with PspA along with IL-12 enhances sIgA and systemic IgG. This combination vaccine delivery strategy results in a dual protection against both carriage and fatal bacteremia, thus precluding the need to physically link different PPS proteins for different modes of infection and clearance (17). In another study recombinant PsaA fused to murine IL-2 or IL-4 is found to induce both IgG1 and IgG2a anti-PsaA antibodies, whereas combination with CFA generates only IgG1 response. Also PsaA plus cytokines confers greater protection to mice challenged with virulence strains than pure PSaA antigen. Thus the protection is 30% with PSaA alone as opposed to 100% with PSaA plus cytokine (122).

Table 1.1: Some Cytokines and their effects on modulating the humoral response to *S. Pneumoniae*

Cytokine	Type	Function in immune response to *S. pneumoniae*
IL-10	**Anti-inflammatory**	**Suppresses pro-inflammatory cytokines, optimal induction is needed for B cell differentiation. Enhances *S. pneumoniae* lethality in a mouse lung infection model.**
IL-12	**Pro-inflammatory**	**Enhances IgG2a and is critical for clearance of *S. pneumoniae* via generation of opsonic antibodies.**
IL-6	**Pro-inflammatory**	**Enhances formation of antibody producing cells against pneumococcal polysaccharide vaccine. Plays a crucial role in the clearance of *S. pneumoniae.***
TNF-α	**Pro-inflammatory**	**Acute early induction is needed for recruitment of leukocytes and clearance of *S. pneumoniae*.**

The adjuvant properties of –C-phosphote-G- (CpG) oligonucleotide are due mostly to this ability to activate both innate (e.g. macrophages, dendritic cells) and adaptive immune cells (e.g. B-cells) to produce cytokines, like IFN-γ, IL-6, IL-12 and TNF-α, that are known to modulate antibody production (Table 1.1). Thus, in a study in which mice are immunized with conjugate vaccine in the presence of CpG, there is an enhancement of polysaccharide specific IgG2A and IgG3, as well as low levels of IgM and IgG1 (69, 189, 334, 388). As a result, a couple of studies show that CpG can be used in combination with DNA vaccine with proteins from *S. pneumoniae*, or with conjugate or polysaccharide vaccines and induces some level of protection against pneumococcal infections (109, 316).

Additional studies show that immunizing mice with TI antigens and subsequently inducing endogenous cytokines like IL-1, IL-4, IL-6, IL-12, IL-2, and IFN-γ via TLR mediated signaling has the ability to activate B cell proliferation, activation, class switching and secretion of Ig (178). Hence, cytokines are potent providers of signal 2 needed to work with TI-2 antigens in activation of B cells. Recently it was shown that the ability of the PPSV23 vaccine to elicit robust responses in some aged groups is due to its contamination with ligands for TLR-2 and 4. This allows the vaccine to mediate production of cytokines that are crucial for the enhancement of Ig secretion and class-switching (178, 317, 331). Another study evaluated the role of monocyte derived cytokines, IL-6, IL-10 and TNF-α, in the increased susceptibility of patients with common variable immunodeficiency disease (CVID) to infection with *Streptococcus pneumoniae*. The results show that the pneumococcal-23 vaccination of these patients produces a less robust response than the normal healthy human controls due to a defect in

the vaccine-induced production of monocyte derived cytokines, IL-6, IL-10 and TNF-α (153). Thus, the levels of cytokines produced endogenously help to regulate the clearance of *S. pneumoniae* and this can be exploited in the immunocompromised host. Exogenous treatment with IL-1 confers protection in mice against lethal challenge with *S. pneumoniae* (167). IL-1 is known to regulate both B-cell expansion and differentiation into antibody forming cells (64, 167, 221).

Challenges with the use of exogenous cytokines

One of the current drawbacks in exogenous administration of cytokines as adjuvants in human studies is due to their pyrogenic properties as shown with IL-1 and IL-12 (259). A human clinical trial that used exogenous recombinant human IL-12 in the presence of the polysaccharide vaccine demonstrates that the cytokines elicit a minimal, but non-significant increase in pneumoccocal IgA, IgM, IgG1 and IgG2 antibodies over the placebo group, but have some adverse effects. These adverse effects include fever and generalized body pain in the participants resulting in discontinuation of the study (140). Another challenge in the use of exogenous cytokines is identifying the route of administration that will elicit a protective response and yet be physiologically relevant to humans (128, 291, 307). A final challenge in using exogenous cytokines as adjuvant is to consider what combinations of cytokines can be given to elicit maximum synergistic effects without side effects (7, 8) .

Endogenous enhancement of cytokines is a biologically relevant alternative adjuvant

There are still key unanswered questions in the use of the current polysaccharide or conjugate vaccines as well as potential challenges with the use pneumococcal protein vaccines or exogenous cytokines. Below, we investigate the plausible means for

enhancing endogenous production of macrophage-derived pro-inflammatory cytokines that provide the signal 2 needed for potent B cell activation, differentiation and anti-PS antibody responses in aged mice. Such an approach is not limited by serotype coverage, aging, T-cell dependency, health conditions or cost. In order to address these challenges with regards to the impaired antibody response to *S. pneumoniae* in the aged, there are four critical questions that must be answered and investigated with depth: (1) how does the age-related cytokine dysregulation in macrophages negatively impinge upon B-cell mediated signaling leading to impairment in class switching, affinity maturation and activation in response the pathogen; (2) How does aging affect the production of opsonizing IgG antibodies for host defense against *S. pneumoniae (36, 157);* (3) What signaling molecules are altered in either B cells, macrophages or both in the elderly that can be pharmacologically targeted to restore the impaired B-cell response to *S. pneumoniae* or pneumococcal vaccine; (4) Can this system be developed as a potential vaccine adjuvant? Answers to these questions will provide critical information for the development of a more efficacious vaccine for pneumococcal infections (309, 366).

Hence, in our laboratory, we are studying TLR-MyD88 signaling in the macrophages in order to elicit endogenous cytokine production to overcome this cytokine dysregulation as a means of enhancing robust B-cell activation, differentiation and anti-PS antibody responses (38, 63). We explore the TLR-cytokine mediated signaling pathways between the aged and the young as a means to identify specific steps in the pathways downstream of the TLR-MyD88 complex that are dysregulated with aging so that they can be pharmacologically exploited to enhance cytokine and anti-capsular antibody response (63). In the studies described in this thesis, we attempt to address these questions in the

context of in vitro cultures of SM and BMDM and splenic B cells from aged and young adult Balb/c and C57BL/6 mice. We ask if an age-associated increased in PI3K-Akt activity plays a role in the cytokine dysregulation in aged splenic and bone marrow macrophages. We wonder further if similar heightened PI3K-Akt activities in B cells explain the age-related B cell defects (267, 268). We also investigate the role of GSK-3, a downstream target of PI3K-Akt pathway in activating both B cells and macrophages.

Role of PI3K-AKT-GSK-3 signaling pathway in macrophage activation

PI3K enzymes form a group of lipid kinases consisting of Class I, II and III that are involved many fundamental physiological processes and also play a role in modulating the activities of phagocytic cells such as macrophages, neutrophils and monocytes that are critical for the clearance of *S. pneumoniae*. PI3K signaling is also important in B and T cell survival and activation. The Class IA PI3K consists of regulatory subunits (p85α, p85β, p55α, p55γ and p50α) that bind to one of three catalytic subunits (p110α, p110β and p110δ) upon activation via a tyrosine-kinase coupled receptor. The Class IB PI3K catalytic subunit lacks the p85 binding domain and is activated by G protein coupled receptors, (173, 223). The PIP3 generated by PI3K activity serves as a docking site for the recruitment of signaling proteins via their pleckstrin homology domains. One of the kinases that is recruited to the membrane by PIP3 is the phosphoinositide-dependent kinase-1 (PDK-1), which then targets the serine-threonine kinase, Akt, for phosphorylation at Thr-308 (361). The activated Akt serves as a master kinase for a plethora of downstream substrates including IKK and GSK-3 (173)(Figure 1.4). The levels of PIP3 can be down-modulated by PTEN (phosphatase and tensin homolog) and SHIP (Src-homology-2 containing inositol 5′-phosphatase), which are phosphatases that

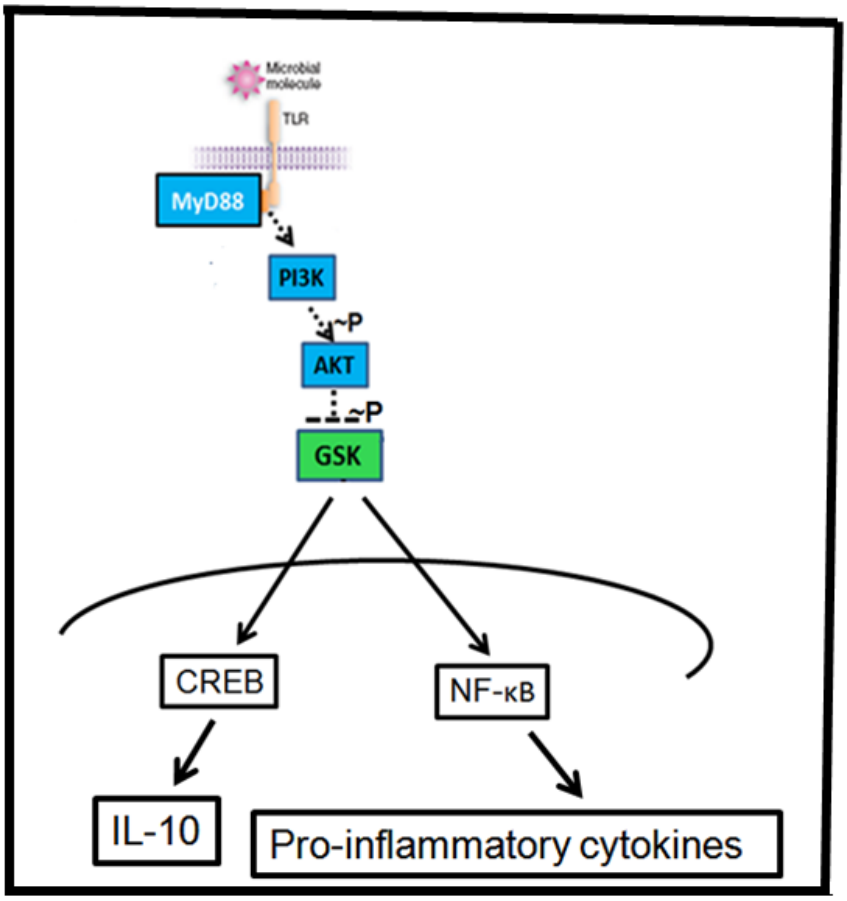
respectively target the D3 and D5 positions of PIP3 converting it to PIP2 (223). Recent pioneering studies show that PI3K signaling is a negative regulator of TLR-induced production of pro-inflammatory cytokines (107). Not only that, but the pharmacological inhibition of PI3K results in the suppression of IL-10 in macrophages and monocytes (108, 226, 308) (290, 308). Also, MyD88 directly interacts with the p85 subunit of PI3K (194). In phagocytes PI3K modulates chemotaxis, phagocytosis, upregulation of activation markers, growth, migration and differentiation (5, 131, 139). The PI3K-Akt signaling pathway differentially regulates pro- and anti-inflammatory cytokines in macrophages by inhibiting glycogen synthase kinase (GSK-3) (139, 225, 382).

GSK-3 is a direct downstream substrate of Akt and is phosphorylated on Serine-9 resulting in its inactivation (225, 382). GSK-3 appears to regulate both the pro- and the anti-inflammatory cytokines by controlling the competitive interaction of either CREB or p65 NF-κB with the CREB binding protein (CBP)(Figure 1.4). Moreover, inhibition of GSK-3 via either siRNA or pharmacological inhibitors results in a cytokine profile that is the reverse of the cytokine profile seen when PI3K is inhibited (225, 382, 393). The ability of GSK-3 to negatively modulate IL-10 allows the induction of IFN-gamma and the associated enhancement in antigen presentation, which has been shown to be critical for the control of mycobateria (60).

Figure 1.4. The PI3K-Akt-GSK-3 signaling pathway in Macrophages.

TLR mediated signaling in macrophages results in an interaction of MyD88 and PI3K resulting in the phosphorylation of Akt. Akt phosphorylates GSK-3 and the latter is inactivated. GSK3 regulates transcription factors like NF-κB and CREB. These transcription factors regulate the anti- and the pro-inflammatory cytokines.

Figure 1.4



(Modified from D.A. Fruman, Current Opinion in Immunology, 2004)(106)

Role of PI3K-AKT in regulating B-cell activation

Several previous studies emphasize the ubiquitous role of the Class IA PI3K signaling in regulating both the innate and the adaptive immune responses (188). The crucial role of PI3K in B-cell development, differentiation, activation, proliferation and survival is amply demonstrated using a combination of both pharmacological inhibitors and mutations that are specific for the PI3K pathway in B cells (268, 343). The class IA PI3K is downstream of signaling via B cell receptor (BCR), B-cell costimulatory molecules, toll like receptors and cytokine receptors. PI3K dependent phosphorylation of Akt is crucial for the regulation of B-cell proliferation and survival (81, 268, 333) (Figure 1.5). A B cell specific conditional knockout of PTEN, a negative regulator of PI3K-Akt signaling, results in enhanced activity of an Akt (343). The hyperactivity leads to a defect in expression of activation induced deaminase (AID), class switching and antibody responses to both TI and the TD antigens (343).

Activated Akt, phosphorylates and inactivates the forkhead transcription factors (Foxo). The suppression of the Foxo protein results in a down modulation of class-switch recombination. When PI3k is pharmacologically inhibited, using either a generic inhibitor like LY294002 or a Class I A (p110δ) specific inhibitor like IC7114, there is an enhancement in the expression of the enzyme AID and class switch recombination (267, 268). Overall, PI3K regulates B cell homeostasis in the periphery, by regulating the different types of B cell subsets, their survival and proliferation (22). On the basis of the foregoing, we attempt to first confirm in our hands that there is a defect in IgG3 in the aged splenic B cells compared to the young under in vitro conditions, and secondly to investigate if PI3K-Akt signaling pathway plays a causal role in this age-associated

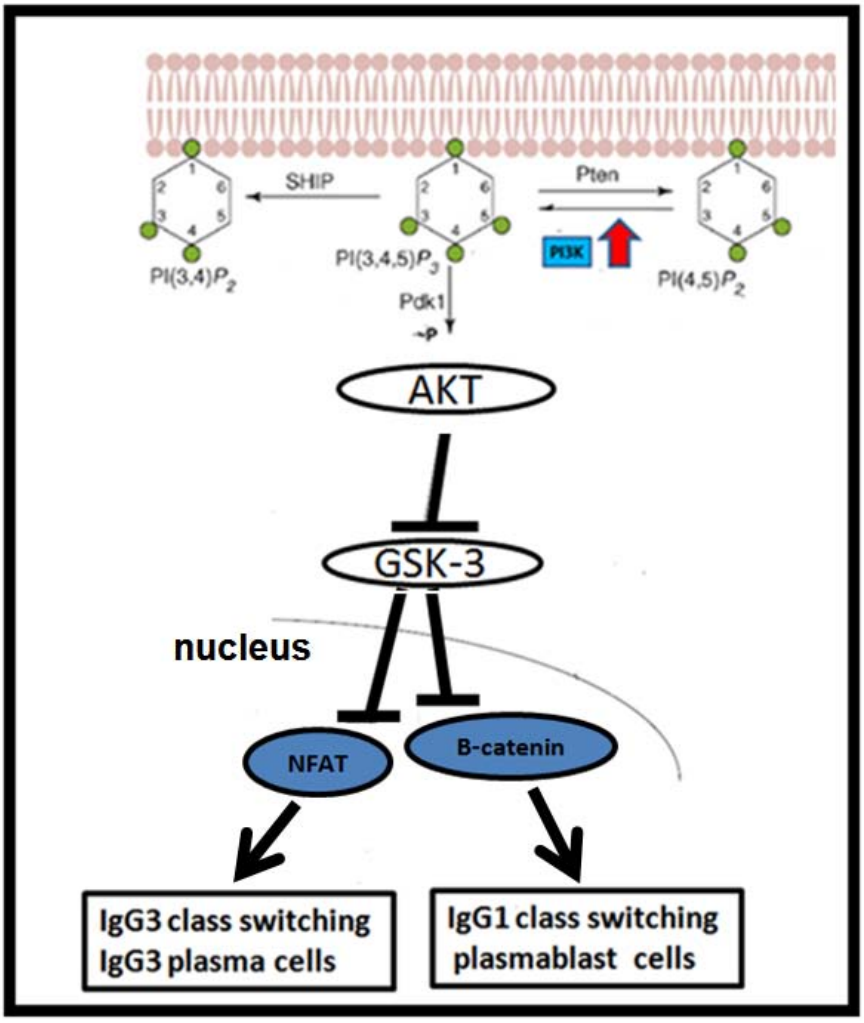
defect in IgG3 expression. IgG3 is crucial for the clearance of *S. pneumoniae* in mouse (227). We decided to use HKSP instead of live *S. pneumoniae* so that we could evaluate the cytokine regulatory effects of cell wall component of the bacteria without any lethal effects.

Role of GSK-3 in modulating B cell responses: GSK-3 is phosphorylated by a PI3K-Akt dependent signaling mechanism via BCR signaling. This phosphorylation inactivates GSK-3 and results in modulation of several of its downstream targets that are essential for B cell function. Two of these transcriptional targets of GSK-3 are NF-AT1c and beta-catenin (118) (Figure 1.5). NFAT1c is a positive regulator of Ig class switch to IgG3 upon immunization with Type-II TI antigens in splenic B cells. It is also a positive regulator of $IgG3^{+}$ plasmablast formation (29). Thus, by regulating NFAT1c, GSK-3 may play a critical role during B cell immune responses. Beta-catenin accumulates in the nucleus of B cells following BCR signaling (391). Studies in conditional knockout of β-catenin specific for B cells showed that it is dispensable in terms of B cell response to both TI and TD antigen challenge in vivo. Nevertheless, these mice show a significant reduction in class switching to IgG1 and plasma cell generation in vitro (391). Under normal unstimulated conditions, GSK-3 is active and thus phosphorylates and degrades β-catenin. However, following BCR stimulation, GSK-3 is inactivated upon phosphorylation which allows β-catenin to accumulate in the nucleus of the B cell and regulate the transcription of several genes that are needed for B cell development and function (29, 68, 158, 391). Since GSK-3 is downstream of Akt and Akt has a role in class switch recombination, we hypothesized that GSK-3 has a role in IgG responses to HKSP.

Figure 1.5. The PI3K-Akt-GSK-3 signaling pathway in B cells.

Signaling downstream of BCR, CD19 or TLR in B cells leads to the activation of PI3K which in turn leads to the phosphorylation of Akt. Akt phosphorylates and inactivates GSK-3 and thus indirectly controls its ability to regulate beta-catenin and NFATc1 that play a role in B cell mediated class switching and plasma cell formation.

Figure 1.5



(Modified from D.A. Fruman, Current Opinion in Immunology, 2004)(106)

In vitro mouse model system of macrophage-derived cytokines on B cell response

We used in vitro model systems to demonstrate that macrophages or macrophage derived cytokines enhance B cell response to the typical TI-2 antigen, TNP-Ficoll (63). Macrophage derived cytokines provide the critical second signal required for TI-2 antigen specific response. We are able to further elucidate that the defective B cell mediated response to either the polysaccharide vaccine or the synthetic TI-2 antigen in the elderly compared to the young adult can be attributed to a defect in macrophage-derived cytokines. We note an enhancement in the anti-inflammatory cytokine, IL-10, but a decrease in the pro-inflammatory cytokines, IL-6, IL-12, and IL-1 in aged macrophages triggered via TLR4. This age-associated cytokine profile is considered a "cytokine dysregulation".

To understand the molecular mechanism of this dysregulation in the aged SM; our laboratory performed a microarray analysis of genes expressed in young and aged macrophages activated via TLR-4. These studies showed that while the TLR expression is similar between the aged and young adult macrophages, there is a decrease in the MyD88 adaptor molecule, as well as several signaling molecules in the pathway that leads to activation of the transcription factor NF-κB (65). Also there is an enhancement in p38 MAP kinase, as well as PI3K subunits in the aged compared to the young. When p38 MAP kinase is pharmacologically inhibited the age-associated cytokine defect is partially overcome (65).

On the basis of the previous findings, our literature search and preliminary data, we advanced the following hypotheses:

(1) Cytokine responses of aged splenic macrophages to TLR2 and TLR9 are similar to those induced by TLR4 as they all share the MyD88 signaling pathway.

(2) An age-associated increase in the PI3K-Akt-GSK-3 signaling pathway has a role in the cytokine dysregulation in splenic macrophages and that inhibiting PI3K can reverse this dysregulation.

(3) The cytokine dysregulation defect may be intrinsic to macrophages from the aged which is tested using BMDM from aged. We further hypothesized that the PI3K pathway will be upregulated in BMDM with age.

(4) We hypothesized that GSK-3, a downstream target of PI3K pathway, will have a critical role in TLR responses of macrophages from the aged.

(5) The impairment in IgG_3 production in the aged splenic B cells compared to young B cells may be due to enhancement in the activity of PI3K.

(6) We hypothesized that inhibiting PI3K or GSK-3 in aged splenic B cells will increase IgG_3 production by enhancing the expression of AID.

CHAPTER TWO: METHODOLOGY

ANIMALS

Female young (8-12 weeks old) and aged (18-22 months old) Balb/c and C57BL/6 mice were obtained from the National Institute of Aging (NIA), National Institute of Health (NIH, Bethesda, MD). The mice were kept in the facility at the Department of Laboratory Animal Research (DLAR) at our university on a 12 h daylight and 12 h night cycle and given food and water ad libitum. The protocols involving the animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

REAGENTS

The TLR-4 agonist LPS (Escherichia coli 055:B5) and the TLR-2 ligands, Pam-3-CSK4 and the GSK-3 inhibitor, SB216763 were obtained from Sigma Chemical Co. (St. Louis, MO), whereas the TLR-2 ligands, Pam2CSK4 and lipoteichoic acid were obtained from In Vivogen (San Diego, CA). LY294002 and wortmannin, the inhibitors for PI3K and the GSK-3 inhibitor 216763 were obtained from Calbiochem (San Diego, CA). FITC-conjugated anti-mouse LY-6G and anti-CD5 antibodies were obtained from BioLegend (San Diego, CA), while the FITC-conjugated anti-mouse CD45R (B220), FITC-conjugated anti-mouse CD11b, PE-conjugated anti-F4/80, biotin-conjugated anti-IgG3, PE-conjugated anti-CD138 antibodies and Strepavidin-conjugated APC were obtained from eBioSciences (San Diego, CA). The antibodies to p-Akt (S473), Akt, p-GSK-3αβ and GSK-3, were from Cell Signaling Technologies; to anti-PTEN and anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The immunoreactive proteins were

detected using horseradish peroxidase coupled secondary antibodies and the PICO Chemiluminescence substrate (Pierce Technology, Rockford, IL). The cytokine OptEIA ELISA kits for IL-10, IL-12(p40), IL-12(p70), IL-6 and TNF-α were all from BD Biosciences, San Diego, CA. The anti-FITC and the anti-B220 antibody coupled microbeads were obtained from Miltenyi Biotec (Auburn, CA). The OptEIA TMB peroxidase substrate for the ELISA was acquired from BD Biosciences (San Diego, CA). We used sterile RPMI 1640 (BioWhittaker) medium which was made complete by supplementing with 25 mM HEPES , 2 mM glutamine (Invitrogen Gibco), and 50 µg/ml gentamicin (Sigma-Aldrich) and 10% Fetal bovine serum (FBS), (Atlanta Biologicals, Lawrenceville, GA). The IgG3 or IgA ELISA kits were from Bethyl Laboratories, (Montgomery, TX), while the IgM, IgG2a, IgG2b or IgG1 ELISA kits were from Southern Biotec (Birmingham, AL).

HEAT KILLED STREPTOCOCCUS PNEUMONIAE (HKSP)

Serotype-2 S. *pneumoniae* bacteria were obtained from Dr. Beth Garvy and plated on BBL pre-made blood agar plates (VWR International). Isolated colonies were grown in Todd Hewitt broth (BD Biosciences) to mid-log phase and collected for counting. Sterility was confirmed by subculture on blood agar plates. After extensive washings the bacterial suspension was adjusted with PBS to give an absorbance reading at 630 nm of 0.59, which corresponded to 1.2 $x10^8$ CFU/ml using a turbidity curve. The bacteria were then heat killed by incubation at 60°C for 1 h or fixed in 10% formalin. Bacteria were then aliquoted at 3.3 $x10^9$ CFU/ml and frozen at –80°C until their use as Ag for macrophage stimulation.

L929 SUPERNATANT

The L929 fibroblast cell line was obtained from Dr. Don Cohen, University of Kentucky, and cultured in 75 mm Flask in 25ml at $1x10^6$ cells/ml of RPMI complete media for 7 days. At the end of the seventh day, the supernatant containing M-CSF was aspirated, spun down and sterile filtered. Aliquots were made and stored at -80^0 C for future use in the generation of BMDM.

CELL PREPARATION:

Splenic Macrophages (SM): Mice were euthanized via carbon dioxide asphyxiation and the spleens aseptically removed and crushed into a single cell suspension. After washing and erythrocyte lysis with lysing buffer (Sigma, Aldirch, St. Louis, MO) at 2ml/spleen for three minutes at room temperature, the FcγR were blocked with normal rat IgG for 15 minutes. The splenocytes were then incubated with a cocktail of FITC conjugated monoclonal antibodies against B220, CD5 and Ly6G in the dark for 30 minutes. This was followed by washing with MACS buffer and incubation with anti-FITC microbeads at 4-8^o C for 15 minutes. The purified macrophages were obtained by separating the magnetic bead bound and unbound cells using the Miltenyi AUTOMACS Cell Separator using the program Deplete-05. The resulting untouched macrophages were found to be 90-95% F4-80 positive cells (Appendix A). The purified macrophages were cultured at $2.5x10^5$ cells/ml in 48 well plates (Costar, Corning, NY) and in RPMI fetal calf serum (FCS) at 37^0C in a humidified 5% CO_2 atmosphere.

Bone marrow derived macrophages (BMDM): Mice were euthanized via carbon dioxide asphyxiation and bone marrow derived cells were isolated from young (8-12 weeks) and aged (18-24 months) Balb/c mice by flushing the bone marrow from the femur and tibia

with HBSS media. After washing and erythrocyte lysis with red blood cell lysing buffer at 2ml/20x10^6 cells, the bone marrow cells were cultured at 2 x10^6 cells/ml in 24 well plates. The cells were cultured in RPMI complete media supplemented with 30% L929 cell supernatant containing M-CSF. On days 4 and 6, the media was completely removed and new RPMI complete plus 30% L929 supernatant was added. On day seven the newly derived BMDM were isolated by removing the entire old media and adding 2ml of trypsin-versene (Lonza Biosceince, Walkersville, MD) per well in a six-well plate and incubating the culture for 30 minutes at 37^0 C. This was followed by adding RPMI complete to reduce the toxicity of the trypsin. The cells were then washed twice, resuspended, counted and used for different experimental conditions. The resulting BMDM were found to be 96% F4-80 and CD11b positive cells. The purified BMDM were cultured in RPMI complete at 37^0C in a humidified 5% CO_2 atmosphere at 2 x10^5 cells/ml/well. Bone marrow derived macrophages (BMDC) were similarly generated except that GM-CSF was used with IL-4 and trysin was not used. The cells were harvested on day 8 which expressed CD11c and other phenotypes of BMDC.

Splenic B cells: Mice were euthanized via carbon dioxide asphyxiation and the spleens aseptically removed and crushed into a single cell suspension. After washing and erythrocyte lysis, the FcγR were blocked with normal rat IgG for 15 minutes. The splenocytes were then incubated with anti-B220 microbeads at 4-8^o C for 15 minutes. The purified splenic B cells were obtained by separating the magnetic bead bound and unbound cells using the Miltenyi AUTOMACS Cell Separator using the program Possel-1. The resulting B cells were found to be 97% B220 positive cells. B220-microbead purified splenic B cells were cultured in RPMI complete medium + 10% fetal calf serum

(FCS) at 37^0C in a humidified 5% CO_2 atmosphere at 2 x 10^6 cells in 2 ml of complete medium and stimulated in the 24 well plates with LPS (10µg/ml), CpG (5µg/ml), Pam3CSK4 (5µg/ml) and HKSP (2 x 10^8 CFU/ml). The stimulation was done with the various ligands alone or together with either the PI3K inhibitors, Wortmannin or LY294002 at various concentration or with the GSK-3 inhibitor SB216763 at different concentrations for 72 h. Optimal culture conditions were determined in preliminary experiments. The supernatants were assayed for IgG3 or IgA as well as IgM, IgG2a, IgG2b or IgG1 by ELISA (Southern Biotech, Birmingham, AL).

FLOW CYTOMETRIC ANALYSES

Control and LPS (1µg/ml) or Pam3CSK4 (1µg/ml), Pam2CSK4 (1µg/ml) as well as LTA(1µg/ml) stimulated splenic macrophages were cultured for 24 hours and gently removed by scraping them off the plate. The cells were over 80% viable. These macrophages from the aged and the young mice were stained for surface expression of CD86 and F4/80 using directly conjugated flourochrome antibodies (BD Pharmingen) for 30 minutes on ice. Newly generated BMDM were stained with anti-CD11b-FITC, anti-F4/80-PE or anti-TLR-2-biotin and then PE-strepavidin. Also the BMDM were either left untreated or stimulated with LPS(1µg/ml), CpG(1µg/ml), P3CSK4(1µg/ml), or HKSP and cultured for 24 hours and gently removed by scraping them off the plate. These macrophages from the aged and the young mice were stained with FITC-anti CD86 or FITC anti-I-E^k class-II (BD Pharmingen) for 30 minutes on ice. B cells from the 72 hour cultures were collected, washed and resuspended in FACS buffer. They were blocked with normal rat IgG to prevent non-specific antigen binding and then biotin-conjugated anti-IgG3 and APC-anti-B220 were added and incubated for 30 minutes in the dark. The

cells were then washed two times and were incubated in dark for 15 minutes with PE-streptavidin. The cells were stained with B220-PE-Cy5, FITC-anti-IgA, FITC-anti-IgG1, FITC-anti-IgG2b, APC-anti-IgM, APC-anti-B220 and PE-anti-CD138. The cells were washed once and analyzed on a flow cytometer.

CYTOKINE ELISA

Cell free supernatants from either basal or activated purified splenic or bone marrow macrophages ($2.5x10^5$ cells/ml) were harvested following culture with or without TLR ligands or HKSP for 24 hours from predetermined optimal dose concentrations. The supernatants were assayed for cytokines, IL-10, IL-12(p40), IL-12(p70), IL-6 and TNF-α, by ELISA using OptEIA kits. The dilution of the capture antibody, the biotin-conjugated detection antibody and the streptavidin-conjugated horseradish peroxidase (HRP) for IL-10, IL-12 (p70), IL-12(p40) and TNF-α was 1:250; while 1: 1000 dilution of the detection antibody for IL-12 (p40), t 1:250. For the IL-6, the capture antibody was diluted at 1:500, the biotin-conjugated detection antibody was diluted at 1:1000 and the strepavidin-conjugated HRP was diluted at 1:2000. All dilutions were done in assay diluents, made from 1X PBS with 10% fetal bovine serum (FBS). The optical densities (OD) were read on an HTS 7000 plate reader (Perkin Elmer, Norwalk, CT) using the 450 and 560nM wavelength. The results were expressed as mean +/-SE of triplicate determinations of supernatants from duplicate cultures.

Ig ELISA

IgG3, IgA, IgM, IgG2a, IgG2b or IgG1 from the 72 hour supernatants were assayed in accordance with a sandwich protocol. Briefly, 96-well flat bottom high binding

polystyrene EIA/RIA microtiter plates (Corning Inc., Corning, NY) were coated with 100 µl of a purified goat anti-mouse capture Ab at a concentration of 1 µg/100 µl coating buffer and incubated at 1 hour at room temperature for the IgG3 and the IgA ELISA. For the IgM, IgG2a, IgG2b or IgG1 ELISA, the coating was done overnight with the goat anti-mouse Ig at 1:2000 dilution in coating buffer (Southern Biotec, #1100-01) in 1x PBS at 4^0 C. Reactions were blocked by adding 200 µl of 1x PBS containing 10% BSA (blocking buffer) to each well for 30 min at room temperature. Wells were then washed three times with 1x PBS containing 0.05% Tween 20 (washing buffer). Serial dilutions of the culture supernatants or of purified mouse IgG3 or IgA, IgM, IgG2a, IgG2b or IgG1, at a concentration of 0.5 µg/ml in blocking buffer, were added to each well (100 µl/well) and incubated for 2 hours at room temperature. Wells were washed thoroughly three times with washing buffer before receiving (100 µl/well) the detecting Ab conjugated with HRP, (Bethyl Laboratories), for IgG3 and IgA, and with alkaline phosphatase (AP) conjugated for IgM, IgG2a, IgG2b or IgG1, (Southern Biotec) at a concentration of 1:10,000 to 1:30,000. After 1 h incubation at room temperature, wells were washed five times and 100µl of TMB substrate for the IgA and IgG3 and PNPP dissolved in phenlylamin buffer (Diethanolamine plusMgCl2, PH9.8) at 1mg of mg/ml for the IgM, IgG2a, IgG2b or IgG1 was added. The TMB substrate was stopped with 2M H_2SO_4. Wells containing the TMB substrate was measured for absorbance at 450 nm, while the PNPP were measured at 405 nm.

WESTERN BLOT

The SM or BMDM from aged and young adult Balb/c mice were plated at 2 x 10^6 cells/well in 500 µl of RPMI 1640 + 2% FCS in 24 well tissue culture plates. These cells

are allowed to rest for 4 hours to allow macrophage adherence and recovery from handling and then stimulated with 1μg/ml LPS or 1μg/ml Pam-3-CSK4, for 15 or 30 minutes. These time points and doses were chosen after performing a detailed kinetic analysis. For inhibition of PI3 kinase pathway, macrophages were pretreated with wortmannin or LY294002. The reaction was stopped by adding 1.0 ml of ice cold 1x phosphate buffered saline (PBS). The cells were washed twice with 1.0 ml 1x PBS. The macrophages were then lysed in a lysis buffer at 100μL/5x10^6 cells (Cell Signaling Technologies, Beverly, MA), to which was added protease inhibitor cocktail (10x), (containing leupetin, EDTA, pepstatin), and phenyl methyl sulfonyl fluoride (PMSF) (1mM) (Sigma Chemical Co. St. Louis, MO). Nuclear and cytosolic fractions from the aged and young BMDM were obtained using the NE-PER nuclear and cytoplasmic extraction kit from Thermo Scientific (Rockford, IL) in accordance with the manufacturer's instructions. The total protein content of the sample was determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels 10% (SDS-PAGE) and were transferred to nitrocellulose membranes for Western blot analyses. The membranes were blocked with 2% milk and were then probed with antibodies to: p-Akt (S473), Akt, p-GSK-3αβ and GSK-3, all of which were from Cell Signaling Technologies; or to PTEN and β-actin from Santa Cruz Biotechnology (Santa Cruz, CA). The immunoreactive proteins were then detected using horseradish peroxidase coupled secondary antibodies and the PICO Chemiluminescence substrate (Pierce Technology, Rockford, IL) and by exposing to Kodak X-omat film. The relative ODs of the protein bands were estimated using the Kodak Image Station software (Eastman Kodak, New Haven, CT). Band

intensities were determined by dividing the phosphorylated protein by the total protein. The blots were stripped with stripping buffer (62.5mM Tris, PH6.8, 2%SDS, 0.1M 2-mercoptoethanol) for 25 minutes at 50^0 C and then probed with anti-beta actin (Sigma, St. Louis, MO) to correct for differences in protein loading. This was done by comparing the protein levels of actin to the various proteins of interest.

REAL-TIME RT-PCR

Purified SM or BMDM ($3x10^6$ cells/3ml) were stimulated with 1 μg/ml LPS for 30 minutes and RNA extracted by TriZol extraction kit and then reverse transcribed to cDNA using the High Capacity cDNA Archive kit (Applied biosystems, Foster, CA). The cDNA was later amplified by real-time PCR in a 25μL reaction volume containing SYBR Green (QIAGEN, Valencia, CA) and analyzed using an ABI Prism Sequence detection system (Applied Biosystems, Foster City, CA). The experimental cDNA was tested in triplicate and normalized to 18S RNA. The RNA levels of p85, p110 and 18S were measured with QuantiTect PCR probes obtained from QIAGEN (Alameda, CA).

LIGHT MICRSCOPY

Newly generated BMDM were evaluated under the Olympus light microscope at a total magnification of 40X and snap shots taken.

STATISTICAL ANALYSIS

Statistical differences between groups were evaluated using an unpaired Student‘s t test, or one way ANOVA. In both tests statistical significance was concluded when $p<0.05$.

CHAPTER 3: **Role of phosphoinositide 3-kinase – Akt signaling pathway in the age-related cytokine dysregulation in splenic macrophages stimulated via TLR2 or TLR 4 receptors**

Introduction

Numerous studies have established the senescence of the immune system in aged human and murine populations resulting in reduced effectiveness of both the innate and the acquired arms of the immune system of the elderly. As a result, older individuals are more susceptible to infections, particularly with Gram positive bacteria such as *S. pneumoniae,* and consistently demonstrate an impaired immune response to pneumococcal polysaccharide vaccine (159, 217, 218, 302). Secreted cytokines like IL-12 and IL-6 help B-cells to produce increased IgG_3 or IgA in the absence of help from T-cells (17, 38, 64, 178, 230). Both IgA and IgG_3 promote opsonization of the bacteria. TNF-α is another pro-inflammatory cytokine that is produced by *S. pneumoniae* activated macrophages and also aids in the recruitment of neutrophils and macrophages, which phagocytose the opsonized bacteria (176, 201).

We have previously shown that upon stimulation with LPS, a TLR-4 ligand, aged SM secrete lower levels of the pro-inflammatory cytokines, IL-6, IL-12, and TNF-α, but higher levels of IL-10, resulting in cytokine dysregulation (62, 63, 65). Similar defects in TLR-induced pro-inflammatory cytokine secretion by aged splenic macrophages are also seen by other investigators (34, 298). As a result, TLR-4 activated aged macrophages or their secreted pro-inflammatory cytokines are not able to effectively provide support to B-cell mediated anti-capsular polysaccharide antibody responses (38, 114). Here we investigated whether or not similar cytokine dysregulation is exhibited when aged macropahges are stimulated with different types of TLR-2 ligands

that are relevant in the context of pneumococcal infections. Unlike other TLRs that form homodimers, the TLR-2 receptor complex is a heterodimer which gives it the ability to recognize a broader spectrum of ligands. Gram positive bacteria contain lipoproteins that are known to be recognized by TLR-2 resulting in the initiation of inflammation and control of bacterial infection (311). We also investigated this phenomenon in the context of heat killed *S. pneumoniae* (HKSP).

The lipoproteins or lipopeptides derived from them are differentially recognized by either the TLR2/6 heterodimer or the TLR2/1 heterodimer depending on whether the cysteine residue is diacylated or triacylated. TLR2/1 recognizes a variety of bacterial lipopeptide/lipoproteins including mycobacterial lipoprotein, meningococcal lipoprotein and synthetic Pam3CSK4, while TLR2/6 recognizes mycoplasma lipoprotein, peptidoglycan and the synthetic Pam2CSK4 (349, 378). However, some studies show that TLR-2 can recognize some bacterial ligands, such as lipoteichoic acids (LTA) independent of TLR-1 or 6 (52). LTAs are more restricted to Gram positive bacteria than synthetic Pam2CSK4 and Pam3CSK4 like lipopeptides that can be found in both Gram positive and Gram negative bacteria (130, 318). LTA is a potent inducer of inflammatory cytokines in macrophages and human PBMC and is important for innate responses to Gram positive bacteria (97, 318, 378).

In spite of the critical role of phosphatidyl inositol-3 kinase (PI3K) in modulating pro-inflammatory and anti-inflammatory cytokines in macrophages and dendritic cells (DC), little research has been done to investigate age-related changes in this important signaling molecule and its potential therapeutic value (173, 223). PI3K is a lipid kinase that phosphorylates the inositide ring of PIP2 at the D3 position to generate PIP3. PIP3

generated by PI3K activity serves as a docking site for the recruitment of signaling proteins via their pleckstrin homology domains. One of the kinases that is recruited to the membrane by PIP3 is the phosphoinositide-dependent kinase-1 (PDK-1), which then targets the serine-threonine kinase, Akt for phosphorylation at Thr-308. The activated Akt serves as a master kinase for many downstream substrates, including IKK and GSK-3 (173). The levels of PIP3 can be down-modulated by PTEN (phosphatase and tensin homolog) and SHIP (Src-homology-2 containing inositol 5′-phosphatase), which are phosphatases that respectively target the D3 and D5 position of PIP3 converting it to PIP2 (223).

Recent studies show that PI3K signaling also plays a role in most TLR signaling as inhibition of PI3K with pharmacological inhibitors results in differential regulation of anti- and pro-inflammatory cytokines (108, 226, 308). In addition, it has been shown that the Class IA PI3K are activated after stimulation via diverse TLRs such as TLR2, TLR3,TLR4, TLR5, TLR9 and also the IL-1R signaling pathway (108). However, the connection between the TLR signaling and PI3K has recently been elucidated in gene knockout studies (215). These resuts show that MyD88 directly interacts with the p85 subunit of PI3K (194). The PI3K pathway is known to negatively regulate the TLR induced production of pro-inflammatory cytokines (108, 226, 284, 308). Moreover, there is some evidence that this pathway is required for IL-10 production (290, 308). Hence, we questioned if PI3K levels are elevated with aging, and if inhibiting PI3K in aged SM restores the production of pro-inflammatory cytokines upon stimulation with either TLR ligands or HKSP. We found that there is an age-associated increase in the amount of PI3K subunit specific mRNA and its activity as shown by increased phosphorylation of

AKT and GSK-3. Inhibition of the PI3K-AKT signaling pathway enhances TLR2 and HKSP induced pro-inflammatory cytokines and decreases the anti-inflammatory cytokine levels in both the aged and young adult macrophages.

RESULTS

Age-associated cytokine dysregulation correlates with an enhancement of Class IA PI3K activity in TLR-4 stimulated macrophages.

We have previously shown that LPS activated aged splenic macrophages exhibited a cytokine dysregulation with a decrease in the pro-inflammatory cytokines but an increase in IL-10, an anti-inflammatory cytokine (63). In these experiments macrophages were purified by adherence or by positive selection with anti-CD11b beads. In order to establish the role of PI3 kinase in this cytokine dysregulation and to avoid the inadvertent activation of splenic macrophages by either adherence or positive selection with CD11b magnetic beads, we purified splenic macrophages using negative selection procedures. This method involved staining splenocytes with anti-CD5-FITC, anti-B220-FITC and anti-LY6G-FITC and using anti-FITC coupled magnetic beads to remove non-macrophages. When these negatively selected macrophages from aged mice were stimulated with LPS they showed an increase in IL-10 secretion (Figure 3.1A) and a decrease in production of pro-inflammatory cytokines, IL-12(p40), IL-6 and TNF-α (Figure 3.1B) compared to similar cells from young adult mice. Thus, these negatively selected SM continued to exhibit the same cytokine dysregulation that was observed with macrophages purified by other methods.

We questioned if the observed dysregulation of cytokine secretion by the TLR-4 activated SM was due to the differential expression of PI3 kinase Class I isoform of PI3K expressed in leukocytes, B-cells, T-cells, macrophages, and consistently shown to be a negative regulator of pro-inflammatory cytokines upon TLR stimulation (139, 188, 194,

219, 308). Hence, we examined our previously published microarray data (65) to determine if there were differences in the expression of the regulatory and the catalytic subunits (p110) of Class I PI3K between the young and the aged. The results indicated that there was a statistically significant increase in the expression of p110δ (Figure 3.1C) and p85β (Figure 3.1D) in aged compared to young adult murine SM, stimulated with LPS for six hours. These findings were confirmed by a quantitative reverse transcriptase real time polymerase chain reaction (qRT-PCR) which showed an increase in both p110δ (Figure 3.1E) and p85β (Figure 3.1F) in aged compared to young adult macrophages. The increased magnitude of differences between young and aged in qRT-PCR versus microarray data could be due to the differences in macrophage isolation between the two studies (positive versus negative selection) and the time of stimulation (30 minutes versus 6 hours). Therefore, we postulated that the age-associated decrease in pro-inflammatory cytokines and increase in anti-inflammatory cytokines was due to increased activity of PI3K.

Age-associated increase in the activity of PI3K as shown by changes in phosphorylation of Akt.

It has been reported that the phosphorylation of Akt was an indirect way to determine the activity of PI3K (135, 308, 346) since PI3K activation resulted in phosphorylation of Threonine-308 and serine-473 on Akt in a temporal manner leading to an increase in its activity (225, 226, 284). Therefore, levels of p-Akt were measured in young and aged SM, as a surrogate measure of PI3K activity. Western blot analysis showed that the phosphorylation of Akt was higher in aged compared to young adult SM stimulated with LPS. The age-associated increase in p-Akt could be seen as early as 15 minutes of

stimulation with LPS and was 2.4 times more in aged than the young adult (Figure 3.2A). The total levels of AKT (normalized to actin) were comparable in the elderly and the young mouse macrophages.

GSK-3 is a direct downstream substrate of Akt and has been shown to be mainly involved in the differential regulation of the anti- and pro-inflammatory cytokines. GSK-3 was phosphorylated on Serine-9 resulting in its inactivation (225, 382). An age-associated increase in the phosphorylation of GSK-3 would provide additional support to our hypothesis that the activity of PI3K/Akt pathway was increased in aged splenic macrophages. We employed western blot analysis to determine if the phosphorylation status of GSK-3 between the aged and the young correlated with the phosphorylation status of Akt. The phospho- GSK-3αβ subunits were normalized to the total GSK-3β subunit. GSK-3 phosphorylation was 3-fold more in the aged than the young by 15 minutes after stimulation with LPS, the TLR-4 ligand (Figures 3.2B). Total GSK-3β levels (normalized to actin) were similar between the young and the aged.

A defect in PTEN can lead to increased levels of PIP3 which in turn can result in increased PI3 Kinase activity (51). Tachado et al. have shown that in asymptomatic HIV positive patients TNF-α production by TLR-4 stimulated alveolar macrophages was suppressed due to a defect in PTEN expression leading to increased PI3K activity (346). Hence, we decided to quantify age-associated differences in total PTEN between the aged and the young adult splenic macrophages by Western blot. Surprisingly, there were no statistically significant differences in the total PTEN at either the basal level or upon activation with LPS (Figure 3.3A). We repeated this experiment using Pam2CSK4 as the ligand for TLR-2. Again, there were no differences in both the basal level or upon

stimulation with the synthetic TLR2/6 ligand (Figure 3.3B). Thus, there was an increase in the activity of PI3K, as demonstrated by increased phosphorylation of AKT and GSK-3, in aged macrophages stimulated with TLR-4 ligand that is independent of the activity of PTEN.

Cytokine dysregulation in aged macrophages stimulated via TLR-2 and HKSP

TLR2 was required for protection against *S. pneumoniae* infection (86, 222). Therefore, we tested if age associated cytokine dysregulation could be shown with Pam3Csk4, a synthetic ligand for TLR2/TLR1 heterodimer. It was observed that the aged splenic macrophages stimulated with Pam-3-CSK4 produced higher levels of IL-10 compared to young adult macrophages (Figure 3.4A). However, there was a decrease in pro-inflammatory cytokines, such as IL-12 (Figure 3.4B) and IL-6 (Figure 3.4C).

Since, there was an increased incidence of pneumococcal infections in the elderly, we inquired if this may be partly due to a similar dysregulation in the cytokine response of macrophages to stimulation with *S. pneumoniae*. To address this question, we stimulated aged and young adult purified SM with inactivated *S. pneumoniae* and assayed the cytokine response. The results demonstrated that there was an age-associated defect in TNF-α and IL-12(p40) production in the aged compared to the young adult macrophages (Figure 3.4D). Thus, age-associated cytokine dysregulation demonstrated with LPS can also be seen in both TLR-2 and HKSP activated SM.

Cytokine dysregulation in aged macrophages is seen with ligands that stimulate TLR-2 receptors

The lipoproteins or lipopeptides derived from bacteria are differentially recognized by either the TLR2/6 heterodimer or the TLR2/1 heterodimer depending on whether the cysteine residue is diacylated or triacylated. TLR2/1 ligands are typified by synthetic Pam3CSK4 while the natural ligands include mycobacterial lipoprotein and meningococcal lipoprotein. TLR2/6 recognizes the synthetic ligand Pam2CSK4, as well as mycoplasma lipoprotein and peptidoglycan (349, 378). However, some studies show that TLR-2 recognized bacterial ligands such as lipoteichoic acid (LTA) independent of TLR-1 or 6 (52, 275, 314, 390). Having shown that aged SM have a defect in their ability to secrete cytokines upon stimulation with Pam3CSK4, we wondered if their responses to Pam2CSK4 and/or LTA were preserved to make them better candidates for adjuvants for the aged. Such an analysis wass important since some studies found that different TLR2 ligands used distinct signaling pathways and had different in vivo effects while others found the two ligands use similar signaling pathways (168).

We stimulated SM from young and aged with the TLR2/6 ligand, Pam2CSK4 for 24 hours and assayed for IL-10 (Figures 3.5A) and IL-12(p40) (Figures 3.5B). As shown in Figure 3.5A, the aged SM produced higher IL-10 than the young adult SM (almost undetectable) when stimulated with Pam2CSK4, but lower levels of IL-12 (p40) than the young (Figure 3.5B). LTA is generally recognized by a TLR2/6 heterodimer (150) in macrophages similar to Pam2CSK4. Accordingly, we demonstrated that LTA was able to induce higher levels of IL-10 (Figure 3.5C) but lower levels of IL-12(p40) (Figure 3.5C) in the SM from aged than young mice. Since TLR4 stimulation induced increased

activation of the PI3-Akt-GSK pathway in aged versus the young macrophages, we tested if stimulation with the various TLR2 ligands also resulted in greater activation of this pathway in the aged. Western blot analysis of lysates from aged and young SM stimulated with Pam2CSK4 showed that there was a 2.7 and 3.5 fold-increase in p-AKT in the aged versus the young adult macrophages stimulated for 15 and 30 minutes respectively (Figure 3.6A). We also investigated the effect of age-associated increase in the phosphorylation of AKT on phosphorylation of its downstream target, GSK-3, in the context of TLR2/6 (Pam2CSK4). The Western blot analysis showed that the levels of P-GSK-3 were 3.3 and 10 fold more in the aged than in the young SM (Figure 3.6B) at zero and 15 minutes of Pam2CSK4 stimulation respectively. Similarly stimulation of macrophages with LTA also demonstrated increased levels of P-GSK-3 in the aged compared to the young (Figure 3.6C). Thus PI3K pathway activity was increased in the aged SM upon stimulation with both TLR4 and TLR2 ligands.

PI3K inhibitors partially rescue the age-associated defect in TLR-4 induced cytokine production.

We wondered if the increase in the activity of PI3K had a role in the decreased production of the pro-inflammatory cytokines and increased IL-10 secretion in the aged SM (284, 308). Hence, SM from aged and young adults were treated with either LY294002 or wortmannin, two well characterized PI3K inhibitors, and then stimulated with LPS, the TLR-4 ligand to initiate cytokine production. At 2 and 5 μM of LY294002 there was a reduction in the levels of IL-10 in both aged and young SM stimulated with LPS (Figure 3.7A). At 50 nM, wortmannin had a more profound inhibitory effect on IL-10 production by aged compared to young macrophages (Figure 3.7A). As predicted,

treatment with LY294002 resulted in the enhanced production of IL-12(p40) in aged SM, but not in the young adult SM (Figure 3.7B). The lack of enhancement in the young SM was surprising but was reproducible. However, when wortmannin was used as an inhibitor, there was a small but significant increase in IL-12(p40) in both age groups at 5 nM and 50 nM (Figure 3.7B) but the magnitude of increase in IL-12 with wortmannin was smaller in the young than in the aged. Thus, cytokine dysregulation in the aged appears to be linked to a defect in the PI3K-Akt pathway, and this can be partially rescued by inhibiting PI3K with either LY294002 or wortmannin.

To be certain that the PI3K specific inhibitors were indeed blocking the PI3K/Akt pathway, Akt activation was evaluated by quantifying p-Akt levels in young macrophages treated with wortmannin or LY294002. LPS stimulation increased p-Akt levels compared to unstimulated cells. Inhibition of PI3K with wortmannin in the presence of LPS resulted in 1.6 fold reduction in the level of p-Akt compared to cells activated with LPS in the absence of the inhibitor at the 30 minute time point (Figure3.7C; Lanes 3 and 4). Similarly with LY294002, another PI3K inhibitor, there was a reduction in LPS induced activation of Akt by 3.7 fold at the 30 minute time point (Figure3.7C; Lanes 3 and 5). To demonstrate that phosphorylation of GSK-3 in SM was indeed dependent on the PI3K/Akt pathway, we determined if inhibition of PI3K with either LY294002 or wortmannin affected p-GSK-3 levels (225). As shown in Figure 3.7D stimulation of macrophages with LPS resulted in an increase in GSK-3 phosphorylation at 15 and 30 minutes of stimulation. Treatment with wortmannin or by LY294002 decreased GSK-3 phosphorylation by 2.1 and 2.8 fold compared to untreated group after 30 minutes of LPS stimulation. Thus, activation of PI3K with TLR-4 ligand resulted in

an increase in phosphorylation of Akt and GSK-3, which was reduced significantly by inhibition of PI3K.

The ability of LY294002 or wortmannin to modulate the age-associated defect in PI3K signaling on cytokine production can also be shown with TLR-2 ligands

We decided to investigate the possibility that decreased secretion of pro-inflammatory cytokines in response to TLR2 ligands might also be rescued by PI3K inhibitors because TLR2 signaling used the same MyD88 adaptor as the TLR4 receptors (86, 134, 332). Purified SM from aged and young adult were pre-treated with LY294002 or wortmannin and then stimulated with Pam-2-CSK4 (1μg/ml) or LTA. Treatment with the LY294002 reduced IL-10 secretion in the presence of both Pam2CSK4 and LTA (Figure 3.8A) and increased IL-12(p40) secretion when either Pam2CSK4 (Figure 3.8B) or LTA (Figure 3.8C) was used as the TLR-2 agonist in the aged SM. Interestingly, the PI3K inhibitor induced very little or no increase in cytokine secretion by young macrophages. Similar results were obtained when Pam-3CSK4 was used to stimulate macrophages in the presence of the PI3K inhibitor. Thus IL-10 was reduced in both aged and young splenic macrophages when PI3K was inhibited, (Figure 3.9A). Moreover, treatment with LY294002 resulted in an increase in IL-6 (Figure 3.9B) and IL-12(p40) (Figure 3.9C) in aged splenic macrophages, an effect that was not seen in the young adult (Figures 3.9 B and 3C).

Thus, PI3K inhibition resulted in reduced secretion of IL-10 but increased pro-inflammatory cytokines in the aged SM when any of the TLR-2 ligands were used to stimulate the macrophages. Moreover, PI3K inhibition regulated cytokine secretion more in the aged than in the young adult.

PI3K inhibition enhanced the HKSP mediated induction of pro-inflammatory cytokines in aged SM

As shown in Figure 3.4D, HKSP induced proinflammatory cytokines were decreased in aged SM. HKSP had been shown to interact with splenic macrophages via TLR-2, 4 and 9 and to induce the secretion of both pro- and anti-inflammatory cytokines (85, 86, 201). Therefore, we tested if the impairment in HKSP-induced production of pro-inflammatory cytokines was also dependent on heightened PI3K activity in aged SM, using LY294002 and wortmannin. Stimulating aged SM with HKSP in the presence of LY294002 resulted in enhanced HKSP induced secretion of IL-6 (Figure 3.10A). Similarly, treatment with wortmannin also enhanced secretion of both IL-6 (Figure 3.10B) and IL-12(p40) (Figure 3.10D) by HKSP stimulated aged macrophages. IL-12(p70) was measured (Figure 3.10C) since the p40 subunit was also shared with IL-23. HKSP alone did not induce much p70 but treatment with wortmannin dramatically enhanced p70 levels. Similarly, induction of IL-12 (p70) by Pam-3-CSK4 was also enhanced by PI3K inhibition (data not shown).

Age-associated cytokine dysregulation involving TLR-2 and HKSP is due to a defect in the Akt-GSK-3 signaling axis

Here we asked if the ability of the PI3K inhibitors to enhance TLR2 induced secretion of pro-inflammatory cytokines was associated with a reduction in the activation of p-Akt and p-GSK-3αβ, two downstream targets of the PI3K pathway. Inhibition of PI3K with 20μM of LY294002 in the presence of Pam3CSK4 (1μg/ml) resulted in 40%

reduction in P-Akt compared to P3C activation without the inhibitor (Figure 3.11A: lanes 2 and 3) when the densitometry of P-Akt was compared to total Akt. Since the loading was unequal between lanes 2 and lane 3, we also compared the band intensity of p-Akt to that of ß-actin band which showed a 76% reduction in p-Akt in LY pretreated cells compared to P3C stimulation without the inhibitor (Figure 3.11A: lanes 2 and 3). Treatment of Pam3CSK4 stimulated macrophages with wortmannin at 30 minutes resulted in a 28% reduction in p-GSK-3αβ (Figure 3.11B: lanes 3 and 4). When LY294002 was used, TLR-2 stimulated macrophages at 30 minutes showed a 56% reduction in p-GSK-3αβ levels (Figure 3.11B: Lanes 3 and 5). Given the consistency in the reduction of p-GSK-3αβ upon stimulation of purified SM with both TLR-2 and TLR-4 ligands in the presence of PI3K inhibitors, it was of interest to evaluate the effect of stimulating aged SM with HKSP in the presence of LY294002. HKSP was effective in activating the PI3K-Akt signaling pathway, as indicated by an increase in the phosphorylation of GSK-3 (Figure 3.11C: Lanes 1 and 2). Inhibition of PI3K with LY294002 resulted in a 52% reduction in the levels of p- GSK-3αβ (Figure 3.11C: Lanes 2 and 3). Thus, activation of PI3K with TLR-2 or HKSP resulted in an increase in phosphorylation of Akt and GSK-3αβ, which was reduced significantly by inhibition of PI3K**.**

Effect of PI3K inhibition on cytokine secretion by SM stimulated with interleukin-1β (IL-1 β) is similar to TLR induction.

Thus far we have shown an age associated cytokine dysregulation using ligands for TLR1/2, TLR2/6 and TLR4 receptors, all of which use the MyD88/Mal heterodimer for signal transduction. One cannot distinguish if signal transduction via MyD88 or Mal

was different in the aged compared to the young. Hence, we tested the effect of aging on IL-1β induced responses of macrophages, since IL-1β had been shown to use MyD88 but not Mal for signal transduction (299). Moreover IL-1β was critical for the clearance of *S. Pneumoniae* via both macrophage activation and anti-polysaccharide antibody production by B-cells (64, 167). Very little was known about the role of the PI3K-AKT-GSK-3 signaling axis in regulating IL-1β-mediated induction of IL-10 and the pro-inflammatory cytokines in the context of aged splenic macrophages. Inhibition of PI3K pathway with LY294002 resulted in a suppression of IL-1 induced IL-10 secretion (Figure 3.12A) but an enhancement of IL-6 secretion (Figure 3.12B) similar to our results with TLR2 and TLR4 ligands. This suggested that major target of PI3kinase-Akt-GSK-3 axis was the MyD88 pathway in macrophages, which was consistent with the recent findings that the p85 subunit of PI3K directly interacts with MyD88 (Laird et al 2009).

Discussion

Immune responses to infection, in particular to encapsulated bacteria, are decreased in the aged. Previously, we have shown that antibody responses to polysaccharide antigens, one of the protective antigens for infections with pneumococcal bacteria, are decreased in the aged, in part due to defects in macrophage function (63). Using TLR-4 activation as a model system we now show that the SM from the aged exhibit a cytokine dysregulation phenotype with a decrease in pro-inflammatory cytokines required for B cell activation. Using gene expression studies we also showed that this is due to a decrease in the activation of the MyD88 signaling pathway leading to a decrease in ERK activation with an aberrant increase in p38 MAP kinase activation (65). Since the immune responses to Gram positive bacteria, such as *S. pneumoniae,* are also dependent on TLR2 activation (86, 318), and since TLR2 ligands also utilize the MyD88 signaling pathway, we hypothesize that SM from the aged would exhibit a cytokine dysregulation phenotype with synthetic TLR2 ligands or intact bacteria similar to that seen with TLR4 activation. Indeed, studies presented here demonstrate that activation with HKSP or any of the TLR-2 ligands, Pam3-CSK4, Pam-2-CSK4 or LTA, induces lower levels of pro-inflammatory cytokines such as IL-12, but higher levels of anti-inflammatory cytokines such as IL-10 in SM from the aged macrophages compared to macrophages isolated from young mice. Together with our previous studies, these results demonstrate that immunosenescence affects responses of SM to both TLR2 and TLR4 ligands.

To understand the signaling mechanism governing this age-associated cytokine dysregulation, we evaluated the activity of PI3K, a well established switch-factor for pro- and anti- inflammatory cytokines (108, 290, 308). An analysis of our previous data on

gene expression in highly purified SM (65) reveals that there is an age-associated increase in expression of genes encoding both the catalytic (p110δ) and the regulatory (p85β) subunits of the Class IA PI3 kinase, which is further validated by qRT real time PCR. There is an increase in functionally active PI3-kinase in the aged macrophages as shown by an increase in the phosphorylation of its downstream target, Akt, in splenic macrophages stimulated by ligands for TLR-2 and TLR-4. Moreover, phosphorylation of GSK-3, an immediate downstream target of Akt (139, 225), is also increased in TLR-2 and TLR-4 stimulated SM from the aged compared to the young. Thus, the PI3K-Akt-GSK-3 pathway that is known to negatively regulate pro-inflammatory cytokine production is upregulated in macrophages from the aged stimulated with TLR2 or TLR4 ligands, as well as HKSP.

We next asked if inhibitors of PI3K could restore pro-inflammatory cytokine production and decrease IL-10 production by TLR-2 or 4 stimulated macrophages from the aged. Accordingly, we show that PI3K inhibitors enhance IL-12(p40) production and decrease IL-10 secretion by aged mouse SM stimulated with either TLR-2 or TLR4 ligands. However, under these conditions PI3K inhibitors do not have much effect on cytokine secretion by young adult SM stimulated with TLR-2 or 4 ligands. Despite previous studies demonstrating specificity of wortmannin and LY294002 to Class I isoform, more recent studies find that one of the Class II isoforms is also inhibited by these reagents, albeit at 50-100 fold higher concentration (135, 238, 335, 338, 367). Currently we are devising an RNAi approach to establish the relative importance of PI3K isoforms in age-related cytokine dysregulation.

Our finding that in vitro stimulation of purified SM produced higher IL-10 but lower pro-inflammatory cytokines in the aged than in young is consistent with other published studies (34, 63, 277, 298). In contrast, there are several reports that IL-6 levels are increased in the elderly and contribute to basal inflammation (47, 102). This apparent inconsistency between the in vitro and in vivo cytokine profile are attributed to the criteria used to select elderly subjects (9). It is known that the frail elderly already have predisposing underlying diseases that impact on basal inflammation, whereas, in the healthy elderly, the inflammation is controlled by the increase in the levels of IL-10 (9, 198). Thus, Njemini and colleagues (2007) show that IL-6 levels in healthy elderly are less than in young adults but the levels of IL-6 were more in elderly than young when there is evidence of infection (257). Additionally, work from Kovacs' laboratory (119) further confirms the in vitro defect in production of pro-inflammatory cytokines by LPS activated macrophages from the aged. Interestingly, IL-6 knockout mice made more TNF-α, IL-1, IL-12 than young wildtype mice suggesting a role for systemic IL-6 in the cytokine defect of aged mice. However, IL-6 regulation is complex since young IL-6 knockout mice makes less IL-1, IL-12 and TNF-α than young wild type mice (119).

Working with monocyte derived dendritic cells (MDDC) from the elderly Agrawal and colleagues (5) show that the age-associated increase in the pro-inflammatory cytokines is due to a decrease in PI3K activity and increased activity of p38 MAP Kinase. These discrepant results could be due to the use of SM in our study; while human peripheral blood monocyte derived dendritic cells are used by Agrawal and colleagues. In contrast to this study with DCs and in agreement with our studies Panda and colleagues report that elderly human myeloid and plasmacytoid DC from peripheral

blood mononuclear cells exhibit a defect in the production of proinflammatory cytokines (277).

Using Zymosan, yeast derived TLR-2 ligand, to stimulate purified SM, Boehmer and colleagues (35) find that IL-6 is decreased in the aged. However, there was no difference in the cytokine levels between the aged and the young adult in terms of IL-10. This apparent contradiction from our findings that IL-10 is higher in the aged compared to the young can be explained by putative differences in the signaling mechanism between Zymosan and the other TLR2 ligands used here. Zymosan interacts with dectin-1 to stimulate production of pro-inflammatory cytokines (84, 87, 90), while Pam-3-CSK4 is not known to signal via dectin-1 to induce cytokine production (113). A second plausible reason for this apparent contradiction could be due to the method by which the macrophages are purified. We used negative selection to isolate the splenic macrophages and thus keep them unperturbed. Boehmer and colleagues used adherence to isolate macrophages, which is known to activate macrophages (80, 175).

Although, we observed the expected negative effects of PI3K on pro-inflammatory cytokines in the aged, the effects of PI3K inhibitors on cytokine secretion by young SM are very modest and are seen only in certain groups. Interestingly most of the studies performed with young macrophages to investigate the role of PI3K and cytokine regulation use human monocytes, murine peritoneal macrophages, alveolar macrophages or macrophage-derived cell lines like THP-1 and RAW 264.7 cells, but not SM (215, 226, 284, 308, 346, 375). It is possible that the PI3K pathway may not have major negative effects on TLR signaling in young adult SM, due to the low expression of

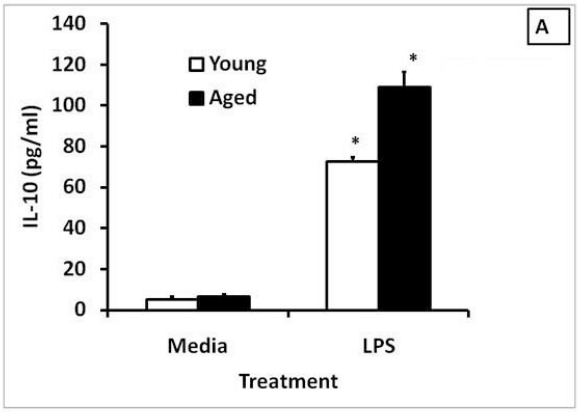
the PI3K enzyme. More profound effects are seen in the aged due to increased expression of PI3K subunits.

The association between the increased activity of PI3K and a decrease in PTEN expression is well established (223). PTEN promotes pro-inflammatory activity of macrophages (56). MDDCs from elderly have increased PTEN levels (5). However, we do not note any difference in the level of PTEN between aged and young adult SM. This apparent contradiction regarding age associated changes in PTEN levels may also be due to differences between macrophages and DCs and/or due to differences between human and mouse.

We propose a model (Figure 3.13) in which there is a heightened activity of PI3K in the aged macrophages (Figure 3.13A) resulting in increased phosphorylation of Akt. The increased activity of Akt results in a further phosphorylation of GSK-3 and reduction in its activity. We propose that the reduction in the activity of GSK-3 affects transcription factors like CREB, AP-1 and p65NFκB via inhibitory phosphorylation or export from the nucleus; resulting in increased IL-10 and a decrease in the pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α. Inhibiting PI3K reverses this phenotype to that of a young adult (Figure 3.12B) with increases in the pro-inflammatory cytokines over IL-10. We are currently investigating the effect of modulating PI3K on transcription factors CREB, AP-1 and p65NFκB. Here, we have included TLR2/TLR6 heterodimers and IL-1R since we find similar results with Pam2CSK4 (TLR2/6 ligand) and IL-1.

The biological significance of our findings is that these inhibitors can reverse the effect of aging on macrophage function. We show that macrophages and macrophage-

mediated cytokine defects in the aged are critical factors responsible for the impaired anti-CPS antibody response in the aged (38, 63). We also show that the humoral response of the aged to PS or TNP-Ficoll and TNP-LPS, representative TI antigens, is enhanced if aged B-lymphocytes are provided with either young macrophages or macrophage-derived cytokines like IL-6 and IL-1β (63, 114). Others show that antibody responses to infection with *S. pneumoniae* are enhanced in the presence of IL-12 (17, 230, 341). We propose that PI3K inhibition can enhance the macrophage-derived cytokines that are known to provide the signal 2 needed to activate B cells and mount an effective antibody response to *S. pneumoniae* and/or PPS vaccines in the elderly. In conclusion, our novel observation that the age-associated increase in PI3K-Akt activity is a signaling defect that contributes to the cytokine dysregulation observed in the aged and that PI3K inhibitors may be useful as unique adjuvants to enhance immune responses in the aged.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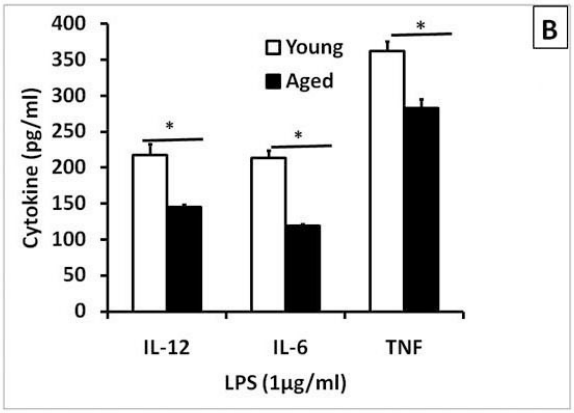
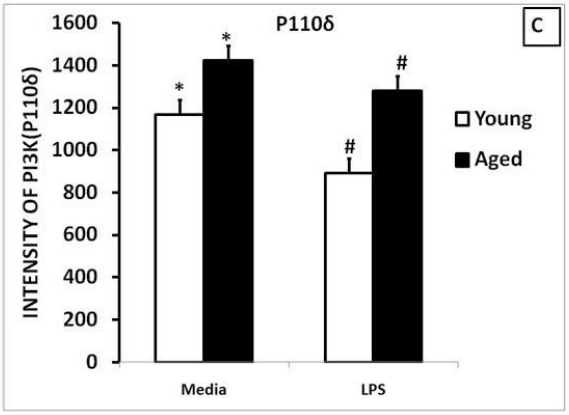
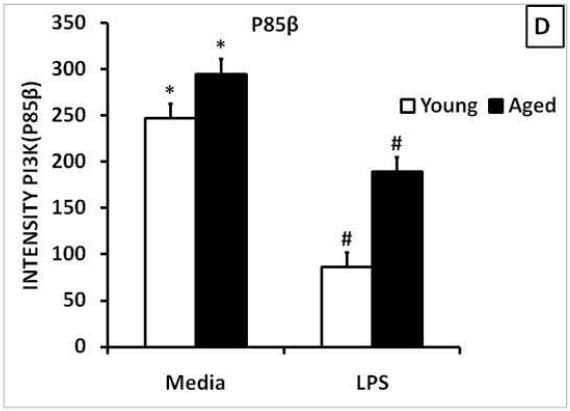
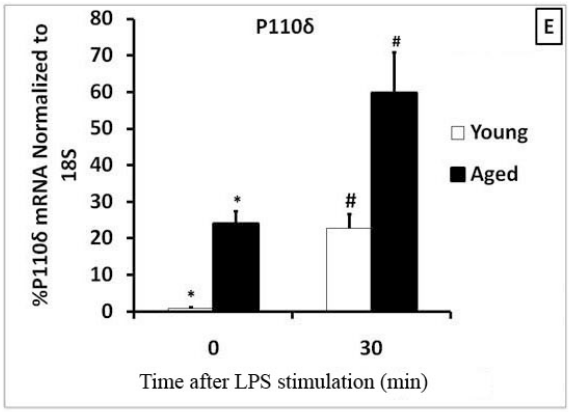
Young
Aged
Media
LPS
Treatment


B
Cytokine (pg/ml)
Young
Aged
IL-12
IL-6
TNF
LPS (1µg/ml)


C
P110δ
INTENSITY OF PI3K(P110δ)
Young
Aged
Media
LPS


D
P85β
INTENSITY PI3K(P85β)
Young
Aged
Media
LPS


E
P110δ
%P110δ mRNA Normalized to 18S
Young
Aged
Time after LPS stimulation (min)

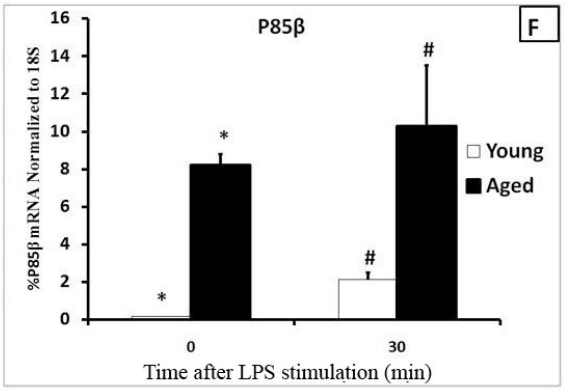

F
P85β
%P85β mRNA Normalized to 18S
Young
Aged
Time after LPS stimulation (min)

Figure 3.1: Age-associated changes in cytokine production and levels of PI3K subunits in SM stimulated via TLR4.

SM ($2.5x10^5$ cells/ml) from young (2-4 months) and aged (20-24 months old) mice purified by negative selection using an AUTOMACS cell separator were stimulated with LPS (1μg/ml) for 24 hours (Panels A and B) and the supernatants were collected and analyzed for IL-10 (Panel A) or pro-inflammatory cytokines, IL-12(p40), IL-6, and TNF-α (Panel B) . Panels C and D represent levels of RNA for p110δ and p85β subunits derived from microarray analysis performed on gene expression in young and aged macrophages. QRT-PCR analysis of mRNA was shown in panels E and F for P110δ and p85β subunits respectively of PI3 kinase in purified splenic macrophages from young and the aged. The C_t values for each probe were normalized to the 18S RNA. The symbols * and $^{\#}$ indicate the statistical significance and was set to $p<0.05$.

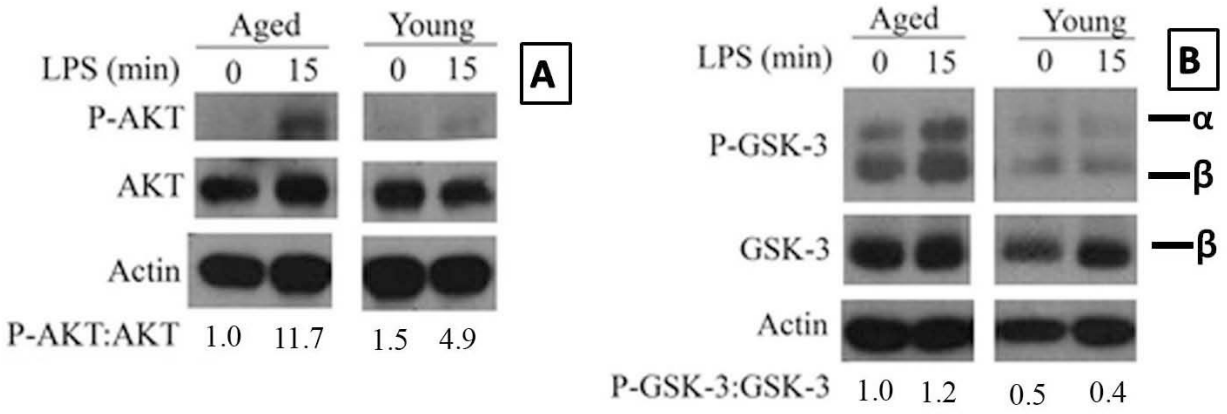


Figure 3.2: The age-associated increase in phosphorylation of AKT and GSK-3 in TLR-4 stimulated macrophages.

Purified young adult and aged SM were stimulated with LPS 1μg/ml for 15 minutes. Panels A and B show the phosphorylation of Akt and GSK-3 respectively in the aged and the young splenic macrophages stimulated with LPS. The blots were stripped and probed for total AKT and actin in panel A and for total GSK-3β and actin in panel B. The numbers represent densities of bands normalized to total AKT with the values for unstimulated aged macrophages set to one.

The figure in panel A is a composite of blots for P-Akt, Akt and actin from the same membrane that was stripped and reprobed. The same method was employed to assemble panel B for P-GSK-3αβ, GSK-3β and actin. The young and aged cell lysates were electrophoresed on the same gel and the membranes were exposed for identical time periods. Although a detailed kinetic analysis of LPS stimulation was performed, only the lanes representing 0 minutes and 15 minutes time points was shown for clarity.

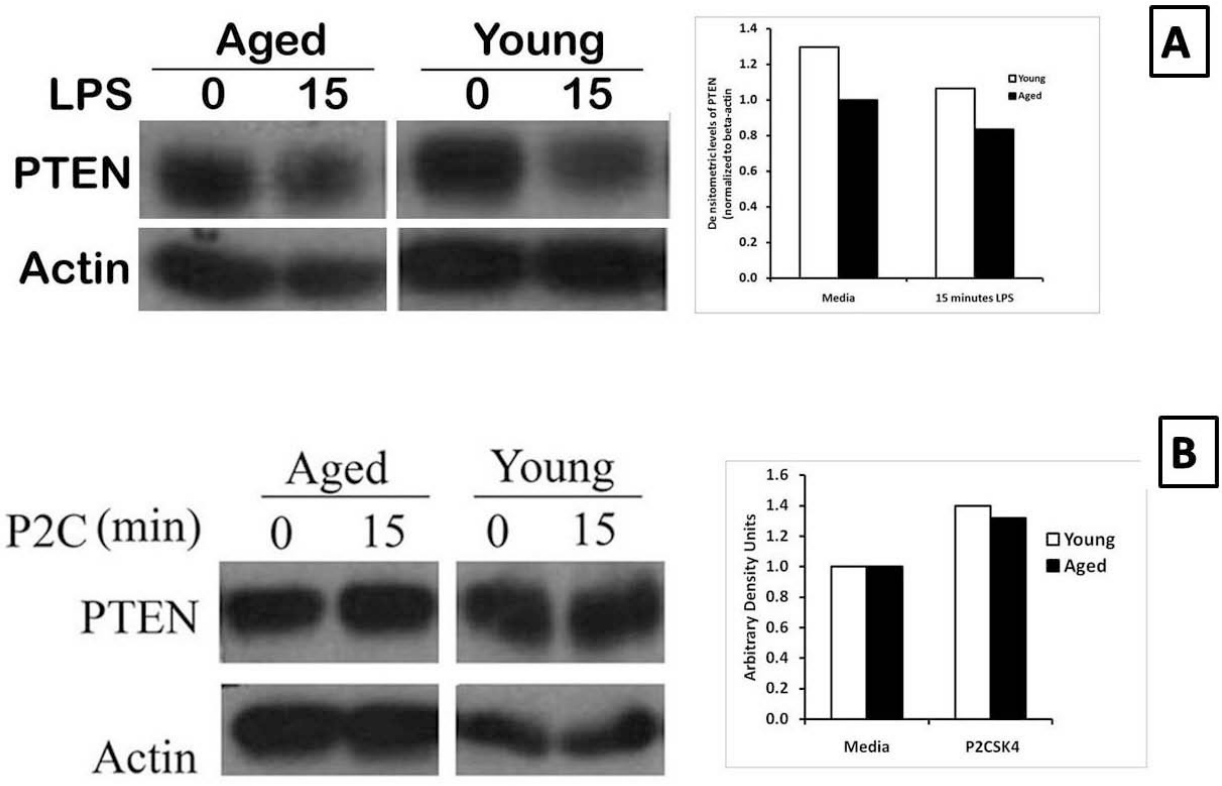


Figure 3.3. Age-associated increase in PI3K activity is not due to changes in the levels of PTEN

Lysates from young and aged SM stimulated with LPS (1µg/ml) for 15 minutes (Panel A) or P2C (Panel B) and analyzed by SDS PAGE and Western blots. The blots were first probed for PTEN and then for actin after stripping. The graphs showed the arbitrary units of band intensities normalized to actin with the values for unstimulated aged macrophages set to one.The final figure was a composite of blots for PTEN and actin for aged and young samples run on the same membrane that was stripped and reprobed. The intervening space, as well as the right end were for the 30 minute time point but were deleted for consistency and clarity.

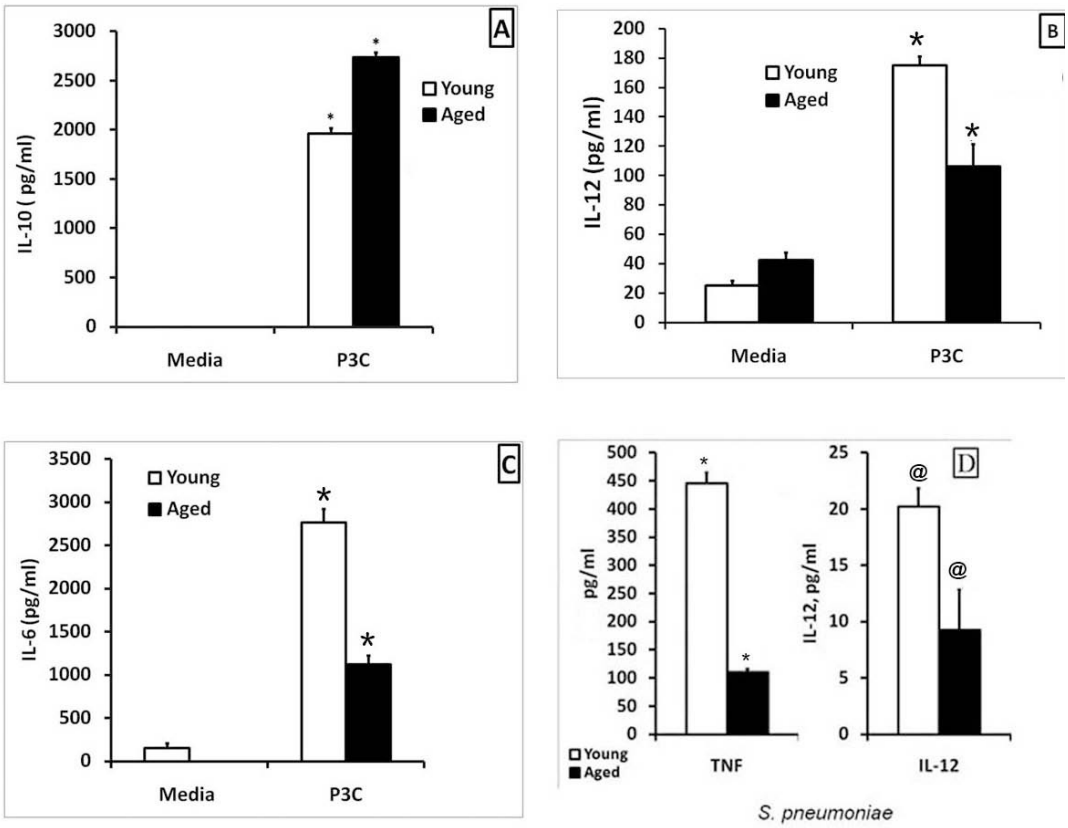


Figure 3.4: TLR-2 and HKSP-mediated cytokine dysregulation in young and aged splenic macrophages.

Macrophages ($2.5x10^5$ cells/ml) purified from the spleens of young and aged mice were stimulated with the TLR-2 agonists, Pam3CSK4 (1µg/ml) (Panels A, B and C) or killed S. *pneumoniae* ($2x10^6$ CFU/ml) (panel D) for 24 hours. The supernatants were collected and analyzed by a sandwich ELISA for IL-10 (Panels A), IL-12(p40) (Panels B and D), IL-6 (Panel C) and TNF-α (Panel D). In panel A, IL-10 levels in unstimulated young and aged macrophages were undetectable. Results from one of three experiments were shown as Mean $\pm$ SE values of 8-12 determinations. The statistical significance of differences in cytokine secretion between the young and the aged was indicated by the symbols * and @ and was set to $p<0.05$.

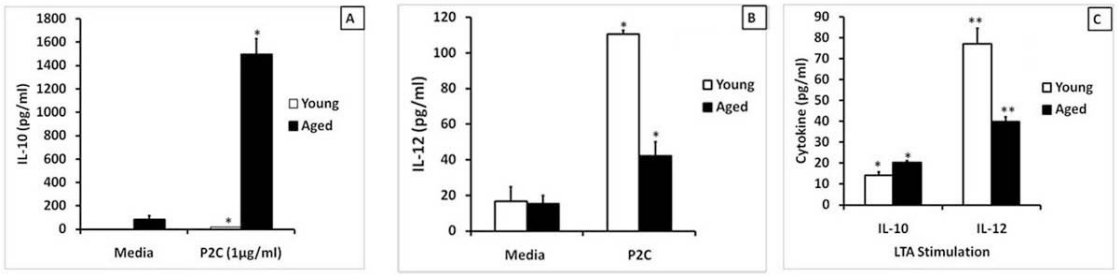


Figure 3.5: Age-related cytokine dysregulation in response to stimulation of macrophages with TLR2 ligands.

SM ($2.5x10^5$ cells/ml) from young and aged Balb/c mice purified by negative selection were stimulated with either Pam2CSK4 (1µg/ml) (Panels A and B) or LTA (1µg/ml) (Panel C). Supernatants were collected at 24 hours and analyzed for IL-10 (Panel A and C) or IL-12(p40) (Panels B and C). The results were shown as mean +/- SE of duplicate determinations from triplicate cultures, and representated three independent experiments. The symbols * and ** indicated the statistical significance (Student's t test) of differences in cytokine secretion between the young and the aged and was set to $p<0.05$.

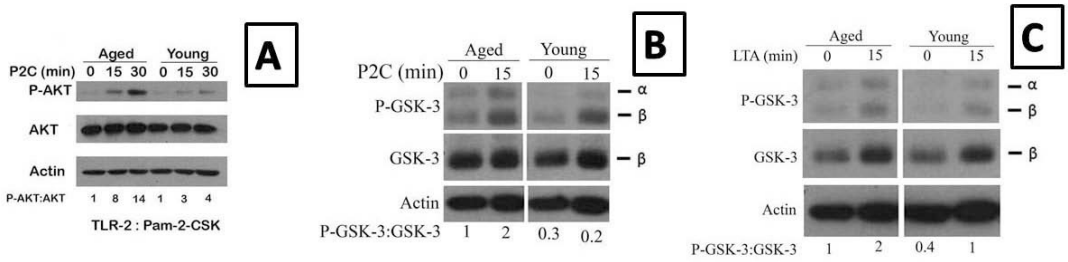


Figure 3.6: Age-related increase in activation of AKT and GSK-3 in splenic macrophages stimulated with TLR2 ligands.

Panel A represents Western blot analysis of levels of P-AKT, total AKT and actin loading control; while Panel B represents P-GSK-3, total GSK-3 and actin loading control in Pam2CSK4 stimulated purified SM from the aged. The blots were stripped and probed for total AKT and actin. The numbers represent densities of bands normalized to total AKT with the values for unstimulated aged macrophages set to one. Panel C represents levels of P-GSK-3, total GSK-3 and actin loading control in LTA stimulated purified splenic macrophages. The final figures in both B and C are a composite of blots for P-GSK-3, GSK-3 and actin from the same membrane that was stripped and reprobed. The intervening deleted space in the middle was for 30 minutes but this was removed for the sake of clarity

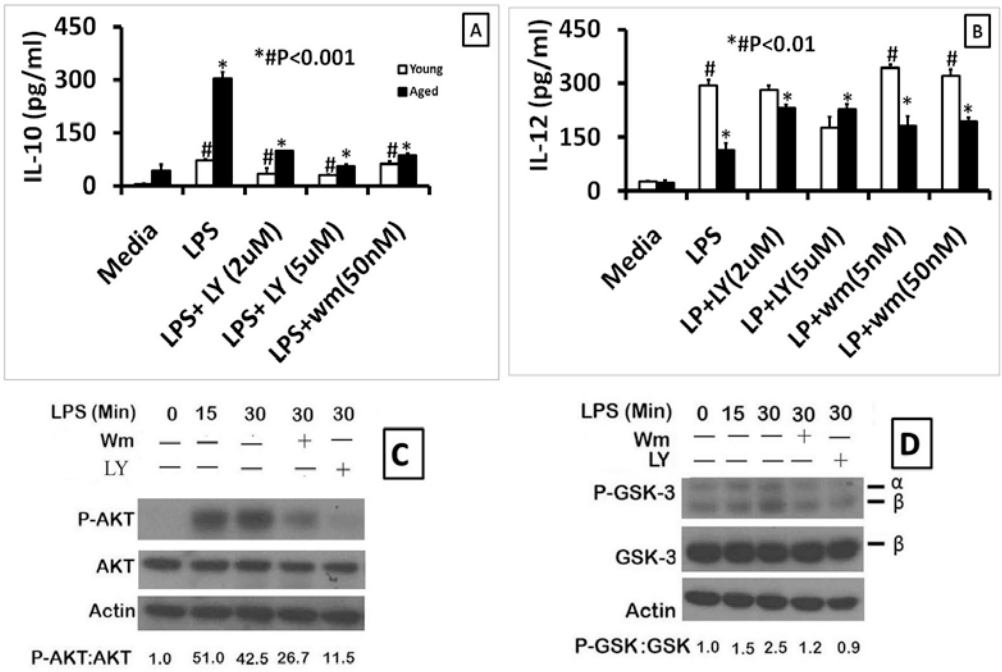

A
IL-10 (pg/ml)
450
300
150
0
*#P<0.001
Young
Aged
Media
LPS
LPS+ LY (2uM)
LPS+ LY (5uM)
LPS+wm(50nM)
B
IL-12 (pg/ml)
450
300
150
0
*#P<0.01
Media
LPS
LP+LY(2uM)
LP+LY(5uM)
LP+wm(5nM)
LP+wm(50nM)
LPS (Min) 0 15 30 30 30
Wm
LY
C
P-AKT
AKT
Actin
P-AKT:AKT 1.0 51.0 42.5 26.7 11.5
LPS (Min) 0 15 30 30 30
Wm
LY
D
P-GSK-3
α
β
GSK-3
β
Actin
P-GSK:GSK 1.0 1.5 2.5 1.2 0.9

Figure 3.7: PI3K inhibitors partially rescue the age-associated defect in TLR-4 induced cytokine production by inhibiting phosphorylation of Akt and GSK-3.

The graphs show cytokine secretion by purified aged and young adult SM pretreated with either 2 or 5 μM LY294002 or 5 or 50 nM wortmannin (panels A and B) for 60 minutes and then stimulated with LPS (1μg/ml) for 24 hours. Supernatants were collected and assayed for IL-10 (panel A) and IL-12 (p40) (panel B) by sandwich ELISA. Data were presented as mean+/-SE values of 6-10 determinations and were representative of three independent experiments. The symbols * and $^{\#}$ indicated statistical significance of differences in responses in groups treated with LPS alone compared to groups treated with LPS + PI3K inhibitor and was set to $p<0.05$. Purified young adult splenic macrophages were pre-treated with either LY294002 or wortmannin for 1 hour and then stimulated with LPS (Panel C) for 15 or 30 minutes. The blots were probed for p-Akt and later probed for total Akt and actin after stripping. The numbers represented densities of bands normalized to total AKT with the values for unstimulated aged macrophages set to 1. Panel D shows relative changes in phosphorylated GSK-3 in young splenic macrophages stimulated with LPS in the presence of PI3K inhibitors. The numbers represent densities of both GSK-3alpha and GSK-3beta bands normalized to total GSK-3β with the values for unstimulated aged macrophages set to one. Results were representative of two independent experiments.

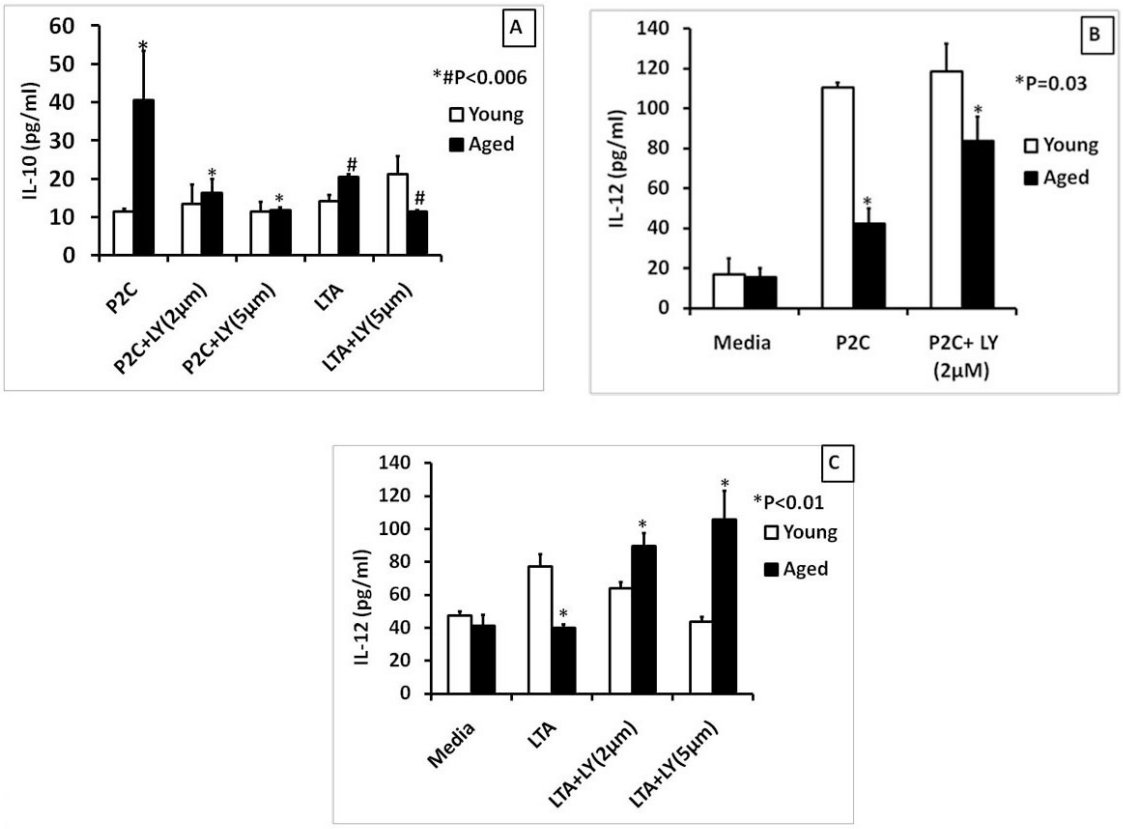


Figure 3.8: Cytokine dysregulation in aged splenic macrophages stimulated with TLR2 ligands can be partially rescued by inhibiting PI3K.

The graphs show cytokine secretion by purified aged and young adult SM pretreated with LY294002 for 60 minutes and then stimulated with Pam2CSK4 (Panels A and B), LTA (1µg/ml) (Panels A and C) for 24 hours. Supernatants were collected and assayed for IL-10 (Panel A) or IL-12(p40) (Panels B and C) by sandwich ELISA. Data were presented as mean +/- SE of duplicate cultures and duplicate ELISA's. The symbols * and # indicated statistical significance of responses in the aged splenic macrophages treated with Pam2CSK4 or LTA with or without the PI3K inhibitor set to $p<0.05$.

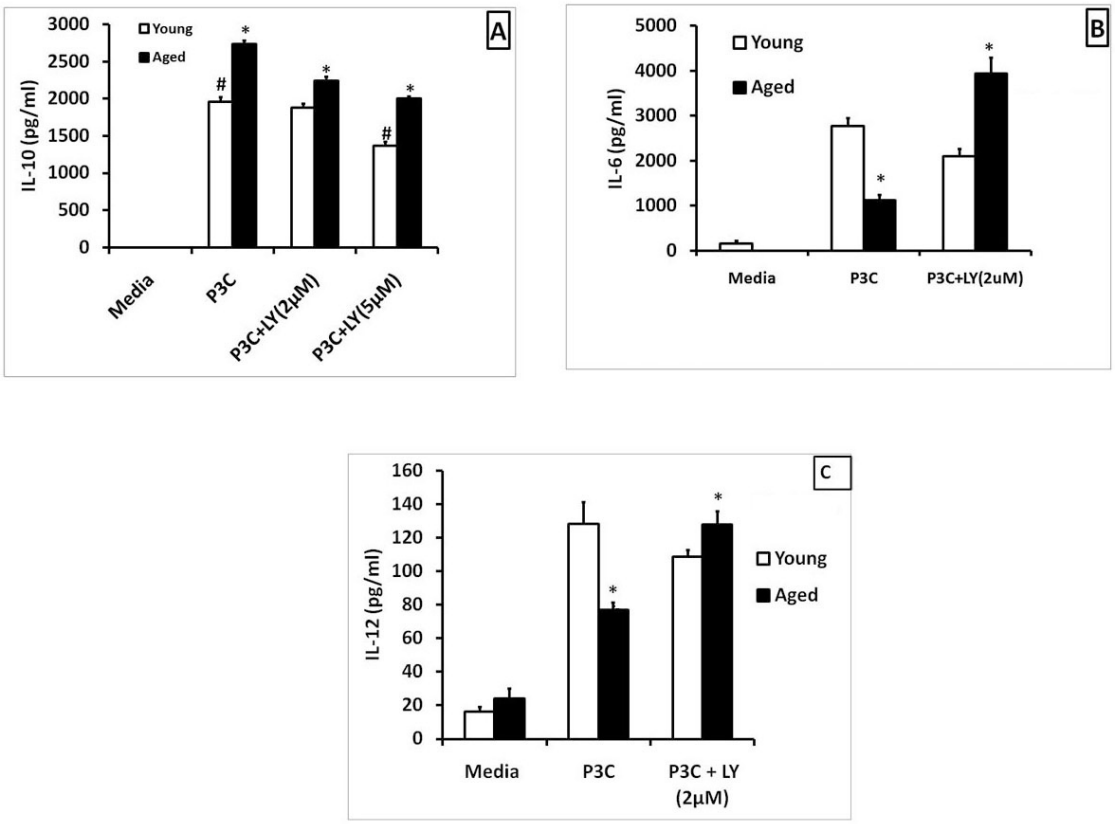


Figure 3.9: The cytokine dysregulation in TLR1/2 stimulated aged macrophages can also be reversed with PI3K inhibition.

Macrophages ($2.5x10^5$ cells/ml) purified from the spleens of young and aged mice were stimulated with the TLR-1/2 agonist, Pam3CSK4 (P3C) (1μg/ml) (Panels A, B and C) for 24 hours in the presence or absence of LY294002. The supernatants were collected and analyzed by a sandwich ELISA for IL-10 (panel A), IL-6 (Panel B) and IL-12(p40) (Panel C). Results from one of three experiments were shown as Mean $\pm$ SE values of 8-12 determinations. The statistical significance of differences in cytokine secretion between the young and the aged macrophage treated with P3C alone compared to groups treated with PI3K inhibitors was indicated by the symbols * and $^{\#}$.

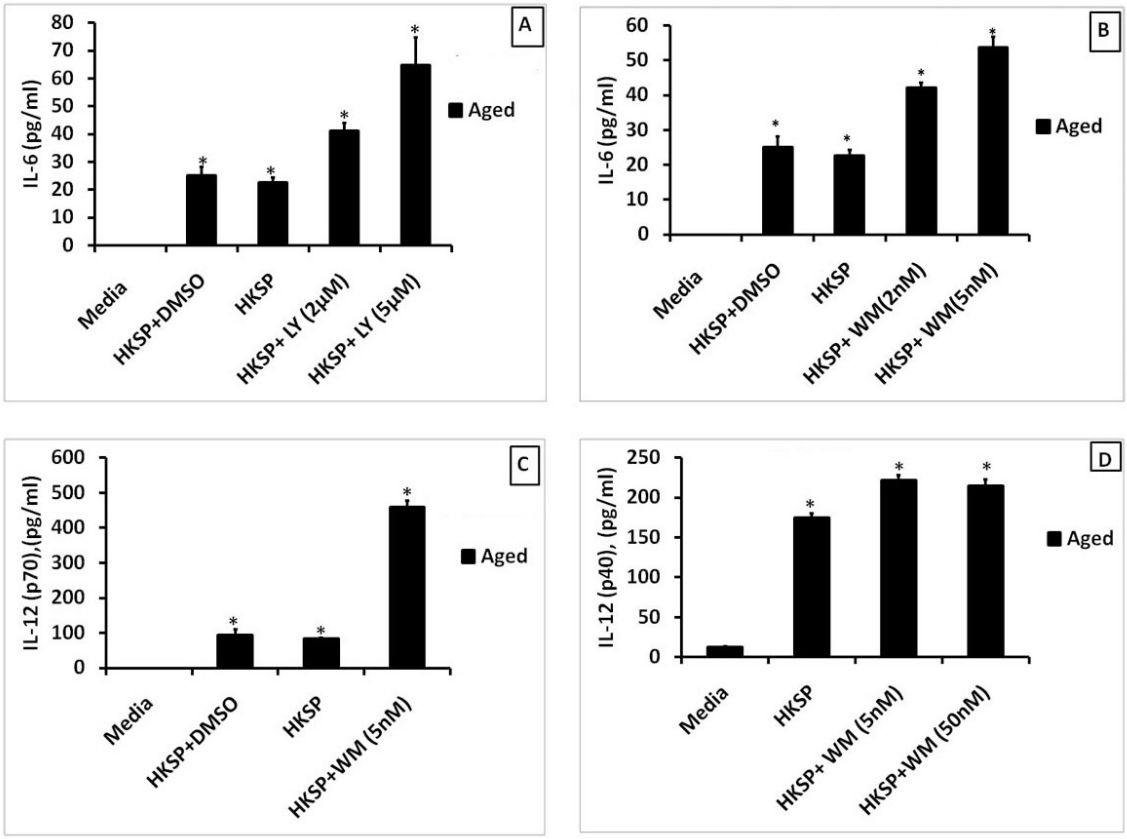


Figure 3.10. The inhibition of PI3K by either LY294002 or wortmannin enhanced the HKSP-mediated induction of pro-inflammatory cytokines in aged splenic macrophages.

The graphs show cytokine secretion by purified aged SM pretreated with LY294002 (panel A) or wortmannin (Panels B, C and D) for 60 minutes and then stimulated with HKSP (2x 10^8 CFU/ml) for 24 hours. Supernatants were collected and assayed for IL-6 (Panels A and B), IL-12(p40) (Panel D) and IL-12(p70) (Panel C) by sandwich ELISA. Data were presented as mean +/- SE of duplicate cultures and duplicate ELISA's. The symbol * indicated statistical significance of responses in the aged SM treated with HKSP alone compared to the responses obtained by treating with HKSP + PI3K inhibitor set to $p<0.05$.

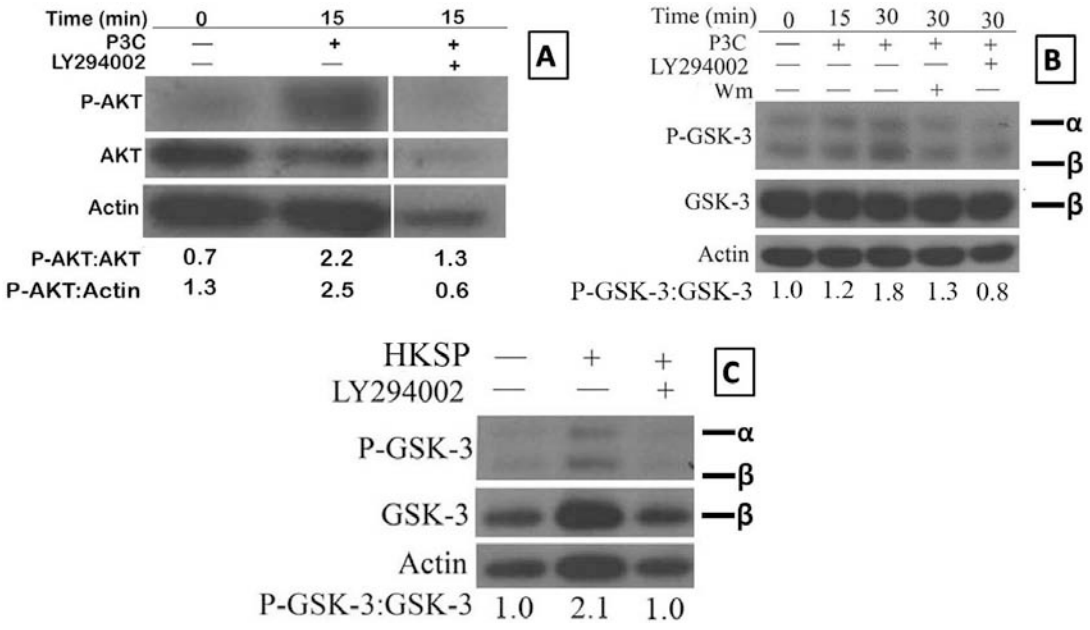


Figure 3.11. Age-associated cytokine defect in TLR-2 and HKSP activated macrophages is due to a defect in the AKT-GSK-3 signaling axis.

Purified aged SM were pre-treated with either LY294002 or wortmannin for 1 hour and then stimulated with Pam-3-CSK4 (Panels A and B) or HKSP ($2x10^8$ CFU/ml) (Panel C) for 15 or 30 minutes, respectively. The blots were probed for p-Akt (Panel A), p-GSK-3αβ (Panels B and C) and later probed for total Akt (Panel A), total GSK-3β (panels B and C) and actin after stripping. The numbers represent densities of bands normalized to total AKT, actin or GSK-3 with the values for unstimulated aged macrophages set to 1. Results were representative of two independent experiments.The final figure in A was a composite of blots for P-Akt, Akt and actin for aged samples run on the same membrane that was stripped and reprobed. The intervening space (between second and third lane) was for lanes loaded with lysates from other time points or treated with other inhibitors but were deleted to focus on the effect of P3C on Akt activation at 15 minutes, which was maximum in this experiment.

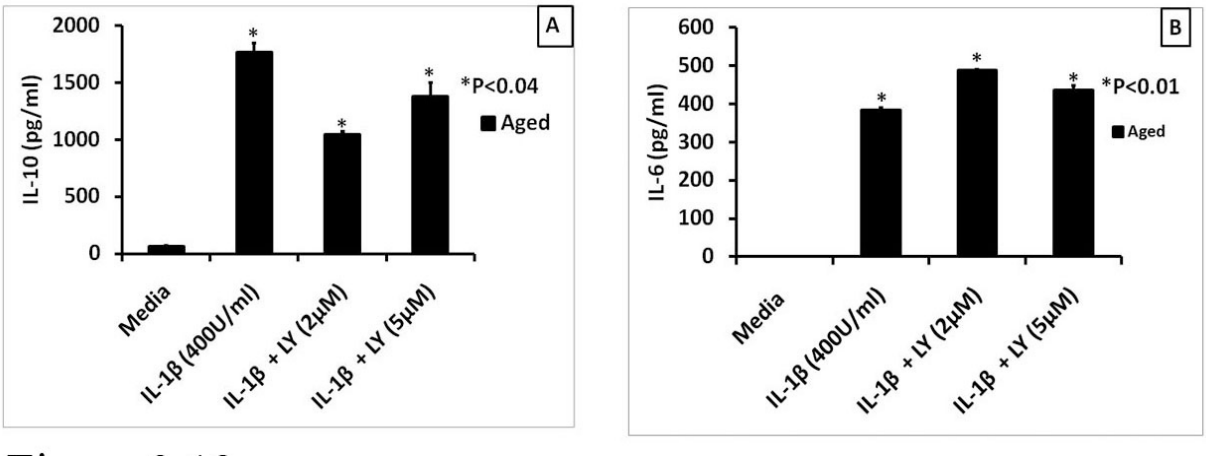


Figure 3.12. The effect of PI3K inhibition on IL-1 β induced cytokine production in splenic macrophages.

Panels A and B respectively showed the levels of IL-10 and IL-6 secreted by IL-1 (400U/ml) stimulated aged SM pretreated with LY294002 for 60 minutes. Supernatants were collected and assayed for IL-10 and IL-6 by sandwich ELISA. Data represent mean +/- SE of duplicate determinations of supernatants from duplicate cultures. The symbol * indicated the statistical significance of differences in cytokine levels between cells treated with IL-1 alone or IL-1 + the PI3K inhibitor set to $p<0.05$.

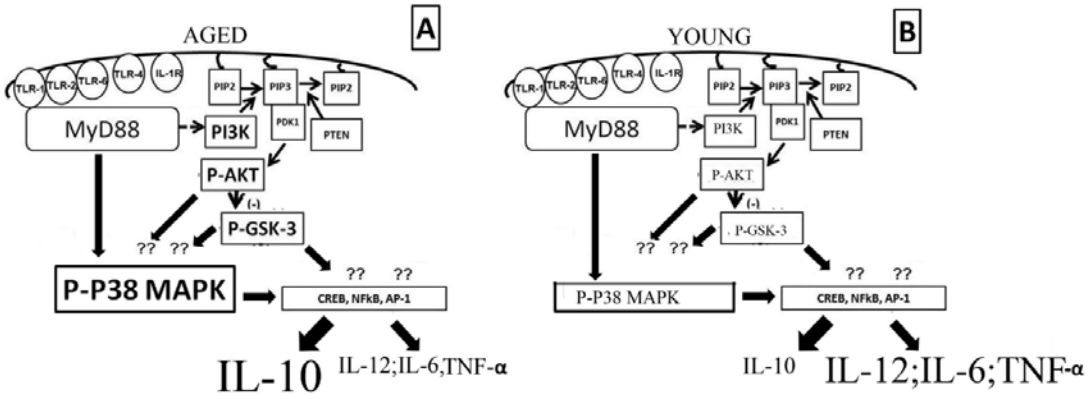


Figure 3.13: Schematic model of differing roles of PI3 Kinase and P38 MAP Kinase pathways in cytokine secretion by young and aged splenic macrophages.

The interaction of aged SM with ligands for TLR-4, TLR-2/1 or TLR2/6 heterodimers, or HKSP induces the activity of an already heightened PI3K, as well as p38 MAPkinase via MyD88 signaling adaptor molecule (Panel A). The activated AKT and GSK-3, as well as the phosphorylated p38 MAP kinase interact with transcription factors like CREB, AP-1 and p65NκB to suppress the pro-inflammatory cytokines but increase IL-10. The effect of this pathway is biased towards pro-inflammatory cytokines in the young due to lower levels of PI3 kinase and p38 MAP Kinase activity (Panel B).

CHAPTER 4: Intrinsic defect in the phosphoinositide 3-kinase – Akt pathway plays a role in cytokine dysregulation in aged bone marrow derivedmacrophages stimulated with TLR ligands or heat killed *Streptococcus pneumoniae*

Introduction

The immune system is dysregulated with age resulting in higher susceptibility to bacterial and viral infections, as shown with an increase in pneumococcal infections, as well as a reduction in the efficacy of pneumococcal and influenza vaccines (236). It has been shown in several species (human, rat and mice) that aging affects the innate components of the immune system, like macrophages and dendritic cells. These effects include impaired antigen presentation and decreased production of critical pro-inflammatory cytokines that help to induce the adaptive immune response (288). The efficacy of the pneumococcal vaccination is low in older adults due to a reduction in opsonophagocytosis of the pneumococcal bacteria as a result of a decrease in high affinity IgM antibody response (278).

Macrophages are known to act in resident tissues to destroy invading pathogens and lead to adaptive immunity by expression of co-stimulatory molecules, cytokine production and antigen presentation to lymphocytes upon activation by Toll-like receptors (TLRs). In a physiological situation, a macrophage sees multiple TLR ligands and thus, may produce pro- or anti-inflammatory cytokines depending on the context (219). Macrophage-derived cytokines, like IL-1, IL-12 and IL-6, help B-cells to produce increased IgG_3 and IgA in the absence of help from T-cells (17, 38, 64, 178, 230). Both IgA and IgG_3 promote opsonization of many bacteria. TNF-α is another pro-inflammatory cytokine that is produced by *S. pneumoniae* activated macrophages and

also aids in the recruitment of neutrophils and macrophages, which phagocytose the opsonized bacteria (176, 201). IL-12 can directly or indirectly act on B-cells to induce B-cell proliferation, activation and differentiation, mostly through intermediaries like Th1 type cells and interferon gamma to enhance production of IgG2a (12, 350). TNF-α and IL-6 play critical roles in the clearance of lung burdens of pneumococcal bacteria (355, 356). TLR2, TLR4 and TLR9 appear to play an important role in host immunity against pneumococcal infections. We have previously shown that upon stimulation with LPS, a TLR-4 ligand; in comparison to the young, aged splenic macrophages secrete lower levels of the pro-inflammatory cytokines, IL-6, IL-12, and TNF-α, but higher levels of IL-10, resulting in cytokine dysregulation (62, 63, 65). We also show a similar cytokine dysregulation when SM is stimulated with ligands for TLR-2/1, 2/6 and TLR-2 homodimer (Fallah et al manuscript in press). Similar defects in TLR-induced pro-inflammatory cytokine secretion by either aged splenic or peritoneal macrophages (resident or thioglycollate induced) are observed in other studies (34, 35, 62, 63, 144, 298). As a result, TLR-2 or TLR-4 activated aged macrophages or their secreted pro-inflammatory cytokines are not able to effectively provide support to B-cell mediated anti-capsular (CPS) antibody responses. Moreover, the increased IL-10 has a role in increased pneumococcal infections in the aged. IL-10 is increased after pneumococcal infection in naïve hosts or hosts previously exposed to influenza virus. Prior influenza virus infection increases lethality of subsequent pneumococcal infection. Anti-IL-10 treatment reduces lethality of *S. pneumoniae* in naïve, as well as influenza in exposed hosts (306, 358).

While investigating the signaling components associated with the defect in TLR-induced activation of aged macrophages, we found that the cytokine dysregulation is in part due to heightened activity of phosphatidyl inasitol 3-kinase (PI3K). Inhibition of the PI3K with two well known pharmacological inhibitors (LY294002 and wortmannin) partially rescues this age-associated cytokine dysregulation (Fallah et al manuscript in press). Presently, it is unclear whether the cytokine dysregulation is due to intrinsic signaling defects or defects in the tissue microenvironment or both in the aged (143, 187, 212, 315, 339, 354).

Current consensus on the effect of aging on hematopoietic stem cells (HSC) points to a dual regulation of HSC by intrinsic factors and cues from the bone marrow microenvironment or stromal niches. Both of these are altered during aging resulting in skewing of the differentiation of HSC towards myeloid lineages. These age-associated effects on HSC have a role in narrowing the immune repertoire and blunting the immune function in the aged (33, 54, 170, 372, 386). Hence, in this study we attempt to reduce the influence of the aging microenvironment by growing a homogenous population of macrophages under in vitro conditions (143, 315). We test the in vitro TLR responses of bone marrow derived macrophages (BMDM) from the young and the aged, thus eliminating the microenvironment of the aged spleen or peritoneum (315).

These in vitro generated BMDM exhibit all of the characteristics of mature macrophages and are phenotypically similar in the young and the aged. Upon stimulating with ligands specific for TLR-4, TLR2/1, TLR2/6 and TLR-9 receptors or HKSP, the BMDM from the aged exhibited cytokine dysregulation which are similar to that seen

with purified SM (62, 63, 65). Furthermore, BMDM from the aged behave like the SM in having an elevation in the activity of the PI3K-Akt signaling pathway and inhibition of this pathway restores pro-inflammatory cytokine production.

RESULTS

The newly derived BMDM from the aged and young adult exhibit similar purity, recovery and activation status.

We first characterized the newly generated BMDM to ensure that they exhibited phenotypes of macrophages and that these phenotypes were similar between the two age groups. There was a similar expression of F4/80 and CD11b markers for macrophages between the aged and the young BMDM (Figure 4.1A) and in both cases greater than 96% of the cells expressed both F4/80 and CD11b. We then showed by light microscopy that the morphologies of the newly generated BMDM from the aged and the young were similar (Figure 4.1B). Although the absolute recovery of BMDM varied from experiment to experiment (Figure 4.1C), the recoveries of BMDM from the bone marrow cell cultures from the young and the aged were similar in each experiments. Thus, we recovered 0.33+/- 0.06 x 10^6 BMDM from the young and 0.28+/- 0.05 x 10^6 BMDM from the aged (n=15 experiments and p=0.52) per 1.0x10^6 bone marrow cells cultured. The extent of activation as determined by CD86 expression was not different between the aged and the young under basal conditions or after stimulation with LPS (TLR-4) (Figure 4.1D), Pam3CSK4 (TLR-2) (Figure 4.1E) and HKSP (Figure 4.1F). These results provided a basis for us to conclude that the newly generated BMDM from young and elderly mice independent of an in vivo microenvironment exhibited similar characteristics.

Cytokine secretion defects in aged BMDM stimulated with TLR4 and 9.

We stimulated the newly derived BMDM from the aged and the young mice with LPS, Pam3CSK4 and CpG for 24 hours and measured the levels of IL-10, IL-12, IL-6 and TNF-α in the supernatants. Similar to our previous data with SM (62, 63), LPS induced higher IL-10 and reduced levels of IL-12, IL-6 and TNF-α in the BMDM from elderly mice compared to the young (Figures 4.2A, 4.2B, 4.2C and 4.2D). We observed a similar age associated increase in IL-10 and decline in IL-12 secretion from BMDM stimulated with Pam3CSK4, the TLR2 ligand (Figures 4.2E and 4.2F) similar to our recent results with SM (Fallah et al manuscript In Press). The TLR9 ligand, CpG, also induced more IL-10 and less IL-6 from the aged than young BMDM (Figures 4.2G and 4.2H). Thus, the cytokine phenotype of newly generated BMDM from the aged was similar to the previously reported cytokine phenotype of SM from the aged stimulated via different TLR receptors suggesting that the splenic microenvironment of the aged was not necessary for the age associated cytokine dysregulation in macrophages.

HKSP-mediated cytokine dysregulation in young and aged BMDM.

Since whole bacteria can stimulate macrophages via several TLR receptors, we decided to test if cytokine response of BMDM to whole pneumococcal bacteria was also affected with aging. After 24 hours of stimulation with HKSP, supernatants from young and aged BMDM cultures were collected and assayed for IL-10, IL-12 and IL-6. In agreement with our previous data on SM activated with HKSP (Fallah et al Manuscript In press) we were able to show an age-related increase in IL-10 (Figure 4.3A) but a decrease in both IL-12 (Figure 4.3B) and IL-6 (Figure 4.3C). Thus, the intrinsic defects in aged macrophage

secretion of pro-inflammatory cytokines observed with individual TLR ligands were also demonstrated with the whole bacteria. Just like with the individual TLR ligands, the decrease in IL-12 was accompanied by an increase in the anti-inflammatory cytokine, IL-10.

The activity of PI3K-Akt pathway is increased in the aged BMDM

We had earlier shown in SM (Fallah et al Manuscript In Press) that the heightened activity of the PI3K/Akt pathway was in part responsible for the cytokine dysregulation in the aged. Hence, we decided to determine if the activity of PI3K-Akt pathway was altered between the aged and the young BMDM grown in vitro without any microenvironmental influences of the aged spleen. The QRT-PCR for the mRNA of the p110δ subunit showed that resting young BMDM had higher levels than the aged BMDM but the message levels declined rapidly upon LPS stimulation in the young cells. In contrast there was no decrease in p110δ mRNA in the LPS stimulated aged BMDM, but there was a small increase with the net result that TLR activated aged BMDM had more p110δ message than the young BMDM (Figure 4.4A). We then measured the activity of the PI3K in the aged and the young BMDM by way of the phosphorylation status of p-Akt. Western blot analysis showed that the phosphorylation of Akt was higher in the aged compared to young adult BMDM stimulated with LPS. The age-associated increase in p-Akt was seen as early as 15 minutes of stimulation with LPS and was 2.45+/-0.6 (n=4) times more in the aged than the young adult cells treated with LPS (Figure 4.4B). The total levels of Akt (normalized to actin) were comparable in the elderly and the young mouse BMDM (data not shown).

PI3K inhibitors partially rescue the age-associated defect in TLR-4 and TLR-9 induced pro-inflammatory cytokine production.

On the basis of our previous findings that inhibiting PI3Kinase with LY294002 or wortmannin could partially reverse the age-related cytokine dysregulation in SM (Fallah et al., Manuscript in press), we attemped to recapitulate this phenomenon in the context of the BMDM. Hence, BMDM was pretreated with either LY294002 or wortmannin and then stimulated them with LPS, the TLR-4 ligand, to initiate cytokine production. Both the PI3K inhibitors (Figures 4.5A and 4.5C) reduced LPS induced IL-10 production by the aged BMDM. The PI3K inhibitors had similar effects on IL-10 secretion by the young BMDM treated with LPS, but the effect of wortmannin was less pronounced. As predicted, treatment of BMDM with LY294002 resulted in the enhancement of IL-12(p40) in the aged but the effect on the young was modest (Figure 4.5B). Treatment of aged and young adult BMDM with wortmannin also induced an increase in IL-12 (p40) (Figure 4.5D) and TNF-α (Figure 4.5E) in both the aged and young adult. Similar results were obtained when aged and young BMDM were stimulated with the TLR-9 ligand, CpG in the presence of PI3K inhibitors (data not shown).

The ability of PI3K inhibitors to rescue the cytokine defect is applicable to aged BMDM stimulated via different TLR-2 heterodimers.

We have shown that TLR-2 ligands behave like TLR4 ligands in their ability to induce high levels of IL-10 and low levels of IL-12-like cytokines from BMDM. Although both TLR2 and TLR4 use the same MyD88 molecule, TLR2 utilizes TICAM/MAL while TLR-4 uses both TICAM and TRIF for complete signal transduction in macrophages (86,

134, 332). Therefore, it was of interest to know if the PI3K/AKT pathway affects the TLR2 mediated cytokine response of aged BMDM similar to the TLR4 response in the aged. BMDM from aged adult were pre-treated with LY294002 and then stimulated with Pam3CSK4 (1μg/ml), the ligand for the TLR2/1 heterodimer (Figure 4.6A and 4.6B) or Pam2CSK4 (1μg/ml), the ligand for the TLR2/6 heterodimer (Figures 4.6C and 4.6D). Treatment with both TLR2 ligands in the presence of the PI3K inhibitors, LY294002, resulted in reduced IL-10 secretion (Figure 4.6A and 4.6C) and increase in IL-12 secretion (Figure 4.6B and 4.6D) in BMDM. Thus, despite differences in adaptors used by TLR2 and TLR4 receptors, the PI3K pathway appeared to play a role in cytokine dysregulation observed in response to both receptors. The reason why P3C induced ten-fold more IL-10 than P2C was not known but a similar result was obtained with young BMDM (data not shown).

Age-associated cytokine defect shown with HKSP activated macrophages can be rescued by inhibiting PI3K

Since TLR2 plays a major role in immune response to HKSP, we predicted that the PI3K pathway will also regulate HKSP induced macrophage activation. Accordingly, we found that PI3K inhibition reduced IL-10 secretion and enhanced IL-12 secretion by HKSP treated BMDM from both age groups (Figures 4.7A and 4.7B). The absolute quantity of reduction in IL-10 was lower in the aged relative to the young (Figure 4.7A), but they had similar enhancement in IL-12 (Figure 4.7B).

Effect of PI3K inhibition on Akt and p65Nf-κB, downstream targets of the PI3K pathway

To be certain that the PI3K inhibitors were inhibiting the PI3K-Akt pathway, we evaluated the phosphorylation status of Akt upon inhibiting PI3K with LY294002 in TLR activated BMDM. The results of the western blot from young BMDM pretreated with LY294002 and then stimulated with LPS were shown in Figure 4.8A. There was a 1.9-fold induction in p-Akt with LPS compared to media treated young BMDM (Figure 4.8A, Lanes 1 and 2). When 5 and 20 μM of LY294002 was used in the presence of LPS, there was a 9.5-fold and 6.3-fold reduction, respectively, in the levels of p-Akt compared to LPS stimulated young BMDM (Figure 4.8A, Lanes 2, 3 and 4). Thus, we confirmed that the proximal signaling defect responsible for the age-related cytokine dysregulation involved the PI3K-Akt signaling pathway.

It was well established in literature that p65N-F-κB was a transcription factor that was capable of regulating the pro-inflammatory and the anti-inflammatory cytokines (55, 219). It was also known that p65NF-κB was regulated by PI3K-Akt signaling at several stages including IkB degradation, Ikkα phosphorylation and p65 translocation (13). Akt enhanced IκB phosphorylation leading to its degradation enabling increased p65NF-κB nuclear translocation. However, inhibition of PI3K in human peripheral blood monocytes (PBM) enhanced LPS-induced translocation of p65NF-κB (226). Similarly, PI3K inhibition enhanced TRIF-dependent NF-κB activation and production of IFN-β (13). When young BMDM were treated with wortmannin in the presence of LPS, there was a 1.9-fold increase in nuclear p65 NF-κB compared to stimulation with LPS alone (Figure 4.8B, Lanes 2 and 3). Thus, murine BMDM behave like human PBM in responding to LPS by increased NF-κB activation upon inhibition of PI3K pathway.

Discussion

SM in the aged exhibit a cytokine dysregulation which impacts on their ability to support antibody responses to polysaccharide antigens that are important for protection against encapsulated bacteria like pneumococci. In the aged, there is a decrease in production of pro-inflammatory cytokines and an increase in the anti-inflammatory cytokine, IL-10, upon stimulation with a variety of TLR ligands that utilize the MyD88 pathway. Previously we showed that alteration in cytokine production in the aged is due to a decrease in the MyD88 signaling pathway leading to a decrease in ERK activation accompanied by an aberrant increase in activation of PI3K/Akt pathway and of p38 MAP kinase (65, 95) (Chapter 3). In this study we determined if the imbalance in the production of pro- and anti-inflammatory cytokines by SM is an intrinsic defect or is due to the exposure of the macrophages to the aged spleen microenvironment. We used freshly generated BMDM from both age groups to address this question. We find that the BMDM generated from the young and the aged are equally pure and have a similar basal state of activation. Interestingly, our studies showed that BMDM from the aged also produce less of IL-12 and IL-6, but more of IL-10 than young BMDM upon stimulation with TLR2, TLR4 and TLR9 ligands recapitulating the cytokine phenotype of the aged SM. Similar results are obtained with HKSP which may utilize both TLR2 and TLR9 to activate BMDM but not TLR4, as pneumolysin, a ligand for TLR4 is inactivated in HKSP. Expression of TLR2 is similar between the young and aged BMDM (data not shown) which is consistent with the report of Boehmer et al (35) who find that expression of TLR2 and TLR4 in peripheral macrophages is not affected by age . Moreover, the PI3K-Akt signaling pathway that is previously shown by us to be elevated in the aged

SM (95)(Chapter 3) is also found to be elevated in the BMDM. Furthermore, just like in the SM, inhibition of the PI3K-Akt pathway enhanced the TLR ligand induced production of pro-inflammatory cytokines and decreased IL-10 in the aged BMDM.

These in vitro generated aged BMDM are a pure homogeneous population of cells that are not exposed to other cells that constitute the microenvironment of the aged animal, especially the spleen but share many of the phenotypic and functional characteristics (secretion of several cytokines in response to stimulation with different TLRs and increased activation of PI3K pathway) of the splenic resident macrophages in the aged (143). Thus, the cytokine dysregulation in the aged SM appears to be due to an intrinsic defect in the BMDM which also manifests in the SM. However, one cannot rule out that bone marrow stromal cells in the aged have some effects on the properties of BMDM (145, 372). It should be noted that multiple phenotypes (secretion of four different cytokines in response to three different TLR receptors, changes in PI3K, Akt and NF-κB and effects of PI3K inhibition) are shared between BMDM and SM. If the macrophage phenotype is due to the microenvironment, then it has to affect similarly several of the phenotypes listed above. Nonetheless, there are many studies demonstrating that the HSCs are altered by a combination of age-associated defects in the bone marrow microenvironment and intrinsic signaling mostly due to epigenetic modifications. The genome integrity is lost due to accumulation of DNA damage with aging and downregulation of genes involved in the maintaining of the integrity of the genome (59, 304, 372). The importance of genetic influence on intrinsic defect in HSC has been shown by the differences in the effect of aging on HSCs in aged mice from different genetic backgrounds (305). A study by Larsson and colleagues showed that an

alteration in the bone marrow microenvironment (niche) may affect the functional properties and homeostasis of HSC (199). Aging results in decreased activity of osteoblasts and increased infiltration of adipocytes with a concomitant increase in osteoclasts which affects the bone marrow microenvironment (303). These age-associated alterations in HSCs and progenitors might precede the downstream age-associated defects in B-cells, T cells, macrophages and overall immunosenescence (246, 372).

We note that the recoveries of BMDM are similar in both age groups. This is rather surprising considering that several studies report an increase in myeloid precursors in the aged bone marrow (25, 208). It is conceivable that despite increased numbers of precursors, the burst size of each myeloid progenitor is less in the aged resulting in similar numbers of macrophages in both age groups. In this context, Sebastian and colleagues show that M-CSF induced proliferation (the assay used does not distinguish between increased progenitors and clonal output) is equivalent in young and aged BMDM whereas GM-CSF induced growth response is substantially lower in the aged due to increased oxidation of Stat-5a transcription factor (315). Their observation that the teleomers are shorter in the aged than in young BMDM is consistent with reduced ability to undergo cell division. Our study emphasizes that despite an increase in numbers of myeloid lineage stem cells with age, the differentiated macrophages are not as immunocompetent as in the young.

The increased levels of PI3K p110delta and of phospho-Akt in TLR activated BMDM with age is consistent with the well known negative regulatory effects of PI3K pathway on TLR induced pro-inflammatory cytokine production (107, 308), as we find an increase in IL-10 and a decrease in IL-12 like pro-inflammatory cytokines in the aged.

Inhibition of Akt pathway with PI3K inhibitors results in a further increase in nuclear p65 (Fig 8B) which is consistent with the observed increase in pro-inflammatory cytokine production. The increase in NF-κB upon inhibition of PI3K pathway could be due to the reported positive effects of GSK-3 (an enzyme inhibited by Akt) on NF-κB activation (225). This is consistent with similar enhancing effects of PI3K inhibitors on the NF-κB activity observed in human embryonic kidney cells lines (HEK293) stimulated via NOD2 (395). Despite the links between Akt, GSK and NF-κB, it is possible that other pathways also modify the TLR induced cytokine secretion. Thus, aging enhances COX-2 mediated induction of prostaglandin-2 (PGE2), which is known to enhance IL-10 but suppress pro-inflammatory cytokines under certain conditions (71, 324, 342, 385). In turn COX-2-PGE2 pathway is enhanced in aged macrophages via upregulation of NF-κB (71, 239, 285, 353, 385). Moreover, PI3K-Akt pathway has been shown to positively regulate COX-2-PGE2 signaling complex which could in turn enhance IL-10 while suppressing pro-inflammatory cytokines (239). The interrelationships between PI3K-Akt pathways and the COX-2-PGE2 axis in aged BMDM have not been investigated thus far and will be a subject of future efforts.

Several previous studies including our own observe defects in cytokine secretion by TLR activated spleen and thioglycollate elicited peritoneal macrophages and support the notion that aged macrophages have intrinsic signaling defects (34, 63, 65, 298)**.** A study by Chen et al (66) came to a different conclusion. In this study nitric oxide production by BMDM is found to be the same in young and the aged, whereas thioglycollate elicited and resident macrophages from the two age groups differ in their ability to produce NO suggesting that the microenvironment in the aged affected

macrophage function. Such conclusions are further supported by the studies by Stout et al. (339, 340) in which exposure of macrophages to different cytokines prior to TLR stimulation altered the cytokine responses. It turns out that Chen and colleagues utilize thioglycollate induced macrophages. But Stout and colleagues use only young BMDM, young thioglycollate elicited peritoneal macrophages and resident macrophages. These studies are unlike ours and other recent studies that focuse on spleen resident macrophages. Although one cannot rule out the ability of cytokines like gamma-interferon to alter the TLR responses to macrophages, the present study establishes that BMDM from the aged behave similar to spleen resident macrophages with respect to responses to three different TLRs and the increased activity of PI3K-Akt enzymes, strongly supporting the concept that the aged macrophages do have intrinsic defects. As noted above aged BMDM have shortened teleomers which is consistent with an intrinsic defect hypothesis for the phenotype of splenic macrophages from the aged (315).

These observations about the ability of PI3K-Akt pathway to modulate cytokine production by aged macrophages are relevant in the context of reduced immune responses of the aged to *S. pneumoniae.* The pro-inflammatory cytokines, IL-6, TNF-α and IL-12 are all critical for antibody response to polysaccharide antigen and the subsequent clearance of *S. pneumoniae* infection as depletion of these cytokines by antibody treatment or gene knockouts increases bacterial load and overall lethality in infected mice (17, 38, 64, 178, 230, 355, 356). Moreover, enhanced IL-10 associates with poor clearance, increased lethality and bacterial load in infected mice (357, 358). IL-10 mediated downregulation of p65NF-κB also correlates with increased susceptibility to *S. pneumoniae* infection (61, 293). Thus, our finding that PI3K inhibition by

pharmacological inhibitors not only reduces IL-10 but also increases p65 NF-κB and critical cytokines, IL-12, TNF-α and IL-6 upon stimulation with individual TLR ligands and HKSP, provides a novel approach to enhance immune responses to pneumococcal infections in the aged.

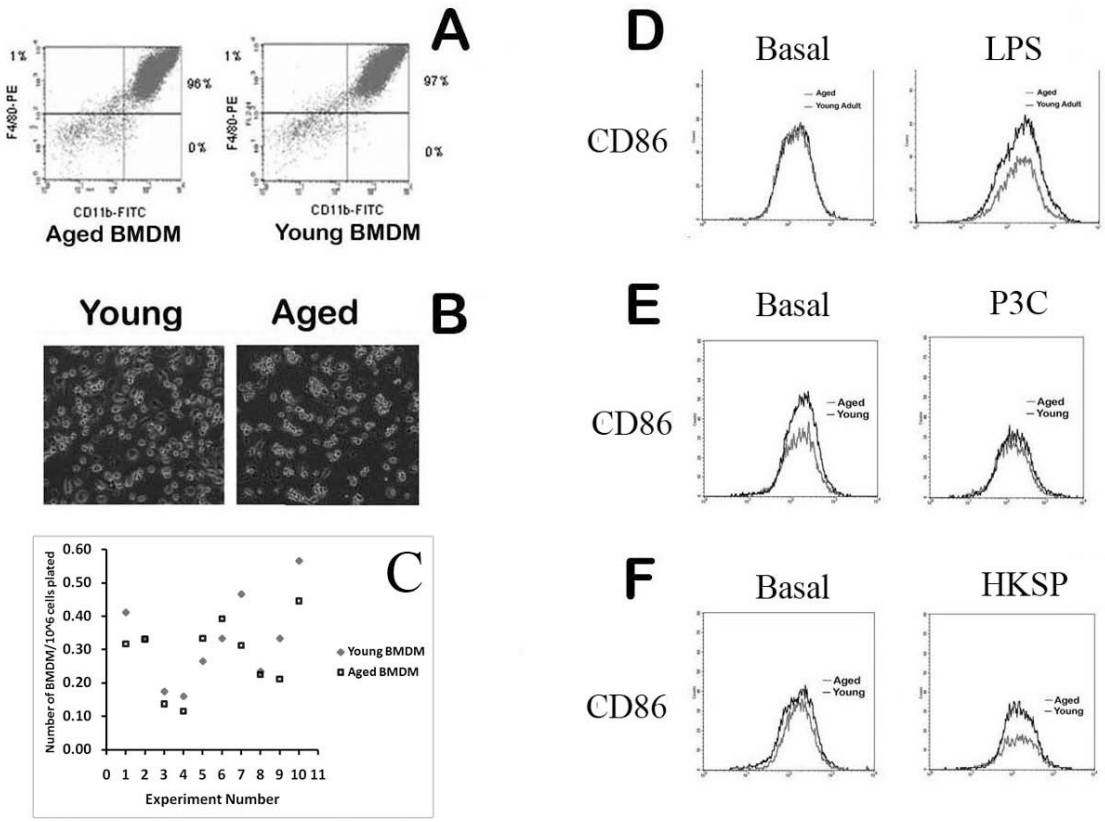
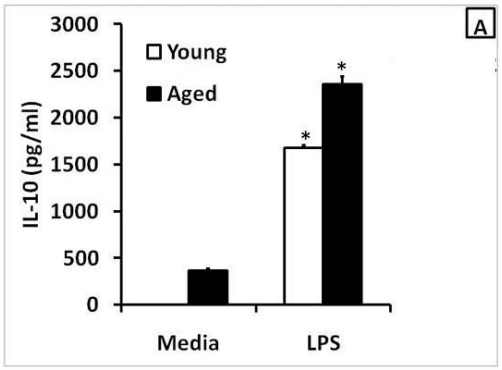


Figure 4.1: The purity, recovery, morphology and activation status of BMDM is similar between the aged and young.

Expression of CD11b and F-4/80 by BMDM from aged and young mice (Figure 1A). Photographs of newly generated BMDM from aged and young Balb/c mice seen at 40X total magnification with an Olympus microscope (Figure 1B). Bone marrow cells ($2.5x10^6$ cells/ml) were cultured for seven days with 30% L929 supernatant. The newly derived BMDM were enumerated and the number of BMDM recovered per 10^6 BM cells was calculated for the aged and the young (Figure 1C). The graph represented results from ten of 15 experiments. BMDM from young and aged mice were cultured in medium or LPS (Figure 1D), or Pam3CSK4 (P3C) (Figure 1E) or HKSP (Figure 1F) and then stained with anti- CD86 FITC.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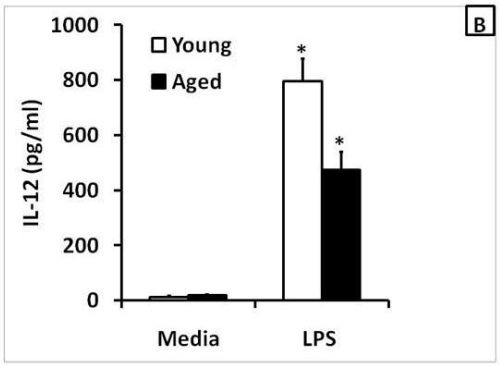
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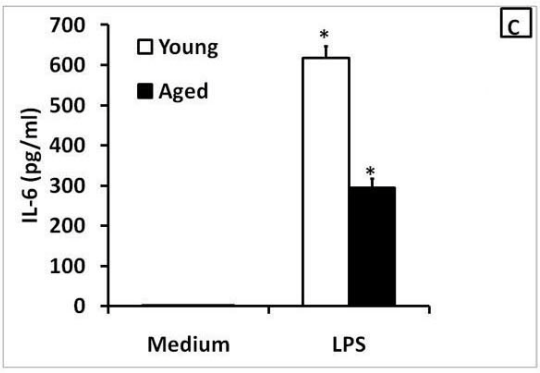
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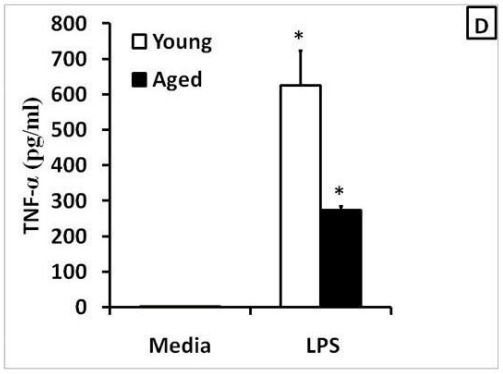
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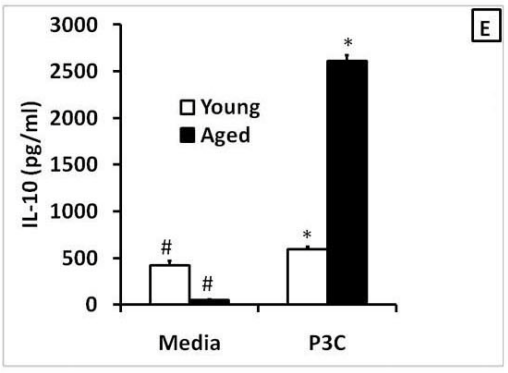
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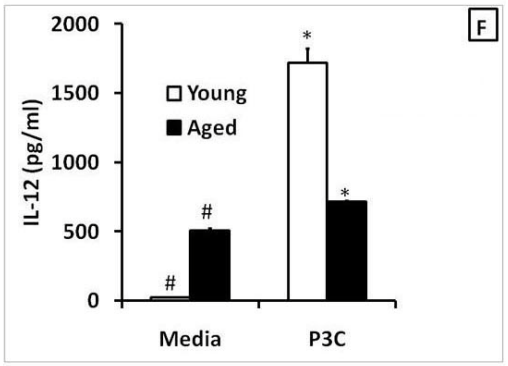
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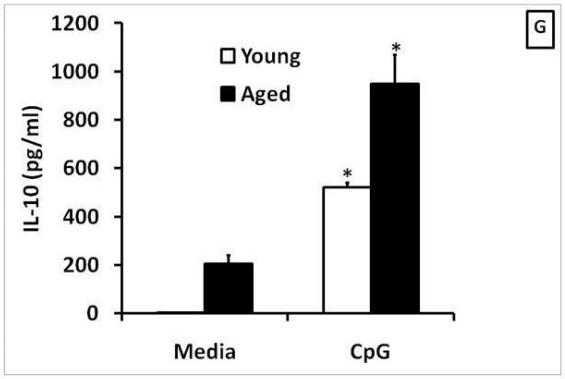
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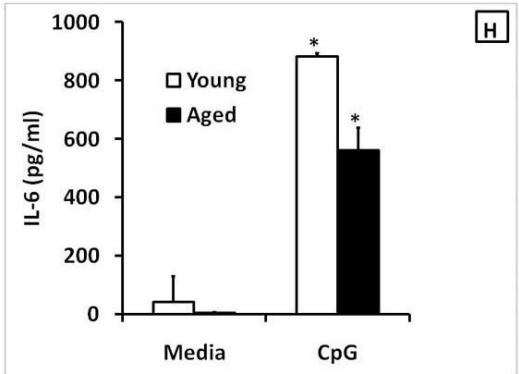
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Figure 4.2: Cytokine production by young and aged BMDM stimulated with TLR2, 4 and 9 ligands.

BMDM ($2.5x10^5$ cells/ml) from young and aged mice were stimulated with the TLR agonists, LPS (1µg/ml) (Panels A, B, C and D), P3C (1µg/ml) (Panels E and F) or CpG (2µg/ml) (Panels G and H) for 24 hours. The supernatants were collected and analyzed by a sandwich ELISA for IL-10 (Panels A, E and G), IL-12(p40) (Panels B and F), IL-6 (Panel C and H) and TNF-α (Panel D). In panels A, C, D, G and H levels of cytokines in unstimulated BMDM from one or both age groups were undetectable. The constitutive production of IL-12 by aged BMDM seen in panel F was not observed in other experiments. Results from one of five experiments were shown as Mean $\pm$ SE values of 8-12 determinations. The statistical significance of differences in cytokine secretion between the young and the aged was indicated by the symbol * and it was set at $p<0.04$.

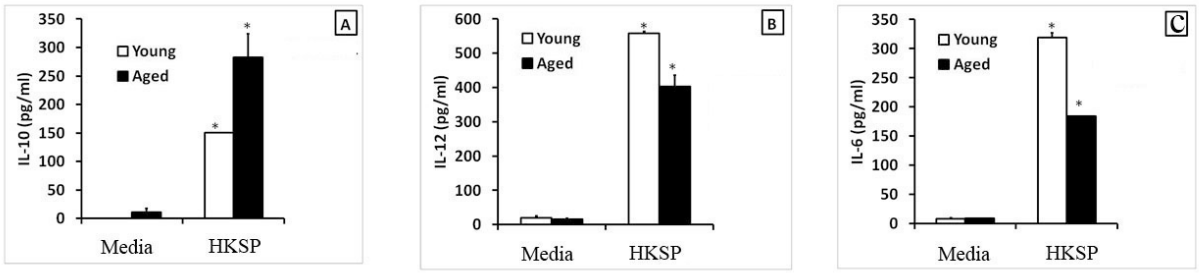

Figure 4.3: Cytokine production by young and aged BMDM stimulated with HKSP

The newly derived BMDM from young and aged mice were stimulated with HKSP ($2x10^6$ CFU/ml) for 24 hours. The supernatants were collected and analyzed by a sandwich ELISA for IL-10 (Panel A), IL-12(p40) (Panel B), and IL-6 (Panel C). In panel A, IL-10 levels in unstimulated young BMDM were undetectable. Results from one of four experiments were shown as Mean $\pm$ SE values of 8-12 determinations. The statistical significance of differences in cytokine secretion between the young and the aged was indicated by the symbol * and it wass set at $p<0.0003$.

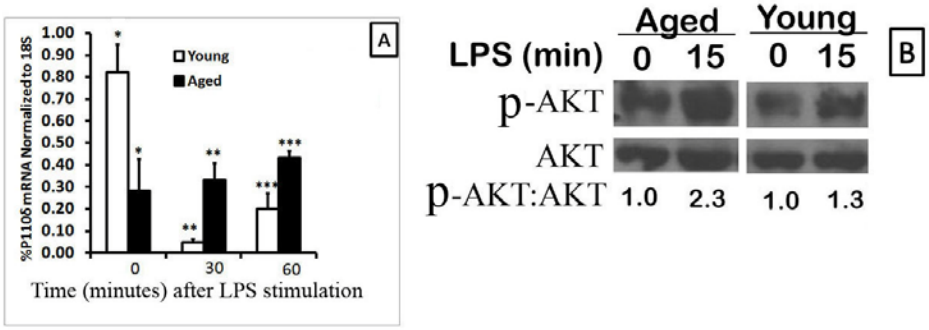

Figure 4.4: Age-associated changes in expression of mRNA for p110δ subunit of PI3K and activation of AKT in TLR4 activated BMDM

Panel A: mRNA was isolated from young and aged mice BMDM ($3x10^6$ cells/ml) after stimulation with LPS (1μg/ml) and converted to cDNA. QRT-PCR analysis of mRNA was shown in panel A for P110δ subunit of PI3 kinase in the newly derived BMDM from young and the aged. The C_t values for each probe were normalized to the 18S RNA. The symbols *, ** and *** indicated the statistical significance ($p<0.05$) of differences in RNA expression between the young and the aged at different time points. Panel B: The BMDM were stimulated with LPS 1.0 μg/ml for 15 minutes, total cell lysates were isolated and immunoblotted for p-Akt. The blots were stripped and probed for total Akt and for beta-actin. The numbers represent densities of bands normalized to total Akt between aged and young with the values for unstimulated cells from both aged and young mice set to one.

The figure in panel B was a composite of blots for p-Akt and Akt from the same membrane that was stripped and reprobed. The young and aged cell lysates were electrophoresed on the same gel and the membranes were exposed for identical time periods. Although a detailed kinetic analysis of LPS stimulation was performed, only the lanes representing 0 and 15 minutes time points were shown for clarity. Results were representative of four experiments.

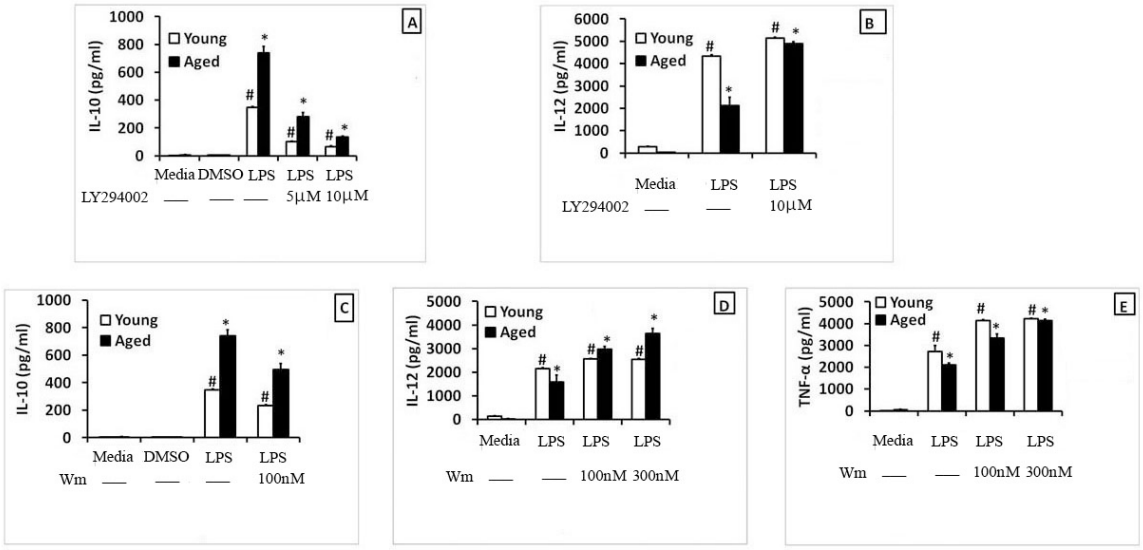

Figure 4.5: PI3K inhibitors partially rescue the age-associated defects in TLR-4 induced cytokine production.

The graphs show cytokine secretion by aged and young adult BMDM pretreated with LY294002 (Panels A and B) or wortmannin (Panels C, D and E) for 60 minutes and then stimulated with LPS (1.0μg/ml) for 24 hours. Supernatants were collected and assayed for IL-10 (panels A and C), IL-12(p40) (panels B and D) and TNF-α (Panel E) by sandwich ELISA. Data were presented as mean+/-SE values of 6-10 determinations and were representative of three independent experiments. The symbols * and # indicated statistical significance ($p<0.005$) of differences in responses in groups treated with LPS alone compared to groups treated with LPS + PI3K inhibitor.

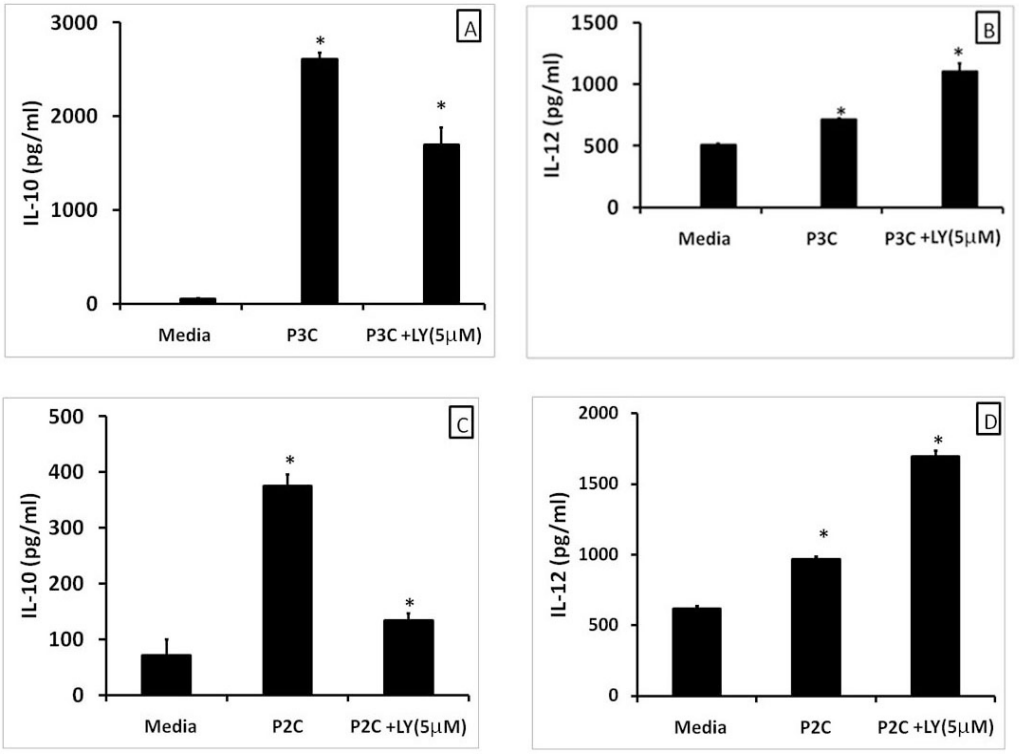

Figure 4.6: PI3K inhibitors alter the pattern of cytokine secretion by aged BMDM stimulated with ligands for TLR-2 heterodimers.

The graphs show cytokine secretion by aged BMDM pretreated with LY294002 for 60 minutes and then stimulated with P3C (Panels A and B) or P2C (Panels C and D) for 24 hours. Supernatants were collected and assayed for IL-10 (Panels A and C) or IL-12(p40) (Panels B and D) by sandwich ELISA and were representative of three experiments. Data were presented as mean +/- SE of quadruplicate wells. The symbol * indicated statistical significance ($p<0.03$) of differences in responses in the aged BMDM treated with Pam3CSK4 (or Pam2CSK4) with or without the PI3K inhibitor.

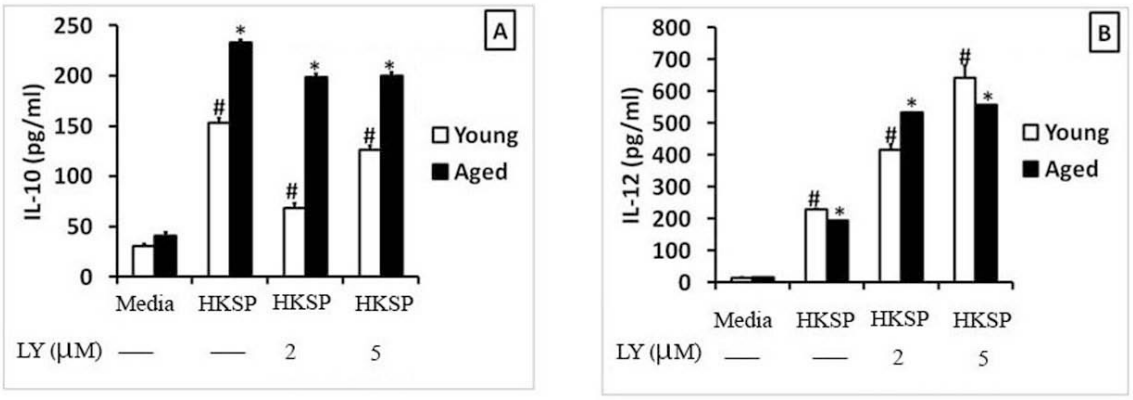

Figure 4.7. The inhibition of PI3K by either LY294002 or wortmannin differentially regulates the HKSP-mediated induction of anti- and pro-inflammatory cytokines in BMDM.

The graphs show cytokine secretion by aged and young BMDM pretreated with LY294002 (Panels A and B) for 60 minutes and then stimulated with HKSP (2x 10^8 CFU/ml) for 24 hours. Supernatants were collected and assayed for IL-10 (Panel A) and IL-12(p40) (Panel B) by sandwich ELISA. Data were presented as mean +/- SE of quadruplicate determinations. The symbols * and # indicated statistical significance ($p<0.03$) of differences in responses in the aged BMDM treated with HKSP alone compared to the responses obtained by treating with HKSP + PI3K inhibitor. Results were representative of four experiments.

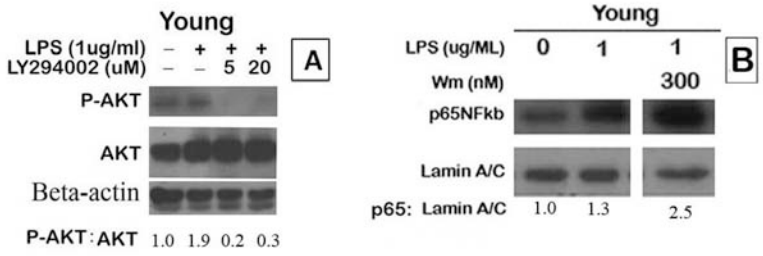

Figure 4.8. PI3K inhibitors reduce activation of Akt but enhance nuclear translocation of p65 NF-κB.

Panel A: The newly derived BMDM from young mice were pre-treated with either LY294002 or medium for 1 hour and then stimulated with LPS (1μg/ml) for 15 minutes. The blots were probed for p-Akt and total Akt and actin after stripping (panel A). The numbers represent densities of bands normalized to total Akt. The final figure in A was a composite of blots for p-Akt, Akt and actin for young samples run on the same membrane that was stripped and reprobed. Panel B: The newly derived BMDM from young were pretreated with or without wortmannin and then stimulated with LPS (1μg/ml) at 30 minutes and the nuclear extract was isolated. The lysate was run on an SDS-PAGE gel, transferred to a membrane and then immnoblotted for p65 NF-κB. The numbers represent densities of bands normalized to LaminA/C, with the values for unstimulated BMDM set to 1. The final figure in B was a composite of blots for p65NF-κB and Lamin A/C for young samples run on the same membrane that was stripped and reprobed. The intervening space (between second and third lane) was for lanes loaded with lysates from other time points or treated with other inhibitors but were deleted to focus on the effect of LPS on p65 translocation at 30 minutes, which was maximum in this experiment. Results were representative of three to five independent experiments.

CHAPTER 5: Novel role of glycogen synthase kinase-3 in secretion of IL-10 and pro-inflammatory cytokines activated via TLR2 and TLR4 receptors

INTRODUCTION

There are several studies exploring the possibility of modulating macrophages or dendritic cells as potential vaccine adjuvants especially for populations like the elderly with impaired vaccine response (94, 301). Some studies are beginning to focus on modulating glycogen synthase kinase-3 (GSK-3) to enhance cytokine production by myeloid cells such as dendtritic cells, human peripheral blood monocytes and macrophages, which are targets for adjuvants (28, 115, 225). GSK-3 is an enzyme that is emerging as a prominant pleoitropic kinase in modulating numerous cellular processes. It is a focal point for an array of upstream signaling cascades. GSK-3 regulates a diverse array of proteins and transcription factors such as CREB and NF-κB (125, 255). It is ubiquitously expressed in B-cells, macrophages, T-cells, NK cells and neutrophils which play crucial roles in both innate and adaptive immunity (28). It has two isoforms, GSK-3α and GSK-3β, which are encoded by different genes. Though the two isoforms share very high homology in their kinase domain, there are some reports of a lack of redundancy between the two isoforms (151).

Macrophages upregulate costimulatory molecules and secrete cytokines to modulate the shift from innate immunity to adaptive immunity (292). Macrophages can also present antigen to T cells in the context of MHC-II and produce cytokines, such as IL-1, which help activate T cells. Macrophages interact with T cells via CD40:CD40 ligands to provide critical co-stimulatory signals (362). DCs are even more efficient in antigen uptake, transport and presentation to T-cells. They are also known to upregulate

costimulatory molecules and secrete some critical pro-inflammatory cytokines that are crucial for both innate and adaptive immunity (161, 326, 337).

Several studies show that inhibition of GSK-3 in either DC or human peripheral blood monocytes in the presence of TLR stimulation results in a suppression of the pro-inflammatory cytokines along with an enhancement in IL-10. While the role of GSK-3 inhibition on the activation of innate cells likes DC and monocytes are thoroughly investigated, there is a conspicuous absence of studies on the role of GSK-3 in cytokine regulation in primary splenic or bone marrow derived macrophages (225, 295, 301). To fill this gap of knowledge, we investigated the role of GSK-3 in cytokine production in SM and BMDM. We earlier established that the PI3K-Akt pathway is more active in TLR stimulated aged SM compared to the young. This led to increased phosphorylation, and that inhibiting PI3K led to decreased phosphorylation of GSK-3 (Fallah et al., manuscript in press). Here we report the surprising result that inhibition of GSK-3 in the presence of ligands for TLR-2 or -4, leads to an increase in both IL-10 and the pro-inflammatory cytokines in macrophages. The increase in cytokines appears to be due to an increase in activation of transcription factor NF-κB.

Results

Inhibition of GSK-3 enhances both pro- and anti-inflammatory cytokines in young and aged splenic macrophages

Purified SM from aged mice were stimulated with either TLR-2 or TLR-4 ligands in the presence of SB216763, a well characterized specific inhibitor of GSK-3, for 24 hours and the supernatant was assayed for cytokines by ELISA. Inhibition of GSK-3 enhanced the production of IL-10 when TLR-4 or TLR-2 ligands were used for stimulation (Figures 5.1A and 5.1D) which is in agreement with previous reports that used DCs (225). However, inhibition of GSK-3 in the presence of LPS, a TLR-4 ligand, significantly enhanced IL-12(p40) and TNF-α production (Figures 5.1B and 5.1C). Similarly, inhibition of GSK-3 in the presence of P3C, a TLR2/1 ligand, resulted in enhancement of TNF-α (Figure 5.1E) and IL-12 (Figure 5.1D). This result was contrary to what had been reported in literature in regards to human blood monocytes or BMDC. Hence, the GSK-3 signaling pathway could be modulating key transcription factors differently than what had been reported in blood monocytes or BMDC (151, 322, 365, 393). We wondered if this effect of GSK-3 inhibition to enhance both the anti- and the pro-inflammatory cytokines was unique to macrophages from aged mice. Therefore, we evaluated the cytokine response of SM stimulated with LPS or P3C in the presence of GSK-3 inhibitors. Similar to the result with aged macrophages, there was an increase in IL-10 and IL-12p40 when young adult SM were stimulated with LPS (Figures 5.2A) or P3C in the presence of SB216763 (Figure 5.2B). Thus, the ability of SB216367 to enhance IL-10 and the pro-inflammatory cytokines in splenic macrophages appeared to be independent of the age of the mice.

GSK-3 inhibition enhances both pro- and anti-inflammatory cytokines in BMDM independent of the age of the mice.

Previously, we had shown that inhibition of PI3K enhanced the pro-inflammatory cytokines but suppressed of IL-10 in either splenic or bone marrow macrophages, indicating that the effect of this signaling pathway on macrophages was independent of the microenvironment. Hence, it was of interest to evaluate the effect of GSK-3 inhibitor on cytokine response of BMDM stimulated with TLR ligands. Interestingly, inhibition of GSK-3 in the presence of LPS in aged BMDM enhanced both IL-10 and the pro-inflammatory cytokines, IL-12 and TNF-α (Table 5.1). When young BMDM were evaluated in the presence of LPS and SB216764, there was an increase in IL-10, IL-12 and TNF-α (Table 5.2). This showed that the ability of GSK-3 inhibition to enhance IL-10 and the pro-inflammatory cytokines was applicable to different types of primary macrophages.

We next asked if the ability of GSK-3 inhibition to enhance both IL-10 and the pro-inflammatory cytokines was influenced by the time of stimulation. Hence, we did a time kinetics analysis of young BMDM treated with SB216763 in the presence of LPS for 6, 24 and 48 hours. We found that GSK-3 inhibitor significantly enhanced IL-10 at all concentrations and at all time points tested (Figure 5.3A). In the case of IL-12, there was a moderate but statistically significant increase at all concentrations of SB216763 at 24 hours, but only at 10 μM at 48 hours (Figure 5.3B).

Enhancement of pro-inflammatory cytokines upon GSK-3 inhibition is independent of its effects on IL-10 production in macrophages

We asked if the ability of GSK-3 inhibitor to enhance IL12 was influenced by IL-10, which was known to inhibit pro-inflammatory cytokines. Accordingly, we cultured young BMDM from IL-10 knockout mice in C57BL/6 background with SB216763 in the presence of LPS for 6, 24 and 48 hours. We then assayed for IL-12 and found that GSK-3 significantly enhanced IL-12 at all concentrations of the inhibitor at 24 and 48 hours (Figure 5.4).

The enhancement of pro-inflammatory cytokines with GSK-3 inhibitor is not seen with BMDC

We have shown that the effects of GSK-3 inhibition on both SM and BMDM were similar irrespective of the duration of the cultures or IL-10 expression. We decided to evaluate the effects of GSK-3 inhibition on the cytokine profile in BMDC. We cultured BMDC in the presence of SB216763 and LPS for 24 hours and measured IL-10, IL-12p40 and IL-12p70 by ELISA. GSK-3 inhibition in the presence of LPS resulted in increased IL-10 production (Figure 5.5A) just like in splenic macrophages and BMDM. However, inhibition of GSK-3 reduced production of IL-12p40 (Figure 5.5B) and IL-12p70 (Figure 5.5C) in agreement with the reported results using rodent and human monocyte derived dendritic cells (210, 301). Thus, the role of GSK-3 in modulating IL-10 and the pro-inflammatory cytokines in both splenic macrophages and BMDM was unique and different from its role in BMDC.

The PI3K-Akt signaling pathway modulates the ability of GSK-3 to regulate cytokines in macrophages

We had shown in SM that Akt phosphorylation via PI3K led to phosphorylation of GSK-3 which was known to result in inactivation of GSK-3 (Chpater 3 Figure 11A, B and C). We asked if the activity of GSK-3 in BMDM was also regulated via PI3K/Akt pathway. Upon inhibition of PI3K in BMDM with LY294002, there was a 30% reduction in p-GSK-3 in the aged BMDM compared to LPS stimulation (Figure 5.6A, lanes 2, 3 and 4). Since, the modulatory role of GSK-3 on IL-10 and the pro-inflammatory cytokines was independent of the age of the BMDM; we extended this study to BMDM isolated from young adult mice. There was a 45% reduction in GSK-3 posphorylation in BMDM treated with LY294002 compared to LPS stimulation at 30 minutes (Figure 5.6B, lanes 3 and 4).

Inhibition of GSK-3 enhances activation of p65NF-κB

We next evaluated the effect of GSK-3 inhibition on nuclear translocation of p65 NF-κB, a known transcription factor that regulates the production of pro-inflammatory cytokines (255). In the presence of SB216763, the stimulation of young BMDM with LPS resulted in a 2.8-fold increase in nuclear localization of p65NF-κB (Figure 7 lanes 2 and 3). Thus, in macrophages, the ability of GSK-3 inhibition with SB216763 to enhance the pro-inflammatory cytokines involved the enhancement of NF-κB translocation, a known modulator of pro-inflammatory cytokines.

Discussion

Using SM purified by negative selection from both aged and young mice, we demonstrated that inhibition of GSK-3 by SB216763 enhanced both IL-10 and the pro-inflammatory cytokines, IL-12 and TNF-α. We further showed that GSK-3 inhibition enhanced these cytokines in vitro generated BMDM from aged and young Balb/c, and from young C57BL/6 mice. The current study indicates that this phenomenon is unique to macrophages as we are able to demonstrate in BMDC that upon inhibition of GSK-3 in the presence of ligand for TLR-4 there is an enhancement in IL-10 with suppression of the pro-inflammatory cytokines, IL-12p40 and IL-12p70. This finding in BMDC is supported by numerous other findings in both human peripheral blood derived dendritic cells and mouse BMDC, which show a reduction in 12p40, IL-6, TNF-α, and IFN-γ with an enhancement in IL-10 (225, 295).

Macrophages and dendritic cells share many similar functional characteristics. BMDC from both Balb/c and C57Bl/6 express CD80, CD86 and produce similar levels of IL-6, IL-12p40 and TNF-α upon stimulation with LPS (292). We demonstrated that upon stimulation of both BMDM and SM with LPS, IL-12p40, TNF-α and IL-6 are produced. Macrophages are able to present antigen and produce relevant cytokines that activate T cells and increase CD80 just like dendritic cells (292).

As a result, one might wonder why we had arrived at seemingly discordant results with monocytes and DC. However, there are studies that point to the fact that cytokine-regulatory capacity of GSK-3 is cell type-specific (166). Thus, Martin and colleagues evaluated the effects of GSK-3 inhibitors on cytokine production in the presence of

ligands to several different TLRs in human peripheral blood monocytes. They showed enhanced secretion of IL-10 with a suppression of pro-inflammatory cytokines (225). Overall, GSK-3 inhibition has anti-inflammatory effects on mature DCS derived from human monocytes (MoDC) (225). This cell-specific modulatory role of GSK-3 is also seen by Liu et al (211). They showed that immature DCs (iDC) generated from human monocytes with GMCSF plus IL-4 in the presence of GSK-3 inhibitor, LiCl, upregulated CD86, IL-6, IL-8, TNF-α, IL-1β with a slight increase in IL-10. However, when these very iDCs are matured by LPS stimulation in the presence of LiCl there is an increase in IL-10 with a reduction in IL-6, TNF-α and IL-1β (210).

Several other studies report results similar to ours but different from the Martin group in the context of different cell types. Using the ST2 Stromal cell line derived from mouse bone marrow, it is shown that IL-17 enhances the production of IL-6 at the mRNA level in the presence of GSK-3 inhibitors, indicating an anti-inflammatory property of GSK-3 (323). It is demonstrated in neonatal cardiomyocytes that GSK-3 is a negative regulator of pro-inflammatory cytokines. Upon LPS stimulation of the cells in the presence of GSK-3 inhibitor there is an enhancement of TNF-α. Not only that but when a dominant negative GSK-3 is over-expressed similar enhancing results are observed, but these could be reversed by over-expressing normal GSK-3 (322). Other studies with fibroblasts and human microvascular cells also support an anti-inflammatory role for GSK-3 (151, 365).

When RAW cells are stimulated with LPS in the presence of GSK-3 inhibitor with or without IFN-γ there is a higher production of IL-10 with suppression of INOS and NOS (206). Another study shows that activating RAW cells with IFNγ plus GSK-3 inhibitors or SiRNA knockdown of GSK-3 results in a suppression of TNF-α, Rantes, INOS and

NOS. The mechanism accounting for this effect is shown to be via phosphorylation of GSK-3α at Tyr-279 and GSK-3β at Tyr-216 by the proline rich tyrosine kinase (Pyk2) (351). There are two central issues that one can garner from this study; one is that the agonist is IFNγ and not LPS. The paper shows that the mechanism involves phosphorylation mediated activation of GSK-3. This is contrary to the findings of LPS mediated regulation of GSK-3, which is a phosphorylation-mediated inactivation at the serine position by the protein kinase family (PKA, PKB, PKC) on the N-terminal serine residues. GSK-3α and GSK-3β are inhibited when phosphorylated by protein kinase B/Akt on serine -21 and GSK-3β on serine-9 (88, 101, 126, 225). Studies show that signaling cascades differ from transformed cell lines and primary macrophages during infections or even between cell lines. This is demonstrated in both P388D1 and RAW264.7, two macrophage cell lines, stimulated with LPS (23, 112, 294). Hence, the findings in RAW cells on the role of GSK-3 on cytokines, though share some similarity with our findings in primary splenic and bone marrow macrophages, they are not comparable because of the inherent signaling differences between these two categories of cells.

In explaining the paradoxical regulation of anti- and pro-inflammatory cytokines by GSK-3 inhibitors in different cell types, one has to take into consideration the uniqueness of this multifunctional kinase. The multi-signaling ability of GSK-3 is due to its unique ability to target and modulate a plethora of transcription factors which include NF-κB, NFATC and CREB (28, 166). Similarly upstream several kinases, such as Akt and mTOR, are known to affect GSK-3 activity. Accordingly, we are able to demonstrate in aged and young BMDM that GSK-3 is regulated by TLR-4 induced PI3K-Akt signaling

pathway. We show in both aged and young groups that inhibition of PI3K results in a decrease in the phosphorylation of GSK-3. Similar results are obtained by Wang and colleagues in TLR dependent regulation of GSK-3 (371) .

Since IL-10 is known to inhibit IL-12 like cytokines, we evaluated if GSK-3 dependent regulation pro-inflammatory cytokines involves IL-10. We showed that the ability of GSK-3 to regulate the pro-inflammatory cytokines is independent of IL-10 using BMDM from IL-$10^{-/-}$ mice. This is in agreement with a study by Ohtani and colleagues, who find that upregulation of IL-12 upon GSK-3 inhibition is independent of IL-10 (266).

When we stimulated young BMDM with LPS in the presence of GSK-3 inhibitor, there is an increase in the translocation of p65NF-κB. The role of GSK-3 in the regulation of p65NF-κB is multi-faceted and is dependent on the type of cell or its activation status. GSK-3 is considered by many studies to be a positive regulator of p65NF-κB activity (322, 365). On the other hand, studies of human intestinal epithelial cells show that LiCl, an inhibitor of GSK-3, enhances p65NF-κB binding and transcriptional activities (255). Schwabe and colleagues show that in hepatocytes stimulated by TNF-α, GSK-3 inhibition induces p65 phosphorylation and upregulates its transctivation in agreement with our studies (313). However, Martin and colleagues do not find any differences in the nuclear translocation of the p50 or p65 subunits in LPS stimulated human monocytes in the presence of GSK-3 inhibitors (225). Hence, the ability of GSK-3 inhibition to induce the production of the pro-inflammatory cytokines in SM and BMDM could be due to the induction of p65NF-κB, which is a transcription factor that regulates pro-inflammatory cytokines.

We can not account for the transcriptional regulation of IL-10 and this will be the focus of future experiments. We propose that IL-10 upregulation depends on the transcription factor CREB and GSK-3 negatively regulates both CREB and p65NF-κB. Hence, inhibition of GSK-3 upregulates both pro- and anti-inflammatory cytokines via increased activities of p65NF-κB and CREB respectively; presently we can not rule out that the effects of GSK-3 on these transcription factors are indirect by its ability to modulate other regulators of these pathways (Figure 5.8).

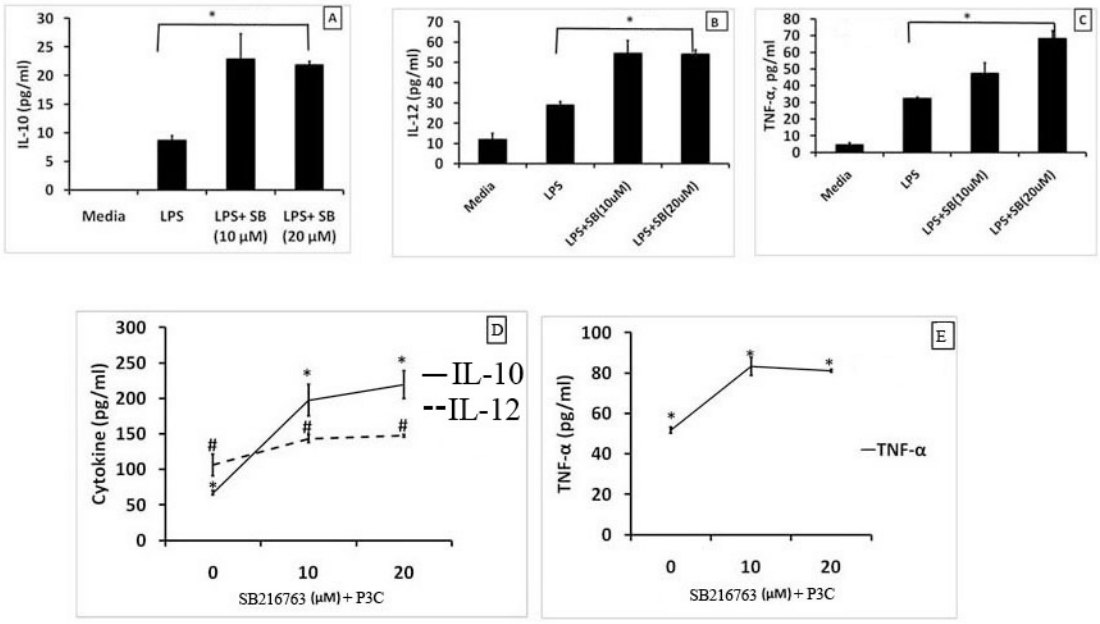

Figure 5.1: Pharmacological inhibition of GSK-3 results in increased production of IL-10 and the pro-inflammatory cytokines in aged splenic macrophages

The graphs showed cytokine secretion by purified aged SM pretreated with SB216763 (Panels A,B, C, D and E) for 60 minutes and then stimulated with either LPS (1.0μg/ml) (Panels A, B and C) or Pam3CSK4 (1.0μg/ml) (Panels D and E) for 24 hours. Supernatants were collected and assayed for IL-10 (panels A and D), IL-12(p40) (panels B and D) and TNF-α (Panels C and E) by sandwich ELISA. Data are presented as mean+/-SE values of 6-10 determinations and are representative of three independent experiments. The symbols * and # indicated statistical significance ($p<0.05$) of differences in responses in groups treated with LPS or P3C alone compared to groups treated with LPS (or P3C) + GSK-3 inhibitor. SB in the graphs represents SB216763.

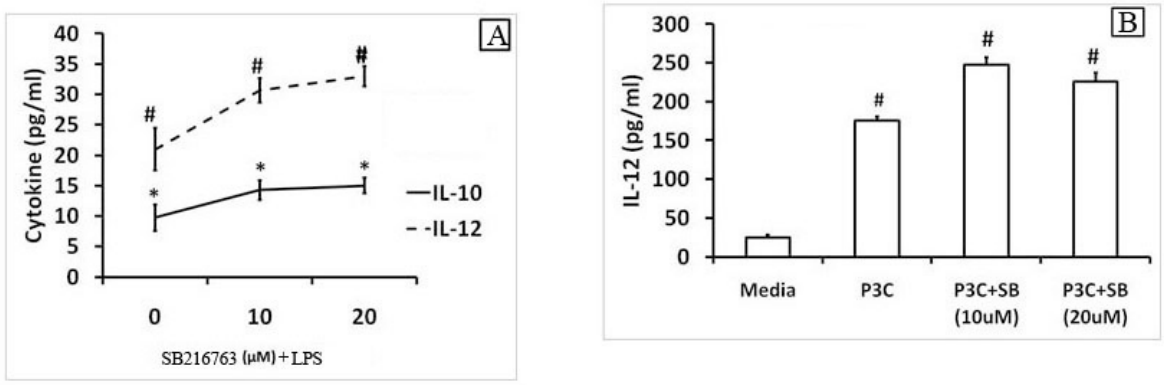

Figure 5.2: GSK-3 inhibition enhances both IL-10 and IL-12 in young splenic macrophages

Young adult purified SM ($2.5x10^5$ cells/ml) were pretreated with SB216763 (Panels A and B) for 60 minutes and then stimulated with either LPS (1.0µg/ml) (Panel A) or Pam3CSK4 (1.0µg/ml) (Panel B) for 24 hours. Supernatants were collected and assayed for IL-10 (panels A) and IL-12(p40) (panels A and B) by sandwich ELISA. Data were presented as mean+/-SE values of 6-10 determinations and were representative of three independent experiments. The symbols * and $^\#$ indicated statistical significance ($p<0.05$) of differences in responses in groups treated with LPS or P3C alone compared to groups treated with LPS (or P3C) + GSK-3 inhibitor. SB in the graphs represents SB216763.

Table 5.1: GSK-3 Inhibition enhanced both IL-10 and the pro-inflammatory cytokines in aged BMDM

	IL-10 (pg/ml)	IL-12 (pg/ml)	TNF-α (pg/ml)
Media	6+/- 2	421+/- 51	45 +/- 27
LPS	740+/- 44	555+/- 11	2114 +/-74
LPS +SB216763	1367+/- 36	1102+/- 15	3204+/-55
P-value	P<0.009	P<0.003	P<0.001

Table 5.1: Inhibition of GSK-3 with SB216763 enhances both pro- and anti-inflammatory cytokines in aged BMDM

Aged BMDM ($2.5x10^5$ cells/ml) were pretreated with SB216763 (Table 5.1) for 60 minutes and then stimulated with LPS (1.0µg/ml) for 24 hours. Supernatants were collected and assayed for IL-10 and IL-12(p40) and TNF-α (Table 5.1) by sandwich ELISA. Data are presented as mean+/-SE values of 6-10 determinations and are representative of three independent experiments. Statistical significance was indicated by $p<0.05$, which compared the differences in responses in groups treated with LPS alone to groups treated with LPS + GSK-3 inhibitor.

Table 5.2: GSK-3 Inhibition enhanced both IL-10 and the pro-inflammatory cytokines in young BMDM

	IL-10 (pg/ml)	IL-12 (pg/ml)	TNF-α (pg/ml)
Media	0 +/- 0.1	135+/-26	2 +/- 1
LPS	347 +/- 6	2305+/- 132	2729 +/-263
LPS +SB216763	495+/-23	2678+/- 43	3585+/-30
P-value	P<0.02	P<0.003	P<0.001

Table 5.2: SB216763 upregulates both pro- and anti-inflammatory cytokines in young BMDM just like the aged BMDM

Young adult purified BMDM ($2.5x10^5$ cells/ml) were pretreated with SB216763 for 60 minutes and then stimulated with either LPS (1.0µg/ml) for 24 hours. Supernatants were collected and assayed for IL-10, IL-12(p40) and TNF-α (Table 5.2) by sandwich ELISA. Data are presented as mean+/-SE values of 6-10 determinations and are representative of three independent experiments. The p-values indicate statistical significance ($p<0.05$) of differences in responses in groups treated with LPS alone compared to groups treated with LPS + GSK-3 inhibitor.

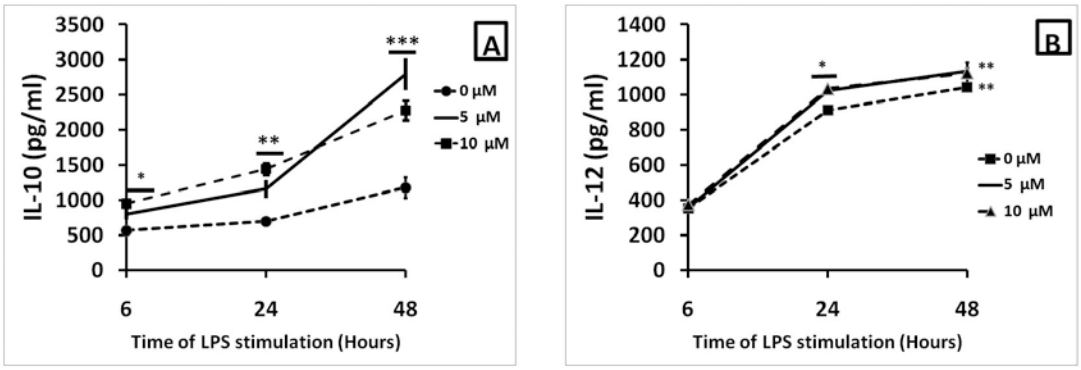

Figure 5.3: Kinetics of cytokine production in young BMDM treated with GSK-3 inhibitor.

Young adult purified BMDM ($2.5x10^5$ cells/ml) from Balb/c mice were pretreated with SB216763 (Panels A and B) for 60 minutes and then stimulated with LPS (1.0µg/ml) (Panels A and B) for 6, 24 and 48 hours. Supernatants were collected at each of these different time points and assayed for IL-10 (panel A) and IL-12(p40) (panel B) by sandwich ELISA. Data are presented as mean+/-SE values of 6-10 determinations and are representative of three independent experiments. The p-values indicate statistical significance ($p<0.05$) of differences in responses in groups treated with LPS alone compared to groups treated with LPS + GSK-3 inhibitor at the different time points.

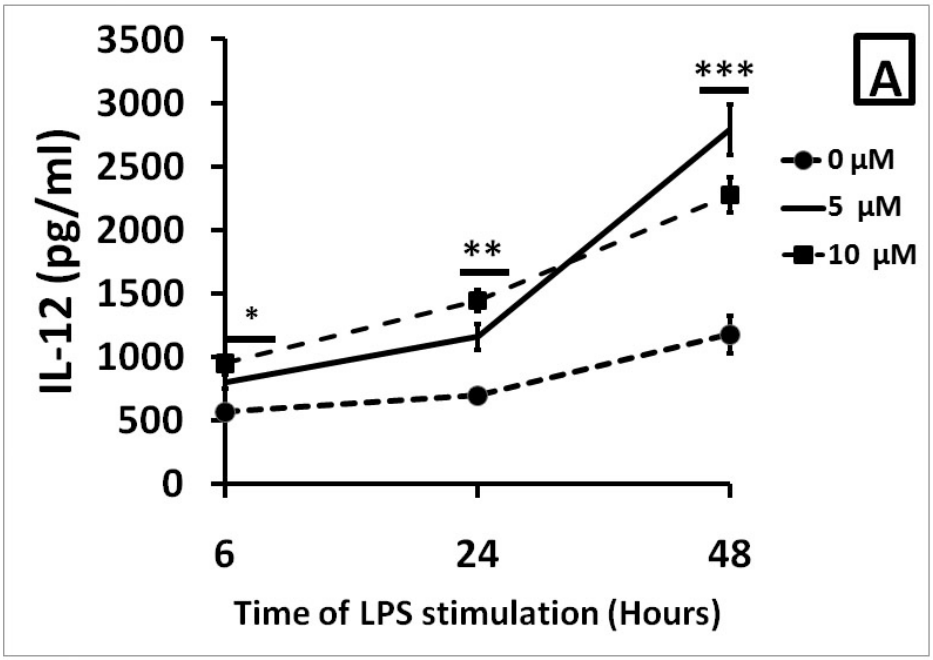

Figure 5.4: The ability of GSK-3 inhibitors of GSK-3 to induce higher production of pro-inflammatory cytokines is independent of IL-10

BMDM ($2.5x10^5$ cells/ml) from young adult IL-10 knockout mice on a C57BL/6 background were evaluated for the production of IL-12 at 6, 24 and 48 hours (Panel A). The BMDM were pretreated with SB216763 (Panels A) for 60 minutes and then stimulated with LPS (1.0µg/ml) (Panels A) for 6, 24 and 48 hours. Supernatants were collected at each of these different time points and assayed for IL-12(p40) (panel A) by sandwich ELISA. Data were presented as mean+/-SE values of 6-10 determinations and were representative of three independent experiments. The symbols *, ** and *** indicated statistical significance ($p<0.05$) of differences in responses in groups treated with LPS alone compared to groups treated with LPS + GSK-3 inhibitor at the different time points.

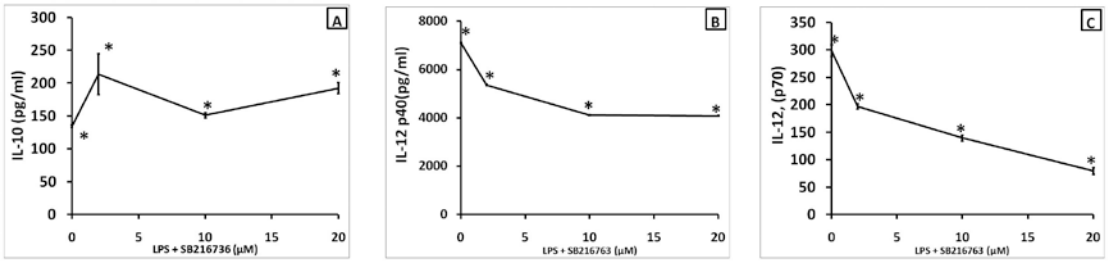

Figure 5.5: GSK-3 inhibitors enhance IL-10 but inhibit IL-12 production by BMDC

BMDC ($2.5x10^5$ cells/ml) from young adult C57BL/6 mice were evaluated for the production of IL-10, IL-12 (p40) and IL-12(p70) at 24 hours (Panels A, B and C). The BMDC were pretreated with different concentrations of SB216763 for 60 minutes and then stimulated with LPS (1.0μg/ml) for 24 hours. Supernatants were collected and assayed for IL-10 (panel A), IL-12(p40) (panel B) and IL-12(p70) (panel C). Data are presented as mean+/-SE values of duplicate cultures and duplicate ELISA of each culture. The symbol * indicates statistical significance ($p<0.05$) of differences in responses in groups treated with LPS alone compared to groups treated with LPS + GSK-3 inhibitor.

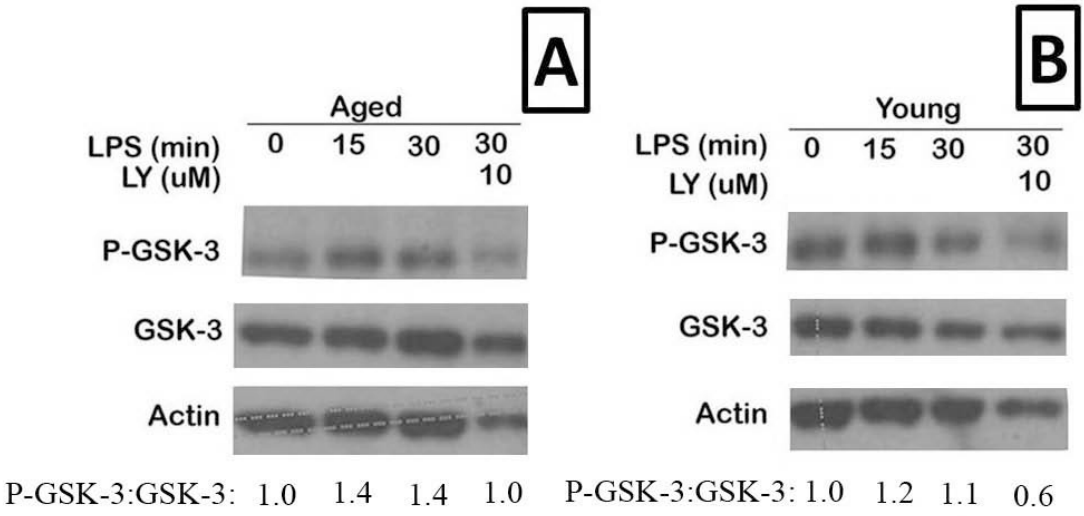

Figure 5.6. The PI3K-Akt signaling pathway modulates the ability of GSK-3 to regulate cytokine production in macrophages

Panels A and B: The newly derived BMDM from aged (Figure 5.7A) and young (Figure 5.7B) mice were pre-treated with either LY294002 for 1 hour and then stimulated with LPS (1μg/ml) for 15 and 30 minutes. The blots were probed for p-GSK and total GSK-3 and actin after stripping (Figure 5A and B). The numbers represented densities of bands normalized to total GSK-3, with the values for unstimulated aged macrophages set to 1. Results are representative of three to independent experiments. The final figure in A was a composite of blots for P-GSK-3, GSK-3 and actin for young or aged samples run on the same membrane that was stripped and reprobed.

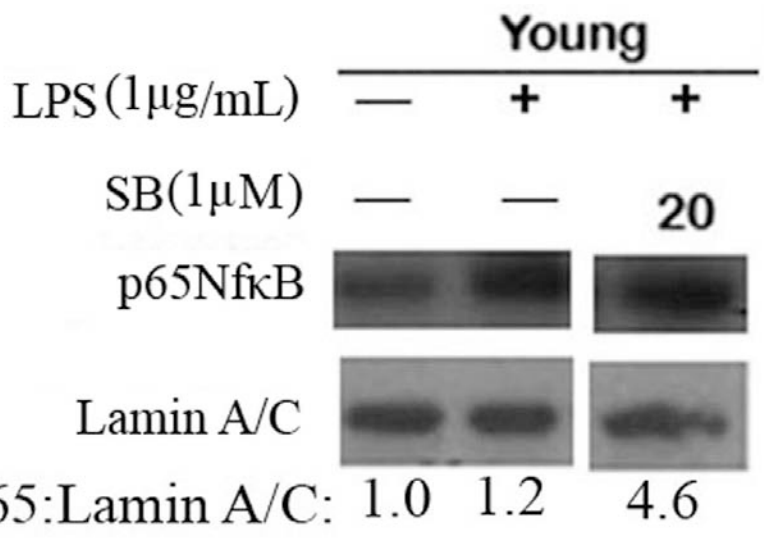

Figure 5.7. GSK-3 upregulates cytokine production in macrophages via p65NF-κB.

BMDM from the young were harvested via trypsin-EDTA, washed and allowed to rest for three hours and then cultures were set up where some were pretreated with SB216763, while others were not treated for one hour. At the end of the hour, the cultures were activated with LPS (1μg/ml) at 30 minutes. The nuclear extract was isolated and run on an SDS-PAGE gel, transferred to a membrane and then immonoblotted for p65 NFκB. The numbers represented densities of bands normalized to LaminA/C, with the values for unstimulated BMDM set to 1. Results were representative of three independent experiments. The final figure was a composite of blots for p65NFκB and Lamin A/C that were run on the same membrane, stripped and reprobed. The intervening space (between second and third lane) was for lanes loaded with lysates from other time points or inhibitors but were deleted to focus on the effect of LPS+ SB216763 on p65 translocation at 30 minutes, which was maximum in this experiment.

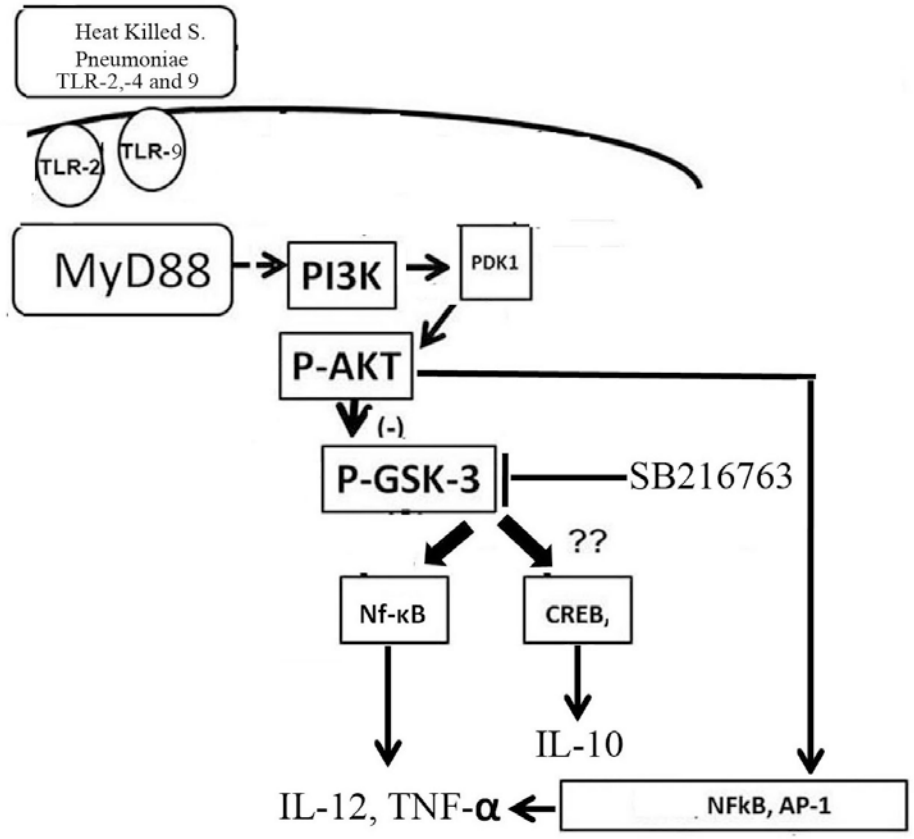

Figure 5.8. Schematic model for GSK-3 inhibition in the regulation of IL-10 and pro-inflammatory cytokine production in splenic and bone marrow macrophages via p65NF-κB and CREB.

Activated GSK-3 negatively regulates p65Nf-κB and CREB: Upon inhibition of GSK-3 both p65NF-κB and CREB functions were enhanced leading to an increase in IL-10, IL-12 and TNF-α. IL-12 and TNF-α can also be induced independent of GSK-3 via Akt-p65Nf-κB or p38 MAP Kinase.

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CHAPTER 6: Role of PI3K-AKT-GSK-3 pathway in TLR Induced B cell activation and differentiation.

Introduction

During aging, the B cell mediated responses, including affinity and avidity of the antibodies, are decreased (40, 103, 220, 327). There is also an age-related decrease in antibody responses to the pneumococcal polysaccharide vaccine in both elderly humans and rodents compared to the young leading to an impairment in opsonophagocytosis of pneumococci (278). To effectively eliminate pathogens the humoral response must be able to effectively class switch from a predominant IgM to non-IgM isotypes. This defect in the elderly is in turn attributed to impairment in class-switch recombination (CSR) to other isotypes, such as IgE, IgG1, IgG2a or IgG3 with specific effector functions (103, 220, 256). The impaired class switching in the elderly mice is shown to be associated with a decrease in the enzyme, activation-induced cytidine deaminase (AID), due to defects in signaling molecules that govern the expression of this molecule (103, 379). Under normal conditions, B cell receptors sense the capsular polysaccharide of *S. pneumoniae*, which is a TI antigen. In the presence of certain cytokines B cells can produce IgG3 via CSR that helps clear the pathogen by opsonophagocytosis (227). However, this is impaired in the aged due to a defect in AID and CSR (103, 105, 220).

PI3K-Akt signaling plays a crucial role during B cell proliferation, survival, activation and differentiation (267, 268). PI3K mediated activation of Akt results in phosphorylation and inactivation of the Foxo-1 transcription factor in B cells. Inactivation of Foxo-1 results in the impairment of B cell development in the bone marrow, fewer lymph node cells in the peripheral and the ability of B cells to undergo

class switch recombination and expression of the enzyme AID (53, 83). Overall, PI3K also regulates B-cell homeostasis by regulating B cell development in the bone marrow, the different types of B-cell subsets, B-cell survival and proliferation (22). Yet no studies have evaluated the PI3K-Akt signaling pathway in the context of the B cells or its role in the impaired class switching in the aged. Since we showed earlier that increased activity of PI3K-Akt pathway is responsible for age-related cytokine defect in macrophages (Chapters 3 and 4), we asked if the PI3K-Akt pathway plays any role in the impaired humoral responses of aged B cells. Glycogen synthase kinase-3 (GSK-3) is a direct downstream substrate of Akt, but its role in B cell immune function like CSR and plasma cell formation is unknown. GSK-3 modulates beta-catenin and NFATc transcription factors as well as cyclin D2 that facilitate B cell survival, proliferation and differentiation (24, 29, 118, 391).

Hence, in this chapter we investigated the role of the PI3K-Akt-GSK-3 signaling pathway in the impaired humoral response with a future goal of pharmacologically modulating this pathway for potent antibody immune responses in the aged. We demonstrate lower production of IgG3 in aged mouse compared to the young upon stimulation with TLR2,-4 and HKSP and that it is in part due to a dysregulation of the PI3K-Akt-GSK-3 pathway.

Results

IgG3 production by splenic B cells activated via TLR2, 4 and HKSP is decreased in the aged.

Aged and young purified splenic B cells were stimulated with LPS (20 µg/ml) (Figure 6.1A), P3C (5µg/ml) (Figure 6.1B) and HKSP (2 x 10^8 CFU/ml) (Figure 6.1C) for three days. Total IgG3 levels were quantified by ELISA. There was lower production of IgG3 in TLR2 (Figure 6.1A), TLR4 (Figure 6.1B) and HKSP (Figure 6.1C) activated aged splenic B cells compared to the young.

Increased activation of Akt in the aged splenic B cells correlates with the defect in IgG3 production

Akt was activated via phosphorylation by the PI3 kinase resulting in suppression of IgG, IgA and IgE production in B cells (269). We inquired if there was an age-related increase in p-Akt which could account for the lower production of IgG3. Levels of p-Akt were determined by Western blot analysis of lysates from aged and young adult splenic B cells stimulated with LPS (20µg/ml). Under unstimulated conditions, there was a higher level (1.5 fold, after normalization to total Akt) of p-Akt in the young compared to the aged, which was suppressed strongly after 120 minutes of stimulation with LPS (Figure 6.2 A, lanes 1 and 4). Reduction of p-Akt levels in young B cells activated with LPS at early time points (15 and 30 minutes, Figure 6.2A, lanes 5, 6 and 7) was very small. In contrast, there was no reduction in p-Akt levels in LPS stimulated aged B cells at early time points (Figure 6.2A, lanes 1, 2, 3). At 120 minutes of stimulation there was only a 20% reduction in P-Akt in aged B cells; unlike young B cells where p-Akt was reduced

by ~87% (Figure 6.2A, lanes 5, 8). Having established that LPS activated aged B cells had higher activated Akt than the young; we next asked if this differential activation of Akt was associated with differences in the protein levels of PTEN, an antagonist of the PI3 kinase pathway. Surprisingly, there was no change in PTEN levels beteen the aged and young B cells (figure 6.2B) at all time points tested.

PI3K inhibition leads to increase in production of IgG3 in both the aged and young adult splenic B cells

We asked if the increased levels of p-Akt had a role in decreased IgG3 in the aged since Akt pathway was known to inhibit IgG3. Since p-Akt activation was dependent on PI3K activity, we tested if inhibition of PI3K enhanced IgG3 expression in aged B cells. We stimulated splenic B cells with LPS (20μg/mL) and HKSP in the presence of wortmannin or LY294002 for 3 days and the supernatants were assayed by ELISA for IgG3 production. There was a 2-fold increase in IgG3 production in the young splenic B cells, while in aged splenic B cells there was a 6-fold increase with LPS stimulation in the presence of wortmannin (Figure 6.3A). When LY294002 was used, there was a two-fold increase at 5μM of LY294002 compared to LPS stimulation in a dose dependent manner (Figure 6.3B). When wotmannin was used in the presence of HKSP, there was a greater increase in IgG3 production in the young B cells at 10 nM of wortmannin compared to the aged, yet both were greater than B cells stimulated with HKSP without the inhibitor (Figure 6.3 C). At 25 nM of wortmannin, the aged B cells had a greater increase in production of IgG3 compared to the young. However, at both concentrations of wortmannin there was a higher IgG3 production compared to HKSP stimulated B cells without the inhibitor (Figure 6.3C). In the case of HKSPstimulation, the enhancement of

IgG3 by PI3K inhibitor was more in the aged than the young (Figure 6.3D). Finally, we asked if the ability of LY294002 to enhance IgG3 production in aged B cells was dependent on a specific strain of mouse. Splenic B cells from C57BL/6 mice were stimulated with LPS in the presence of LY294002. There was a significant increase in IgG3 production in the presence of LY294002 (Figure 6.3 E). Thus, inhibiting PI3 kinase partially rescued the age-related defects in IgG3 production.

Inhibition of PI3K with wortmannin induces AID.

PI3K-Akt pathway regulates immunoglubulin production by inhibiting the expression of the enzyme AID which facilitates class switch recombination (268). On the basis of this finding and the fact that wortmannin elicited enhanced IgG3 production by both aged and the young B cells at all concentrations evaluated (Figure 6.3 A), we measured the expression of AID in young splenic B cells stimulated with Pam3CSK4, a ligand for TLR-2/1 that is associated with HKSP lipoproteins, in the presence of different doses of wortmannin for 72 hours. There was a 1.3 fold increase in AID levels at 25nM and 1.8-fold increase at 50nM of wortmannin+ P3C activated B cells compared to P3C stimulation alone (Figure 6.4). Thus, PI3K inhibtion induces increased expression of AID that plays a role in IgG3 production.

GSK-3 inhibition in the presence of LPS induces higher production of IgG3 and IgA in young splenic B cells.

Glycogen synthase kinase-3 had been shown to influence B cell survival, proliferation and activation (391). Since GSK-3 was an immediate downstream substrate of Akt, we evaluated the effect of GSK-3 inhibition on LPS induced IgG3 and IgA secretion. Splenic B cells from young mice were stimulated with LPS in the presence of SB216764, a well

characterized inhibitor of GSK-3 (Figures 6.5 A and B). There was a 2.6-fold increase in IgG3 production in the young splenic B cells at 5 and 10 µM of the inhibitor (Figure 6.5A), while there was a 3 and 6-fold increase in production of IgA at 5 and 10 µM respectively (Figure 6.5B).

SB216763 enhances secretion of IgA and surface expression of switched isotypes of young mouse B cells stimulated with Pam3CSK4, a TLR2/1 ligand.

IgA immunoglubulin conferred protection against *S. pneumoniae* in aged mice at mucosal sites like the lungs (110, 111, 172). Hence, we asked whether GSK-3 inhibition enhanced production of IgA if we use Pam3CSK4, the TLR2/1 ligand. We demonstrated that SB216763 enhanced the production of IgA (Figure 6.6A) in splenic B cells stimuated with Pam3CSK4. We next asked if this effect could be shown via surface expression of the IgA. B cells were stimulated with Pam3CSK4 in the presence of SB216763 and cultured for 3 days. Figure 6.6 B showed that SB216763 increased the surface expression of IgA compared to B cells treated with LPS alone.

Pharmacologically inhibiting GSK-3 results in increased production of IgA and IgG3 as well as enhanced expression of surface IgA in young splenic B cells stimulated with CpG, a TLR-9 ligand.

TLR9 signal has been implicated in immune response to *S. pneumoniae* presumably via bacterial DNA containing CpG motifs (14). Therefore; it was of interest to know if TLR9 mediated IgG3 expression was enhanced by GSK-3 inhibition. We demonstrated an enhanced production of IgA (figure 6.7A) and IgG3 (Figure 6.7B) at all concentrations of the GSK-3 inhibitor tested. We then asked if this effect could be shown via the surface

expression of switched isotypes of IgA. B cells were stimulated with CpG (Figure 6.7 C) in the presence of SB216763 and cultured for 3 days. The B cells were collected and analyzed by flow cytometry. Figure 6.7C showed that SB216763 increased the surface expression of IgA compared to B cells treated with LPS alone.

Activation of young adult splenic B cells with HKSP in the presence of SB216763 increase the production of IgG3 as well as surface expression of IgA and IgG3.

Now that we had shown that synthetic ligands for TLR-2,-4 and -9 which were associated with *S. pneumoniae* enhanced the production and surface expression of different immunoglobulin, we decided to approximate as closely as possible the normal host-bacterial interaction. For this purpose, *S. pneumoniae* was grown to mid-log phase, heat killed and used to activate B cells in the presence of SB216763. We cultured the B cells for 3 days and assayed the supernatants by Ig ELISAs. There was enhanced in the production of IgG3 (Figure 6.8A) at all concentrations of SB in HKSP stimulated B cells. We tested if GSK-3 affected switching to IgA and IgG3 isotypes. Therefore, surface expression of IgA and IgG3 was analyzed by flow cytometry. B cells expressing IgA (Figure 6.8B) and IgG3 (Figure 6.8C) switched isotypes were enhanced when GSK-3 was inhibited. Please note the magnitute of the HKSP induced IgG3 response in these experiments was significantly lower than in experiments shown in Figures 6.3. We attribute this to a new batch of HKSP which was not as potent as the one used in PI3K inhibitor studies. However, the enhancement of IgG3 response by the GSK-3 inhibitor was highly reproducible.

GSK-3 inhibitor enhances plasma cell formation in TLR-2,-4,-9 and HKSP stimulated splenic B-cells

Previous studies by Omori and colleagues showed that PI3K inhibition enhanced class switching at the expense of plasma cell formation via differential regulation of BLIMP and FOXO trancription factors (268, 269). Since, GSK-3 was a downstream substrate of PI3K-Akt and had multi-factorial roles in regulating B cells we evaluated the effect of GSK-3 inhibition on plasma cell formation. We stimulated young adult splenic B cells with LPS, P3C, CpG and HKSP in the presence of SB216763 for three days, collected the B cells and quantified the expression of the plasma cell marker, CD138 by flow cytometry. Surprisingly, there was an increase in plasma cells ($B220^{lo}$ $CD138^{hi}$) upon B cell activation with LPS (Figure 6.9 A), P3C (Figure 6.9 B, upper panel) and CpG (Figure 6.9 B, middle panel) at all concentrations of SB216763 tested. Interestingly, LPS and HKSP stimulation induced CD138 expression without reducing B220 levels. Though the significance of this difference between P3C and CpG versus LPS and HKSP was unclear, $B220^{hi}$ $CD138^{hi}$ cells represented an early stage of plasma cells.

SB216763 modulates B cell activation and differentiation in the presence of ligands for TLR-2, 9 and HKSP without any toxicity.

Since inhibition of GSK-3 imposed greater metabolic demands on the B cells to both class switch and form plasma cells, we wondered if this was at the expense of viability or proliferation of the B cells. The viability of the young splenic B cells was examined by trypan blue count following 3 days of culture in the presence of SB216763 with CpG (Figure 6.10 A), Pam3CSK4 (Figures 6.10 B), and HKSP (Figure 6.10C). The recovery

of viable B cells increased by 3-4-fold upon stimulation with CpG and Pam3CSK4, which was further enhanced slightly by GSK-3 inhibition. Stimulation with HKSP had only a modest effect on B cell recovery, which was enhanced at one concentration of the GSK-3 inhibitor.

SB216763 enhances the expression of AID in TLR-4 stimulated B cells.

GSK-3 regulates beta-catenin and NFATc1, transcription factors that play a role in CSR, which can be determine by the expression of AID. B cells were stimulated with LPS with our without SB216763 for 72 hours and the probed for AID. Our results show that SB216763 increased AID expressions at all concentrations (Figure 6.11, lanes 2, 3, 4 and 5). We conclude that the ability of SB216763 to increase IgG3 and IgA may be via CSR.

DISCUSSION

Our ultimate goal is to establish a possible link between alteration in the PI3K-Akt pathway in the aged mice compared to the young that explains the impaired IgG3 production in aged splenic B cells and whether we can rescue this defect pharmacologically. We also wanted to create a proof of principle that pharmacologically inhibiting GSK-3, a downstream kinase of the PI3K-Akt signaling induces greater B cell differentiation, activation and thus, serves as a potential vaccine adjuvant in the aged mice. We demonstrate that aged splenic B cells produce lower levels of IgG3 compared to the young and that this is associated with an increase in the activation of Akt, which is known to downregulate AID and class switching (268). We establish a causal role of the activated Akt in blunting the IgG3 production as we are able to rescue the age-related defect in IgG3 production upon inhibition of PI3K in the context of TLR-4 and HKSP. We make a notable finding that inhibiting GSK-3 with SB216763 results in an enhanced class-switching and plasma cell formation. Inhibitors of PI3K and GSK-3 indicate that both signaling molecules are able to upregulate AID expression.

Aging is associated with impaired humoral and vaccine responses to pathogens such as the Gram positive *S. pneumoniae* that is the leading etiological agent for community acquired pneumonia (CAP) (278). The impaired humoral response in the aged associates with a decrease in AID, class switch recombination and other signaling molecules like protein tyrosine kinase (PTK) and protein kinase C isoforms and transcription factors like E 47 (105, 379). Hence, these aging markers can be pharmacologically modified to create a close approximation of the effective young adult immune responses.

We established in vitro conditions which mimicent the defective B cell response to pneumococcal polysaccharide vaccine or TNP-Ficoll, a model TI-II antigen and showed that the response can be enhanced by macrophage derived cytokines (38, 114). We also demonstrated that the age-associated defect in B cell antibody responses to these antigens is due to a cytokine dysregulation in the aged macrophages (63). We further showed that inhibiting PI3K in aged macrophages rescues this cytokine defect and serves as a potential target for providing endogenous cytokines needed for B cells to overcome the defective humoral response (Chapter 4). The in vivo use of PI3K inhibitor also affects B cells. Hence, we established that PI3K-Akt is also dysregulated in the aged B cells and can be rescued by pharmacological inhibition.

The Class IA PI3K plays a very important role in modulating B-cell immune functions and activities (89). It is one of the key signaling molecules downstream to B cell receptor (BCR), B-cell costimulatory molecules, toll like receptors and cytokine receptors and can be activated by one or a combination of these receptors. Once activated, it phosphotylates Akt, which in turn modulates B-cell proliferation and survival (81, 268). The p110 delta (p110δ) catalytic subunit is critical for the phosphorylation of Akt. Phosphorylated Akt then phosphorylates and inactivates Forkhead box-1 containing transcription factor, Foxo1 (205). Genetic or pharmacological inactivation of p110δ prevents B cells from entering the cell cycle by inhibiting upregulation of cyclin D2 (30, 267).

In this study we show that p-Akt is increased with age and explains the defective IgG3 production observed in the aged. Our finding is supported by studies showing that activation of Akt via a B cell specific PTEN conditional knockout leads to a defect in the production of most isotypes of IgG upon B cell stimulation by either TI or TD antigens

(343). Though we show a correlation between increased p-Akt and defective IgG3 production in aged B cells, we still need to establish causation. Hence, we demonstrate that inhibition of PI3K-Akt signaling pathway by either LY294002 or wortmannin is able to rescue the age-associated defect in IgG3 production. This finding is supported by several studies that demonstrate that pharmacological or genetic inhibition of PI3K-Akt signaling enhances Ig production (30, 81, 268, 269). AID plays a crucial role in CSR and somatic hypermutation which are critical for a potent humoral response to pathogens (248). Our studies indicate that inhibition of the PI3K-Akt signaling pathway resultes in a higher expression of AID and thus increased production of IgG3.

Having established that PI3K-Akt can be pharmacologically modulated to shift from a decreased AID and IgG3 production, a hallmark of aging, to enhanced AID expression and IgG3 production, a marker of young adult mediated immune response, we decided to look at downstream of this pathway. A downstream molecule can be modulated under in vivo conditions with fewer side effects. Hence, we evaluated the role of GSK-3 in enhancing Ig production, AID expression and plasma cell formation in a young splenic B cell in vitro model. BCR signaling triggers PI3K-Akt activation resulting in the phosphorylation and inactivation of GSK-3 activities. Since GSK-3 negatively regulates several of its downstream transcription factors, the phosphorylation mediated inactivation of GSK-3 leads to an increase in the activities of transcription factors such as NF-ATc and beta-catenin. These transcription factors are important in modulating the immune functions of B cell (118, 391).

We inhibited GSK-3 with SB216763 in B cells that are then activated via TLR-2,4 and 9, as well as HKSP and demonstrated an enhanced production of different isotypes of IgG,

as well as IgM and IgA. This is the first study to show that GSK-3 inhibition can lead to increased production of immunoglobulin. In support of this notion, a conditional knockout of beta-catenin, one of the downstream targets of GSK-3 shows a significant reduction in class switching to IgG1 and plasma cell generation in vitro (391). Since the activity of unphosphorylated GSK-3 is known to phosphorylate and degrade beta-catenin, thus inhibiting GSK-3 by SB216763 results in increased beta-catenin which in turn may enhance Ig production and plasma cell generation. In the future we will evaluate the role of GSK-3 inhibition on beta-catenin in B cells and evaluate its effects on Ig production. We wonder if the increased production in IgA and IgG3 is due to class switching or expansion of an already class-switched group of B cells. To address this question we evaluated the generation of B cells with switched isotypes IgG3 and IgA on their surface by FACS. We consistently show that SB216763 enhances the surface expressions of the IgG3 and IgA isotypes indicating that this phenomenon is due to class switching. Similar results are obtained for surface IgG1 and IgG2b positive B cells (data not shown). We further validate that GSK-3 is mostly inducing increased production of the immunoglobulin by class switch recombination by Western blot for AID expression. As mentioned earlier AID upregulation is a very good indicator of class switch recombination (249).

It is known that plasma cell formation is the terminal and irreversible differentiation state of B cells where they lose their B cell characteristics and become potent antibody producing cells. This process is aided by antagonistic interactions of transcription factors like BLIMP1, BCL6, Pax5 (320). Since switched cells can eventually become plasma cells, the CSR may correlate with plasma cell development in a sequential manner (270).

Recently, Omori and colleagues show that PI3K inhibition leads to increase CSR at the expense of plasma cell production (268, 269). Yet in our hands, we show that GSK-3 inhibition upregulates both CSR and plasma cell formation at the end of three days. This is supported by other studies that show that CSR is a prelude to plasma cell formation (181).

In spite of this novel finding with GSK-3, there is a caveat we must point out in our work. We have not as of yet clearly delineated the transcription factors that are being regulated by either PI3K-Akt or GSK-3 inhibition in our studies. GSK-3 is known to regulate transcription factors like NF-κB (p65 and p105), beta-catenin and NFATc1 that are known to regulate CSR or plasma cell formation (166). Hence, a future challenge for us will be to clearly delineate the downstream signaling pathway to Akt and GSK-3 that are inducing CSR and plasma cell formation, respectively. Our observation can be summarized in a model wherein increased activity of the PI3K-Akt pathway in the aged suppresses AID and CSR and resulting in reduction of IgG3 production (Figure 6.12 A). Inhibiting PI3K-Akt relieves this suppression and enhances AID, CSR and IgG3 production (Figure 6.12B). TLR-2, 4 or 9 and HKSP mediated stimulation of B cells in the presence of SB216763 reduces p-GSK-3β, leading to increased GSK-3β activity, and increased AID, CSR and immunoglobulin production through transcription factors that are yet to be charaterized (Figure 6.12B).

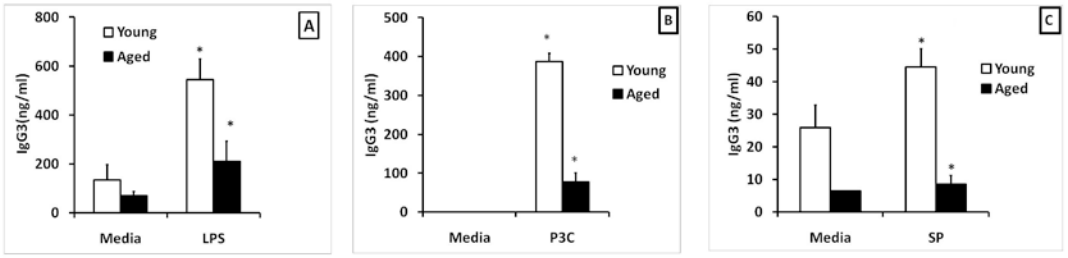


Figure 6.1. **Age-associated decrease in IgG3 production by splenic B cells activated via TLR2, 4 and HKSP.**

Splenic B cells (1.0 x 10^6 cells/ml) from young (open box) and old (filled black box**)** mice were cultured with LPS (20 µg/ml) (6.1A), P3C (5µg/ml) (6.1B), and HKSP (2 x 10^8 CFU/ml) (6.1C), for 3 days. At the end of three days supernatants were collected and assayed for IgG3 by ELISA. Results were expressed as means ± SE which were representative of four independent experiments. The symbol * indicated statistical significance ($p<0.03$) of differences in responses of young and aged B cells.

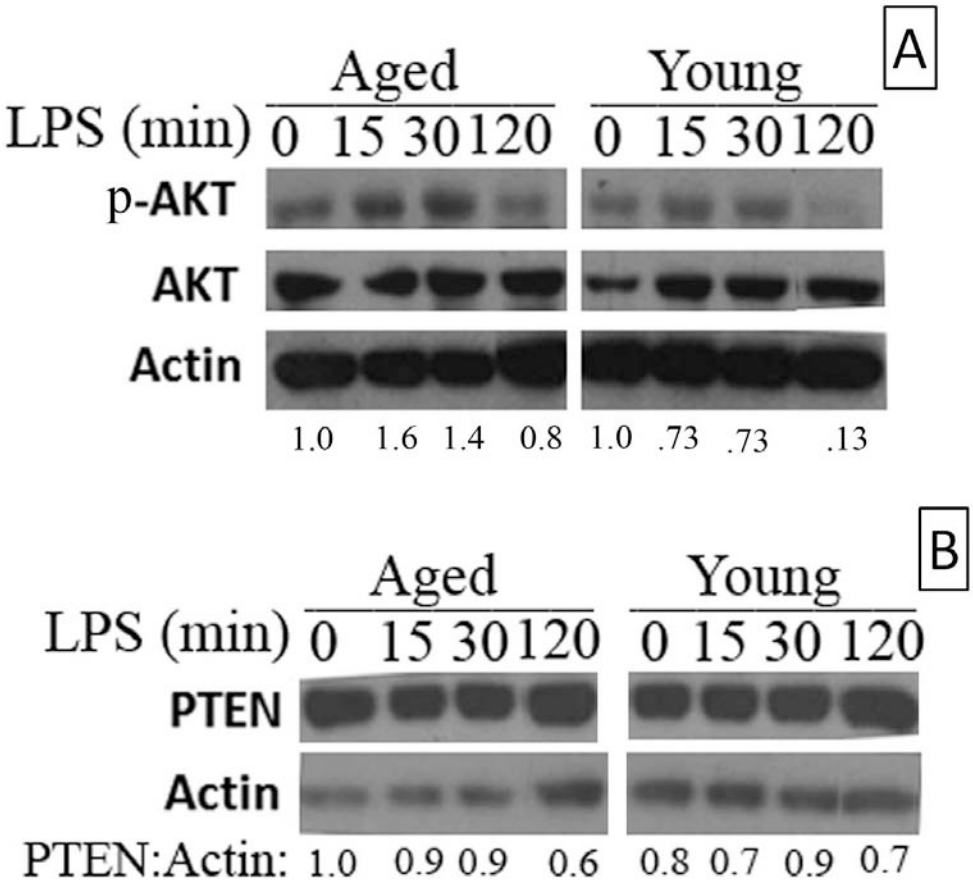
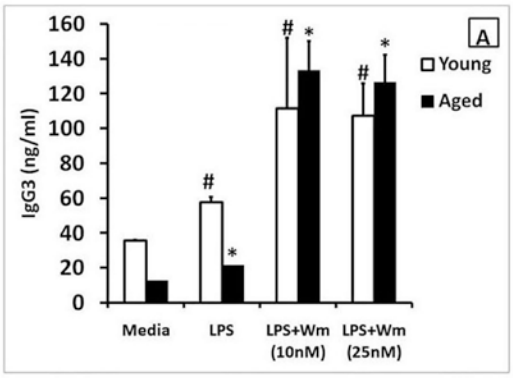
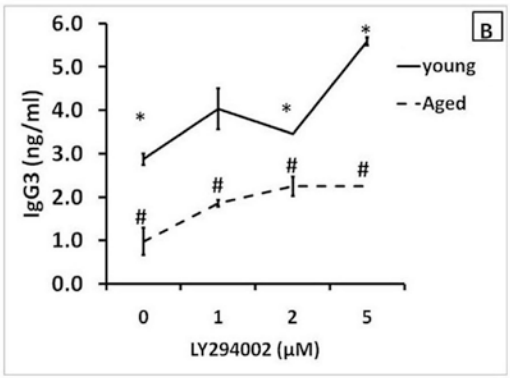
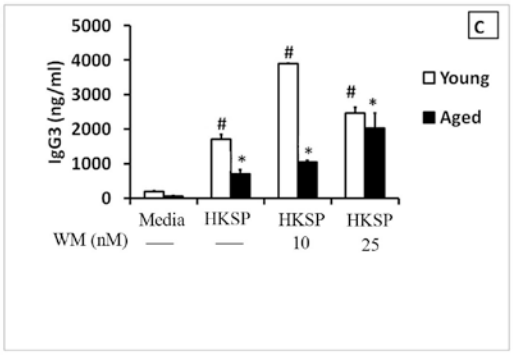


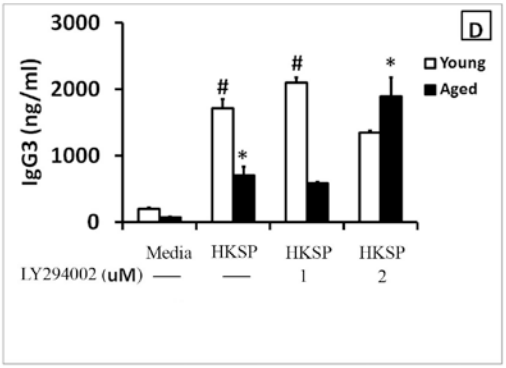
Figure 6.2. Age-associated increase in activation of Akt without changes in PTEN in splenic B-cells.

Purified splenic B cells (2.0 x10^6 cells/ml) from aged and young adult B cells were stimulated with LPS (20 µg/ml) and total cell lysates were isolated and immunoblotted for p-Akt (Figure 6.2A) and PTEN (Figure 6.2B). The blots were stripped and probed for total Akt and for beta-actin. The numbers represented densities of bands normalized to total Akt with the values for unstimulated aged and young B cells set to one.


A
IgG3 (ng/ml)
160
140
120
100
80
60
40
20
0
Young
Aged
Media
LPS
LPS+Wm (10nM)
LPS+Wm (25nM)


B
IgG3 (ng/ml)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
young
Aged
0
1
2
5
LY294002 (μM)


C
IgG3 (ng/ml)
5000
4000
3000
2000
1000
0
Young
Aged
Media
HKSP
HKSP
HKSP
WM (nM)
10
25


D
IgG3 (ng/ml)
3000
2000
1000
0
Young
Aged
Media
HKSP
HKSP
HKSP
LY294002 (uM)
1
2

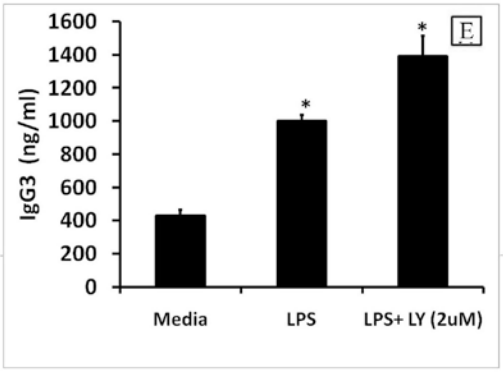

E
IgG3 (ng/ml)
1600
1400
1200
1000
800
600
400
200
0
Media
LPS
LPS+ LY (2uM)

Figure 6.3. Inhibition of PI3K activity increases the production of IgG3 in both the aged and young adult splenic B cells

Splenic B cells (1.0 x 10^6 cells/ml) from young (open box) and old (filled black box**)** Balb/c mice were pretreated for 1 hour, with the PI3K-inhibitors, LY294002 (Figures 6.3 B,D and E) or wortmannin (Figures 6.3A and C) at different concentrations that were previously tested and optimized. The B cells were then activated with LPS (20 µg/ml) (Figures 6.3 A, B and E) or HKSP (2x 10^8 Cfu/ml) (Figures 6.3 C and D). After three days of culture the supernatant was collected and assayed by ELISA for IgG3. (E) IgG3 ELISA from splenic B cells of aged C57BL/6 mice activated by LPS in the presence of LY294002. Results were presented as mean ± SE of four independent experiments. The symbol * and $^\#$ indicated statistical significance ($p<0.04$) of differences in responses in the aged and young B cells treated with LPS (or HKSP) with or without the PI3K inhibitor.

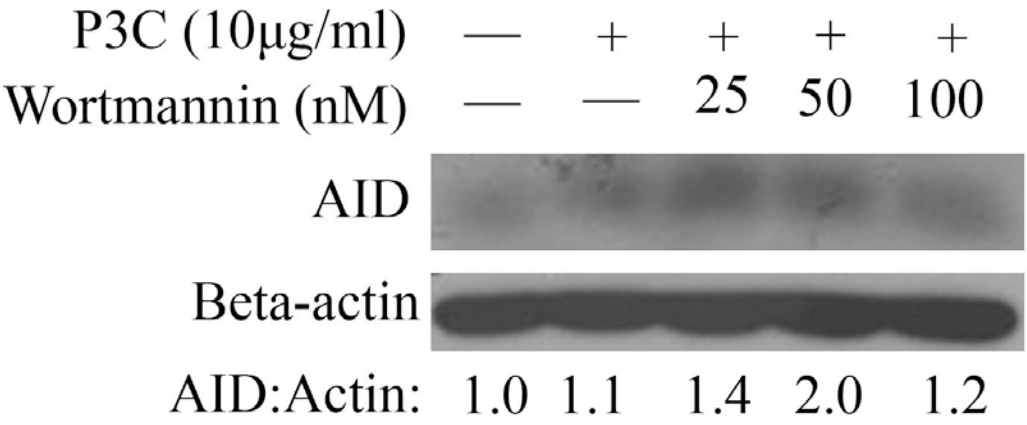


Figure 6.4. Wortmannin, a PI3K inhibitor, enhances AID expression in B cells.

Purified splenic B cells (2.0 x10^6 cells/ml) in 5 ml of RPMI complete from young adult mice were stimulated with P3C (10 µg/ml) for three days and total cell lysates were isolated and immunoblotted for AID (Figure 6.4A) . The blot was stripped and probed for beta-actin. The numbers represent densities of bands normalized to beta-actin with the values for LPS stimulated young B cells set to one.

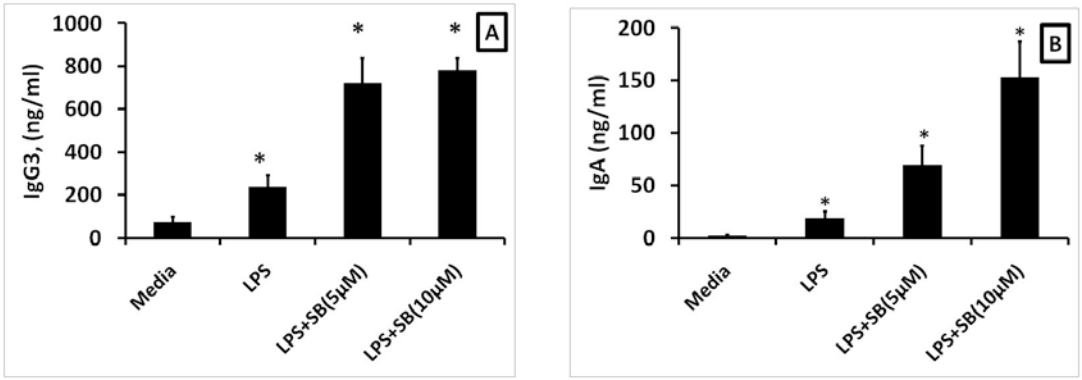
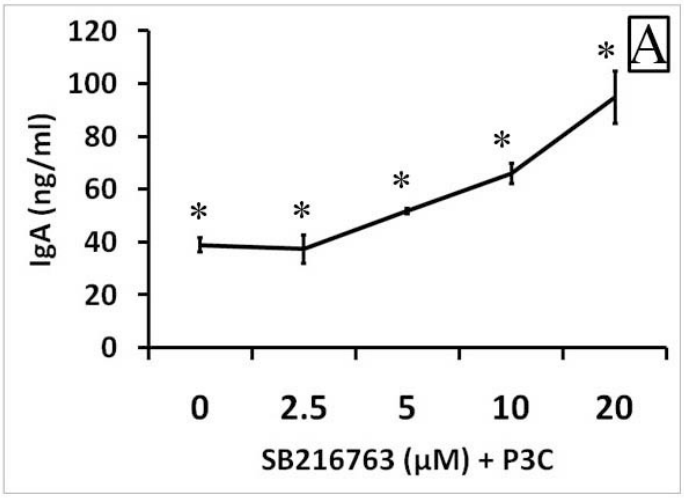


Figure 6.5. GSK-3 inhibition induces higher production of IgG3 and IgA in young splenic B cells stimulated via TLR-4.

Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with LPS (20 μg/ml) for 3 days and the supernatant was collected and evaluated by sandwich ELISA for IgG3 (Figure 6.5A) and IgA (Figure 6.5B). Results were means ± SE of four independent experiments. The symbol * indicated statistical significance ($p<0.01$) of differences in responses in the young B cells treated with LPS with or without the GSK-3 inhibitor.



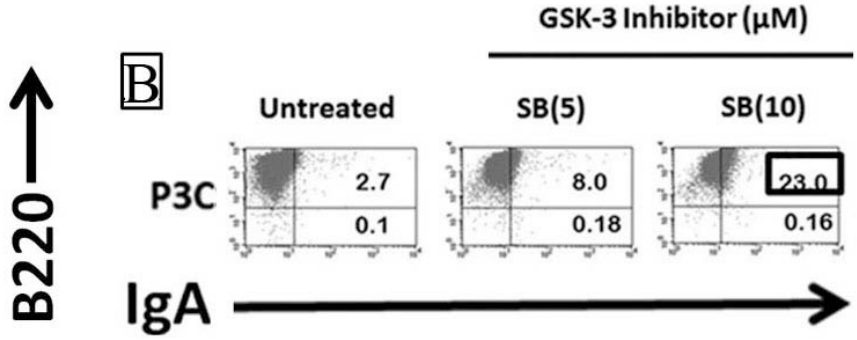


Figure 6.6. Inhibition of GSK-3 enhances secretion of IgA and surface expression of IgA in young mouse B cells stimulated with Pam3CSK4.

Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with P3C (5μg/ml) for 3 days and the supernatant was collected and evaluated by ELISA for IgA (Figure 6.6A). Results were means ± SE of four independent experiments. The symbol * indicated statistical significance ($p<0.03$) of differences in responses of young B cells treated with LPS with or without the GSK-3 inhibitor. (Figure 6.6B) After culture for 3 days B cells are removed, washed and then analyzed by FACS for the expression of membrane IgA using PE-Cy5-labeled anti-B220 Abs and FITC-labelled IgA anti-mouse Ab. Results were representative of five independent experiments.

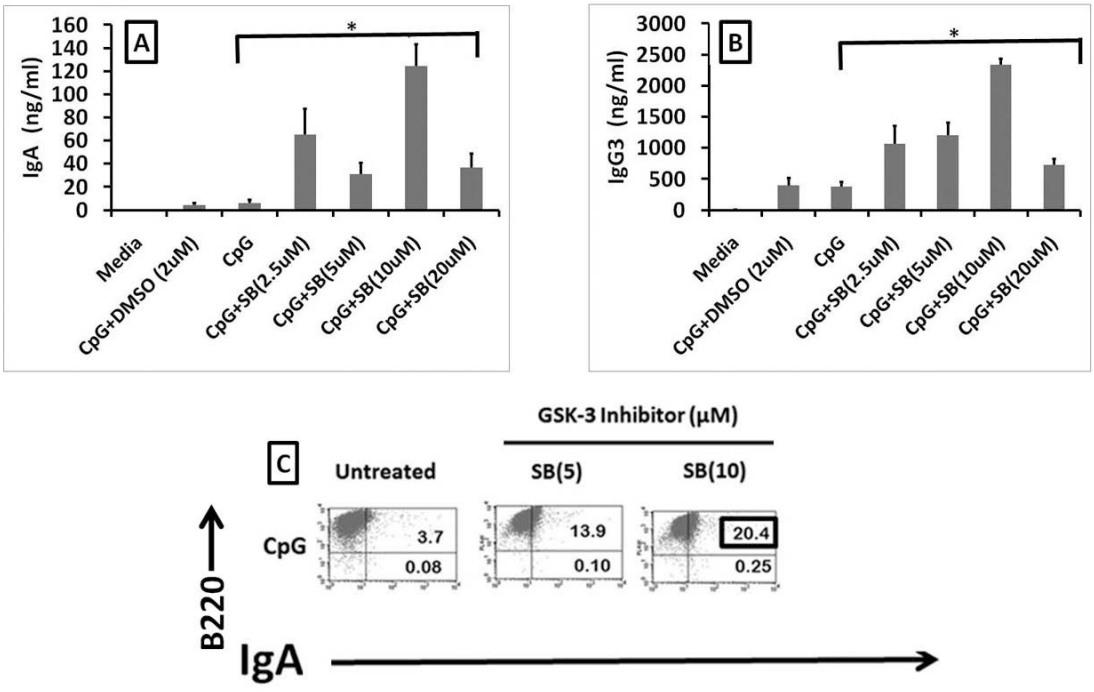
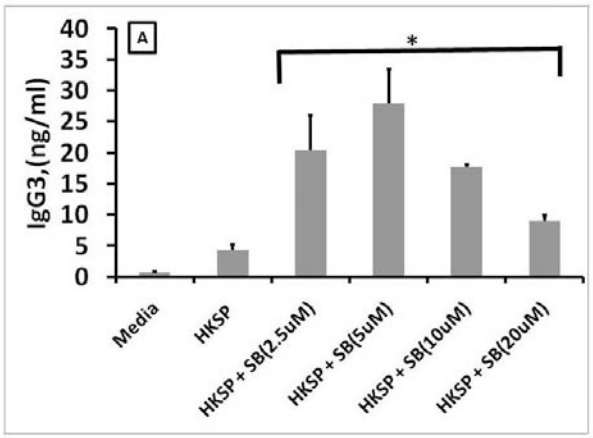
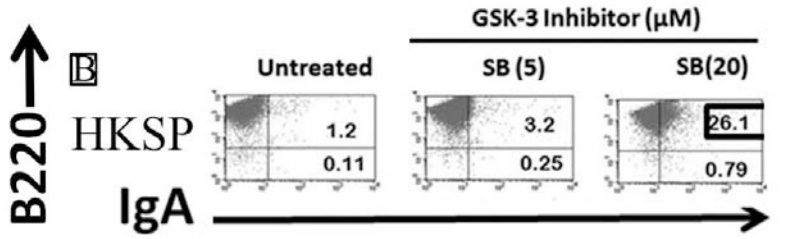


Figure 6.7. GSK-3 inhibition leads to increase in production and expression of surface IgA and IgG3 in young splenic B cells stimulated with CpG, a TLR-9 ligand. Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with CpG (5μg/ml) for 3 days and the supernatant was collected and evaluated by ELISA for IgA (Figure 6.7A) and IgG3 (Figure 6.7B). Results were given as means ± SE and are representative of four independent experiments. The symbol * indicated statistical significance ($p<0.03$) of differences in responses in the young B cells treated with LPS with or without the GSK-3 inhibitor. (Figure 6.6C) FACS analysis was done on B cells cultured for 3 days for the expression of membrane IgA using PE-Cy5-labeled anti-B220 Abs and IgA FITC-labelled IgA anti-mouse Abs. Results were representative of five independent experiments.


A
IgG3,(ng/ml)
40
35
30
25
20
15
10
5
0
*
Media
HKSP
HKSP + SB(2.5uM)
HKSP + SB(5uM)
HKSP + SB(10uM)
HKSP + SB(20uM)


GSK-3 Inhibitor (μM)
B
Untreated
SB (5)
SB(20)
HKSP
B220
IgA
1.2
0.11
3.2
0.25
26.1
0.79

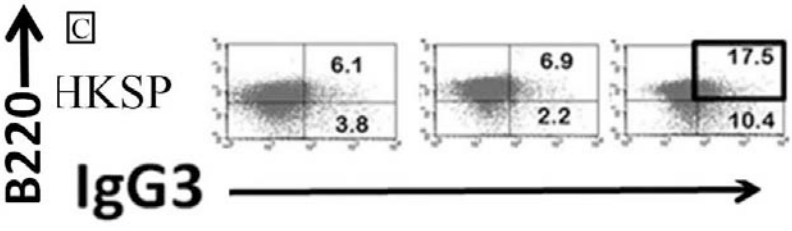

C
HKSP
B220
IgG3
6.1
3.8
6.9
2.2
17.5
10.4

Figure 6.8. HKSP activates young splenic B cells in the presence of SB216763 to increase the production of IgG3 as well as surface expression of IgA and IgG3. Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with HKSP (2.0×10^8 CFU/ml) for 3 days and the supernatant was collected and evaluated by ELISA for IgG3 (Figure 6.8A). Results were indicated as means ± SE and were representative of four independent experiments. The symbol * indicated statistical significance ($p<0.03$) of differences in responses in the young B cells treated with HKSP with or without the GSK-3 inhibitor. FACS analysis was done on B cells cultured for three days for the expression of membrane IgA (Figure 6.8B) and IgG3 (Figure 6.8C) using PE-Cy5-labeled anti-B220 Abs, FITC-labelled to antibodies to IgA and biotin-labelled anti-IgG3 Abs. After incubation for 30 minutes, the biotin-labeled cells were washed two times and resupended in APC-strepavidin for additional 15 minutes. All the cells were finally washed once and were analyzed on FACS Calibur. Results were representative of five independent experiments.

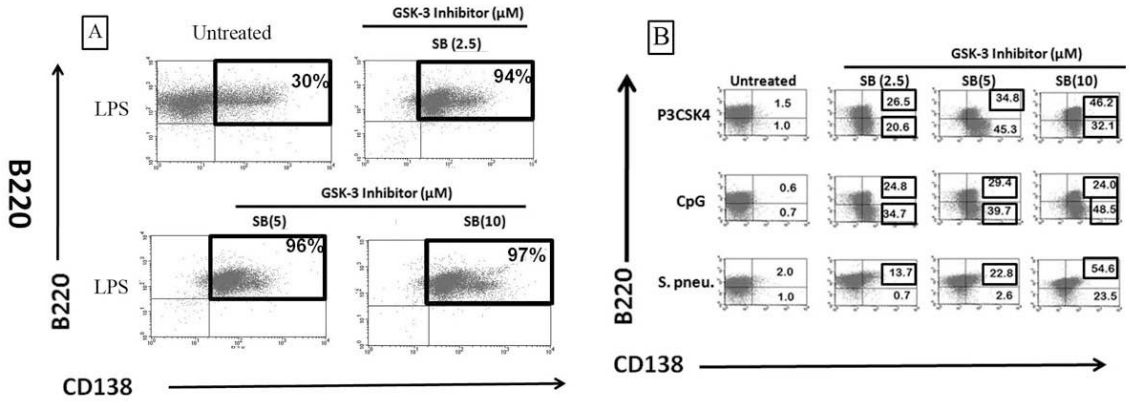


Figure 6.9. SB216763 induces increased plasma cell formation in TLR-2,-4,-9 and HKSP stimulated splenic B-cells

Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with LPS (20 μg/ml) (Panel A), Pam3CSK4 (5μg/ml), CpG (5μg/ml) and HKSP (2.0×10^8 CFU/ml) (Panel B) for 3 days. FACS analysis was performed on B cells stained with PE-Cy5-labeled anti-B220 and PE-labelled anti-CD-138 antibodies. Results were representative of five independent experiments.

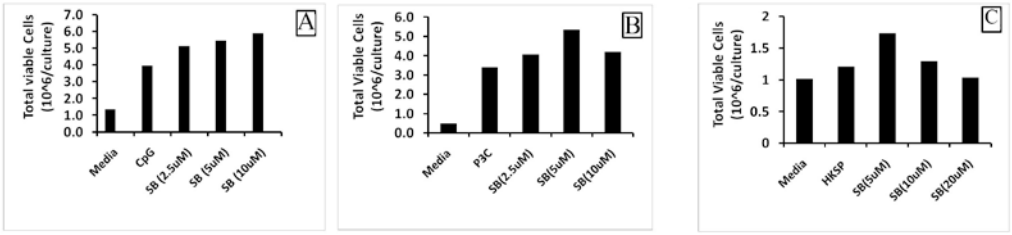


Figure 6.10. SB216763 is not toxic to young splenic B cells activated via TLR-2, 9 and HKSP

Splenic B cells (1.0^6 cells/ml) from young Balb/c mice were cultured with SB216763 for 1 hour, then stimulated with CpG (5µg/ml) (Figure 6.10 A), Pam3CSK4 (5µg/ml) (Figures 6.10 B), and HKSP (2.0×10^8 CFU/ml) (Figure 6.10C) for 3 days. The cells were harvested and washed twice with 1X PBS and then resuspended in RMPI. The viable cells were counted by trypan blue-mediated enumeration. Results were representative of three independent experiments.

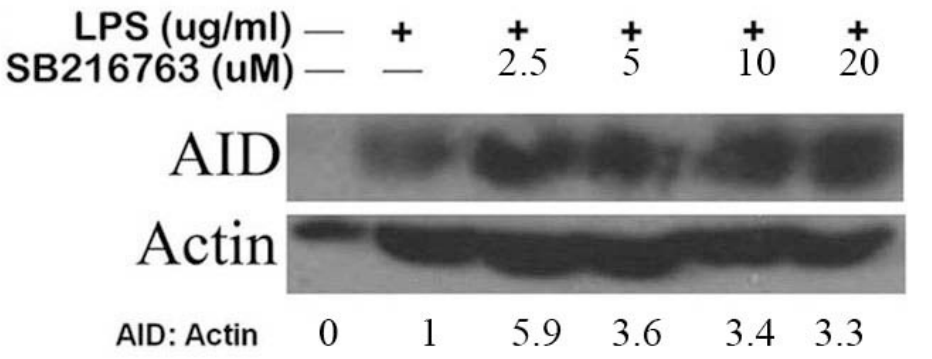


Figure 6.11. SB216763 enhances expression of AID in young splenic B cells activated via TLRs.

Purified splenic B cells (2.0 x10^6 cells/ml) in 5 ml of RPMI complete from young adult mice were were either treated with SB216763 or left untreated for 1 hour and then stimulated with LPS (20 µg/ml) for three days and total cell lysates were isolated and immunoblotted for AID (Figures 6.11) . The blot was stripped and probed for beta-actin. The numbers represented densities of bands normalized to beta-actin with the values for unstimulated young B cells set to one.

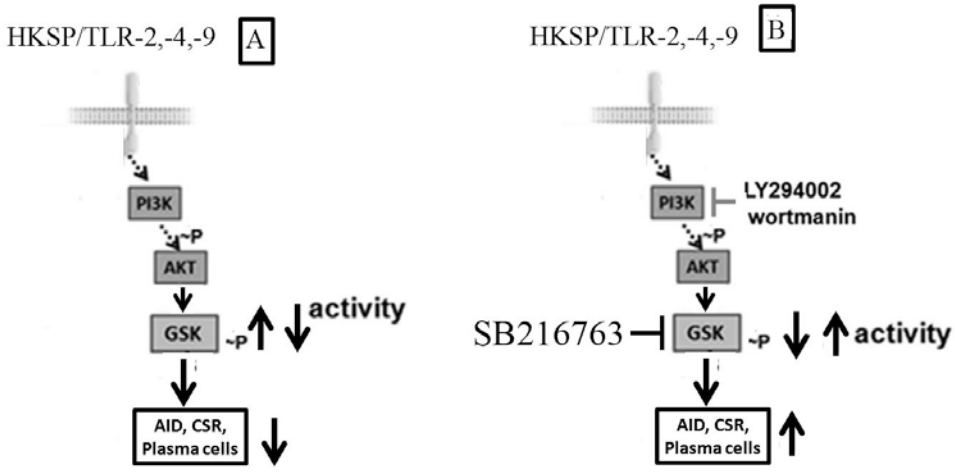


Figure 6.12. Schematic model for PI3K-Akt and GSK-3 inhibition in positively modulating B cell immune functions.

The HKSP or TLR-2,-4 and -9 activation of aged splenic B cells triggers activation of PI3K, increased activity of PI3K-Akt pathway (increased p-Akt, p-GSK-3) leading to suppression of AID, CSR and IgG3 production (Figure 6.12A). This age-related defect can be rescued by inhibiting PI3K or GSK-3 leading to reduction in p-Akt relieving the suppression of AID which then promotes CSR and IgG3 production at levels similar to the young (Figure 6.12B).

CHAPTER 7: OVERALL DISCUSSION

Biomarkers of aging- macrophages: Aging has become a challenging global health concern as the development in health and technology is giving rise to an ever increasing elderly population. This population exhibits a unique aging phenomenon referred to as immunosenecence, which is associated with impaired innate and adaptive immune functions (197, 220, 228). As a result of these defects, this population is affected with pathologies that are neurological, metabolic, plastic and systemic. There is increased incidence of dementia, Alzheimer disease, diabetes, cancers and infections resulting in higher morbidities, mortalities and costly hospitalization (58, 204). These age-related pathologies have led to frantic efforts to identify markers of aging so that effective therapeutics can be developed. In any disease entity the identification of biomarkers is very relevant as it leads to the ability to (1) predict the disease progression and intensity (2) develop effective therapeutics and (3) effectively measure the efficacy of the existing and newly developing therapeutics. In the aged humans and rodents, a couple of these markers are known and are paving the way to (1) predict the aging population that are more susceptible to age-related disease syndromes, (2) develop therapeutics, such as exogenous cytokines, ligands for TLRs such as CpG, that show promises in mouse models to rescue these aging defects; and (3) lead the way in measuring intervention in age such as increase in CSR and antibody responses that are of higher affinity and avidity (78, 196).

In the context of immunosenecence, there are still many challenges in adequately defining such markers, tailoring patented therapeutics and developing assays that will give a good measure of outcomes. One of the challenges is that aging markers are not

restricted to one type of cell or tissue as aging is known to affect all innate and adaptive immune cells. Hence, a marker in one group of cells may only provide for partial therapeutics and outcome measure of disease state and progression. For instance, while the level of AID could serve as a very good marker for B cell immunosenescence, it will not serve similar purposes in macrophages or T cells (196).

Macrophage and macrophage-derived cytokines are crucial in modulating both the innate and immune responses, and a dysregulation in cytokines is a chief predisposing factor for age-abnormalities like Alzheimer's disease, cancer and increased susceptibilities to infectious diseases. Age-related defects in macrophages are a contributing factor to the diminished immune responses in the host (186, 282, 288). The sentinel functions of macrophages in the respiratory tract are defective which contribute to mortality from pneumonia and influenza among the elderly (186, 397). In the context of immune responses to *S. pneumoniae*, a dysregulation in IL-6, TNF-α, IL-1β in alveolar macrophages increases the susceptibility to the disease leading to increased lethality (355, 357). The age-related decline in vaccine responses has also been attributed to defect in macrophage function (58, 186). The prognosis of elderly patients with acute pneumonia are very poor if they produce lower TNF-α and IL-1β compared to their young adult counterparts (121). IFN-γ has also been shown to cause hypo-responsiveness to *S. pneumoniae* in the post-influenza recovery period.

If macrophage and macrophage-derived cytokines are crucial biomarkers for aging, and modulating their age-related defects could provide effective measurable therapeutics, why is there an absence of research to therapeutically modulate macrophage and macrophage-derived cytokines to restore immune functions? There are several challenges

such as (1) establishing the dichotomy between cytokine dysregulation and "inflammaging"; (2) uniform characterization of aged macrophages in terms of phenotypes and signaling molecules; and (3) evaluating age-related impairment in terms of intrinsic and extrinsic defects.

Cytokine-dysregulation and inflammaging:In this thesis, we report in chapter 3 that upon stimulating negatively selected splenic macrophages with ligands for TLR-4, TLR2/1 and TLR2/6 there is a reduction in pro-inflammatory cytokines, IL-12, IL-6 and TNF-α, but an increase in IL-10. We repeat this with a ligand for the IL-1 receptor and heat killed *Streptococcus pneumonia*e (HKSP) that contain ligands for TLR2 and TLR9. This is established in literature as "cytokine dysregulation", and is reported in both peritoneal and splenic macrophages by many other investigators (34, 63, 277, 298). However, this finding runs contrary to the concept of "inflamm-aging" which indicates increased basal inflammation is the norm in the elderly. IL-6 and TNF-α are the two common cytokines that gave rise to this basal inflammation and predispose such individuals to several inflammatory disease such as atherosclerosis (47, 102). This low grade inflammation has several contributing factors including: frailty of the elderly, obesity, lack of exercises and prior infections among others (9). The frail elderly already have predisposing underlying diseases that impact on basal inflammation, whereas, in the healthy elderly, the inflammation is controlled by the increase in the levels of IL-10 (10). For instance IL-6 is less in normally healthy elderly than the young adult, but this is reversed in elderly having infections (257).

A recent study shows that the apparent discrepancies in age-related macrophages in terms of cytokine-dysregulation and to age-related inflammation are due to differences in

strains, sex, site and methods of macrophage collection and experimental conditions (186). But we are able to show in chapter 4 that cytokine dysregulation can also be demonstrated in in vitro generated BMDM stimulated with several TLR ligands and HKSP. Since two different groups of macrophages (spleen and BMDM) are isolated under different conditions and treated similarly to yield cytokine-dysregulation, we conclude the method of macrophage isolation or the site of macrophages does not explain this deviation in cytokine prolife. If cytokine dysregulation represents an aging marker for effective therapeutics, there must be a careful selection of healthy elderly candidates to account for other co-morbidities.

Another issue with the use of cytokine dysregulation in macrophage as a marker for aging for the development of effective therapeutics is the fact that there are differences in macrophages in terms of phenotype, function and effects on the surrounding cells (245). Thus, human intestinal macrophages do not produce inflammatory cytokines and do not express complement receptor (CR/CD11b) (330). There is a classification of M1 and M2 macrophages, in which the M1 macrophages are associated with the Th1 strain of mice (C57BL/6) and produce high levels of nitric oxide (NO) in response to LPS or interferon gamma (IFN-γ), while the M2 are associated with Balb/c, the Th2 strain and produce arginase which converts arginase to L-Ornithine in response to LPS alone (237). The most commonly identified macrophages are the classically activated macrophages (CAM/M1) and the alternatively activated macrophages (AAM/M2) (123). The CAM and the AAM are relevant to our studies as each of these classes of macrophages is influenced by different cytokines and they in turn produce different cytokines. Not only that, they can each respond to different classes of pathogens and thus the appropriate

microenvironment must be created to tailor a response for a specific pathogen. In our case, the goal is to design therapeutics that precisely orchestrate a particular phenotype of macrophages that target extracellular pathogens like *S. pneumoniae*; as in the case of the CAM which are effective against intracellular pathogens like Mycobacterium tuberculosis (245, 258).

The CAM are activated by a dual signaling involving first a priming by IFN-γ, produced by Th1 cells, and then a subsequent signal by microbial LPS, but the AA macrophages don't need a priming as IL-4 and/or IL-13 produced by Th2 cells can activate these cells (123). The CAM have higher phagocytic and antigen presentation functions than AAM. They produce chemokines like IL-8 and Rantes that help in the recruitment of NK cells, neutrophils, T cells and even immature DCs. In addition, they produce pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α (245, 258). However, they have a tendency to produce massive inflammation and uncontrolled activation that can result in tissue damage, type I autoimmunity and tumors (123). The AAM also upregulate MHC-II and a distinct set of chemokines like CCL-17, 18 and 22, which help in the recruitment of leukocytes. Unlike CAM, AAM do not produce NO from L-arginine (247). Once activated they can pinocytose antigens via their mannose receptor (MR), which are specific to the AAM, and present these antigens to T cells in the context of MHC-II. They also upregulate the enzyme arginase-I, a hallmark of AAM, that help produce proline required for rebuilding of extracellular matrix that is destroyed by the overly activated CAM (123, 247). AAM can down-modulate the massive pro-inflmmatory cytokines produced by the CAM via the production of anti-inflammatory cytokines like IL-10, TGF-β and IL-1Ra. They are also involved in would healing, angiogenesis and tissue

repairs (245, 258). The AAM like the CAM, have beneficial and pathological effects. While the AAM are involved in mediating humoral immunity and anti-parasitic responses, such as Shistosomiasis, they are associated with common pathologies like allergy and asthma (123).

In this thesis we demonstrated that both in splenic and bone marrow derived macrophages inhibiting PI3K-Akt signaling pathway orchestrates a macrophage population that is specifically producing the pro-inflammatory cytokines, while suppressing IL-10, one of the anti-inflammatory cytokines used by the AAM to down-modulate pro-inflammatory cytokines. We could probably say that PI3K-Akt inhibition produces a macrophage population that is closer to CAM in terms of the cytokines profile. A recent study indicates that PI3K is needed for the development of AAM, where a deficiency of SHIP, a negative regulator of PI3K, leads to the production of AAM, but that SHIP-mediated negative regulation of PI3K had a propensity for CAM (296). Hence, by modulating PI3K-Akt we can drive the AAM or CAM dichotomy for rescuing the age-related defects in macrophage and macrophage-derived cytokines for potent immune response to specific type of infections. However, we cannot clearly identify whether these pharmacological inhibitors are able to produce either CAM or AAM. We will need additional assays to determine NO and arginase-I for CAM and AAM respectively under these conditions. Our microarray shows that neither iNOS nor arginase is selectively increased in the aged versus young macrophages (65).

Finally, for the use of cytokine-dysregulation in macrophages as a marker for aging in the development of therapeutics, there is the challenge of clearly establishing the contribution of the intrinsic and extrinsic factors (186). Until these can be resolved, it becomes

difficult to develop effective therapeutics and to rely on reversing cytokine-dysregulation as treatment marker. In the context of viral infection, it is known that macrophages can exert both intrinsic (limiting viral growth in vivo) and extrinsic (preventing viral production in other cells via the induction of interferons or TNF-α) modulatory effects (229, 243, 381). Macrophage function in terms of cytokine production and antiviral resistance can be positively modulated by exercise, which is an extrinsic modulatory effect (146, 186). To address this important question, we used negatively selected SM and BMDM that are grown under in vitro conditions and treated them with ligands for TLR-2, 4 and/or -9 as well as HKSP with or without PI3K inhibitors. By demonstrating similar signaling mechanisms and cytokine profile in BMDM and SM we showed that the age-related defect in macrophages is most likely due to intrinsic signaling defects. We also demonstrated that PI3K-Akt inhibition can rescue this cytokine dysregulation in macrophages from either of these sources. Thus, we established that the cytokine dysregulation can be considered an aging marker, which can be treated in the context of intrinsic signaling defect in the PI3K-Akt pathway (17, 38, 64, 178, 230, 355, 356). We further show that inhibiting GSK-3, a downstream kinase of Akt, can potently activate macrophages to secrete IL-10 and pro-inflammatory cytokines needed for B cell mediated humoral responses to *S. pneumoniae,* as well as co-stimulatory molecues that are necessary for activation of the adaptive immunity (213, 325). Our studies do not address the reasons for elevation of PI3 kinase subunits in the aged SM and BMDM. Presently little is known about how genes for the p85 and p110δ are regulated (116). It is conceivable that epigenetic changes have a role in increased expression with ageing.

Future studies will have to address the kinetics and mechanism of regulation of such genes with increasing age.

Biomarkers of aging-B cells: Even, if we assume that AID levels are a very significant all-encompassing marker for aging, there is the additional challenge to establish whether the B cell immunosenescene is due to age-related intrinsic defects or extrinsic environmental defects or a combination. If it is possible to show that both intrinsic and extrinsic defects are affecting B cells in the aged it will be difficult to develop a unified therapeutics that modulates both the intrinsic and the extrinsic defects simultaneously. One group of studies show that over expressing transcription factors like E47 or genetically down-modulating Akt increases AID, CSR and upregulates antibody production and thus serves as a potential method to rescue the age-related intrinsic defects in B cells. We and others show that providing an extrinsic microenvironmental support like macrophage-derived cytokines could similarly rescue B cells in terms of antibody production under in vitro conditions (38, 63, 114, 196, 268).

Our laboratory has been studying the effect of aging on B cell response in the context of *S. pneumoniae,* a Gram positive bacterium that affects the elderly more than any other pathogen and is responsible for very long expensive hospitalization, morbidity and mortalities (218). We show that B cells from the aged Balb/c mice have an impaired response to both pneumococcal polysaccharide vaccine (Pnu-Immune) and the TNP-Ficoll, a typical TI-2 antigen. These age-related defects can be rescued by macrophages or macrophage-derived cytokines from young adult mice. We further demonstrate that the age-related defects in B cell responses are due to extrinsic defects due to cytokine dysregulation in aged macrophages (38, 63, 114). However, these studies still have some

unanswered questions, such as (1) is the increase in antibody responses due to CSR and to what extent is AID involved? (2) are there intrinsic signaling pathways that are defective in the aged B cells that are contributing to the B cells defect? (3) is the modification of the extrinsic cytokines that positively upregulate the B cell antibody response also regulate a signaling pathway in B cell? (4) can we modify B cell responses by modifying the age-related cytokine dysregulation in macrophages? (5) is cytokine-dysregulation a good aging biomarker that can be used to design therapeutics and to reverse immunosenence in B cells and thus enhance immunity to *S. pneumoniae* and other pathogenic infections? and (6) can we correlate this cytokine dysregulation with a signaling pathway that can be pharmacologically modulated to reverse age-related B cell defects?

As alluded to earlier, the immunsenescence markers in B cells include impaired AID and CSR leading to a decrease in the quality and quantity of antibodies that are needed for the clearance of infections (196). These defects have been shown to be both intrinsic and extrinsic by us and many other laboratories and can be potentially rescued by modulating either the intrinsic factors or the extrinsic factors like the cytokine milieu (38, 114, 196). On the basis of literature on the role of the PI3K-Akt-GSK-3 pathway in modulating B cell immune function (103, 105, 268, 269), we investigated this pathway. We showed in Chapter 6 that the age-related heighted activity of the PI3K-Akt pathway correlates with a defect in the production of IgG3 in the aged splenic B cells and that inhibiting this pathway can rescue this pathway. We further show that inhibiting this pathway can enhance the AID marker for CSR. The finding that PI3K-Akt is an inhibitor of AID and

that this pathway is crucial to pathway for humoral responses including CSR is supported by many studies (22, 30, 81, 343).

Finally, we show that GSK-3 has comparable effects to the PI3K-Akt pathway in young adult splenic B cells. Inhibiting GSK-3 pharmacologically induces higher production of different isotypes of IgG, IgA and IgM, as well as increased production of plasma cells. The inhibition also leads to the induction of AID and CSR. The transcriptional factors responsible for these effects are not presently known, but on the basis of previous studies we are postulating that β-catenin, NFATc1 and NF-κβ may be involved and will be included in future studies (29, 82, 391).

In conclusion, we show in this thesis that the age-asscociated cytokine dysregulation in macrophages and impaired humoral response of B cells when activated via TLR-2, 4 and 9 and HKSP are crucial biomarkers for aging. We showed that PI3K pathway plays a critical role in both macrophages and B cells. We will have to test the effect of the inhibitors in models of challenge with live *S. pneumoniae*. Also, these biomarkers and the effects of PI3K and GSK-3 inhibitors have to be further validated in aged humans. We propose an overall model in Figure 7.1 to summarise the findings from the work of this thesis. We demonstrate that the cytokine dysregulation in aged macrophage is a factor that can possibly account for the inability of macrophages to provide extrinsic support to the already intrinsically defective aged B cell resulting in overall impaired B cell function which may have a role in the clearance of *S. pneumoniae*.

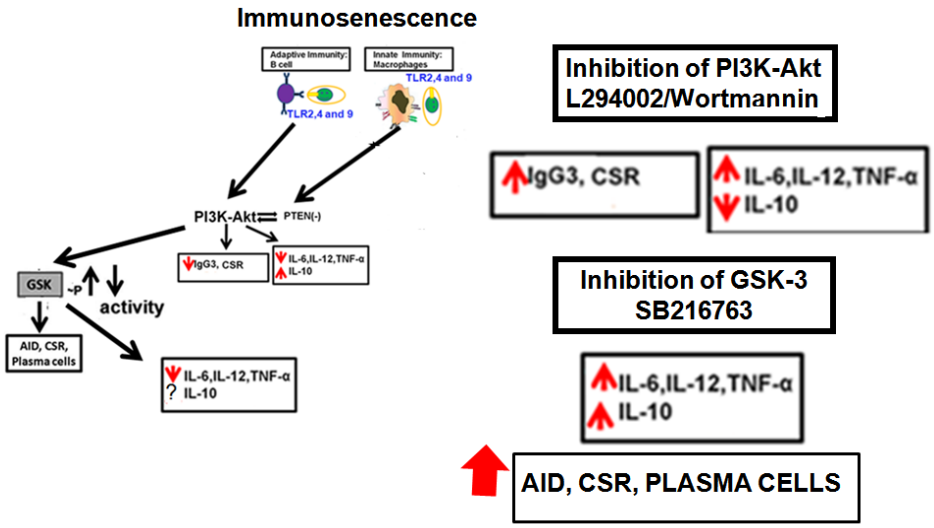
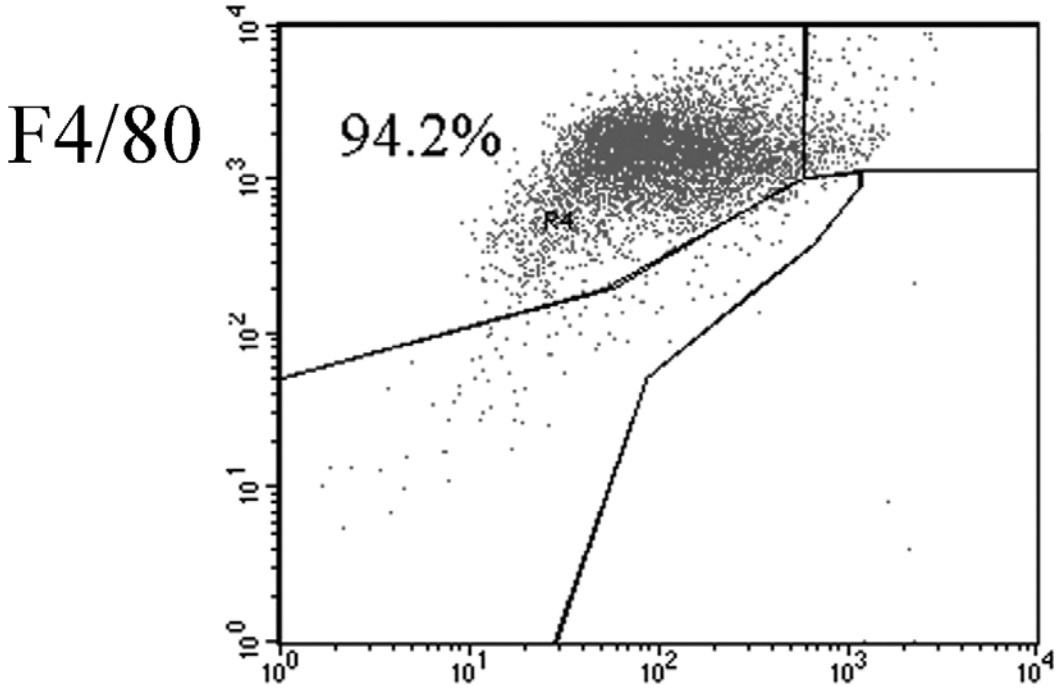


Figure 7.1: Age-related cytokine dysregulation provides a less robust support for B cell humoral response to *S. pneumoniae*. This can be overcome by inhibiting the PI3K-Akt-GSK-3 pathway.

Upon inhibiting the PI3K-Akt pathway in the presence of TLR-2, 4, and 9 or HKSP the cytokine dysregulation in macrophages is reversed and B cells are able to induce AID and CSR that may be more effective in pathogen clearance.

Appendices

Appendix A – Purity of splenic macrophages: (A) FACS staining by PE-conjugated F4/80 on the Y-axis and X-axis is CD11b negative. (B) Average purity of macrophages from seven experiments.



B

Experiment #	% F4/80 positive
1.	94.2
2.	97.3
3.	93.5
4.	92.9
5.	88.7
6.	85.0
7.	89.0
Average	91.5

Appendix B - Abbreviations

AID	activation-induced cytidine deaminase
AP-1	Activator protein-1
AAM	alternatively activated macrophages
BMDM	bone marrow derived macrophages
BCR	B cell receptor
BAFF	B cell activating factor
BLyS	B lymphocyte stimulator
BAFFR	B cell activating factor receptor
COX-2	cycloxyenase-2
CSR	class switch recombination
CR	complement receptor
CAM	classically activated macrophages
CBP	choline binding protein
CVID	common variable immunodeficiency disease
CREB	cAMP-response element-binding protein
DCs	dendritic cells
DTH	Delayed type hypersensitivity
ERK	extracellular-signal-regulated kinase
ELISA	enzyme-linked immunosorbent assay
FOXO3a	forkhead transcription factor/Forkhead box 3a
FcγR	Fc γ receptor
FITC	Fluorescein isothiocyanate (FITC)
GM-CSF	granulocyte-macrophage colony stimulating factor
GSK-3	Glycogen synthase kinase-3
HSC	Hematopoietic stem cell
HKSP	Heat killed *Streptococcus pneumoniae*
IPD	Invasive pneumococcal disease
IRAK-4	Interleukin-1 receptor-associated kinase 4
IRAK1	Interleukin-1 receptor-associated kinase 1
IL-1β	Interleukin -1 beta
IKK	Inhibitor of nuclear factor kappa B kinase
IRF3	Interferon regulator factor 3
IFN- γ	Interferon-gamma
LPS	Lipopolysaccharide
LY	LY294002
LTA	Lipoteichoic acid (LTA)
LRR	leucine-rich repeats
MyD88	Myeloid differentiation primary response gene 88
MAPK	Mitogen activated protein kinase
MDDC	Monocyte derived dendritic cells
MZ	Marginal zone
MHC-II	Major histocompatibility complex II
MPL	Monophosphoryl lipad A sIgA
NF-ATc	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B

Pam-3-CSK4	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-C-S-Lys4
PMSF	phenyl methyl sulfonyl fluoride
PBP	penicillin binding protein
PCV-7	the seven valent conjugate vaccine
PI3K	phosphatidyl inositol 3-kinase (PI3K)
PTEN	phosphatase and tensin homolog
PspA	pneumococcal surface protein A
PsaA	pneumococcal surface adhesion protein A
PRR	pattern recognition receptors
PPS-23	23-valeent pneumococcal polysaccharide vaccine
PTK	Protein tyrosine kinase
PKC	Protein kinase C
pDC	plasmacytoid dendritic cells
PGE2	prostaglandin E2
PPS	polysaccharide capsule
PH	pleckstrin homology domain
PDK-1	phosphoinositide-dependent kinase-1
PAMPs	pathogen associated molecular patterns
PBM	peripheral blood monocytes
qRT RT-PCR	quantitative real time reverse transcriptase polymerase chain reaction
RPMI	Roswell Park Memorial Institute media
SAC	Staphylococcus aureus cowan
SM	splenic macrophages
S. pneumoniae	*Streptococcus pneumoniae*
TCR	T cell receptor
TLR	Toll like receptor
Treg	regulatory T cells
TIR	Toll/interleukin-1 (IL-1) receptor homology
TIRAP	TIR domain containing adaptor protein
TRIF	TIR domain containing adaptor -inducing interferon-β
TNP-Ficoll	trinitrophenol conjugated to Ficoll
TNP-LPS	trinitrophenol conjugated to LPS
TICAM1	TIR domain containing adaptor molecule likes MyD88
TRAM	TRIF-related adaptor molecule
TRAF-6	TNF receptor associated factor-6
TD	T-cell dependent
TGF-β	transforming growth factor beta

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Manuscripts in preparation:
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Mosoka P. Fallah, Michael Baker, Eric Rogier, Beth Garvy and Subbarao Bondada, "PI3 kinase inhibition rescues age-related defects in IgG3 and class switch recombination via TLR-2,-4 and heat killed Streptococcus pneumoniae"

Mosoka P. Fallah, Michael Baker and Subbarao Bondada. "Regulation of B cell activation and differentiation by the GSK-3 signaling via TLR-2,-4, -9 and heat killed Streptococcus pneumonia".

Scientific Meetings (First author presentations)

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Poster presentations:
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