REGULATION OF TRANSCRIPTION FACTOR NFKAPPA B IN IMMUNE SENESCENCE

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TABLE OF CONTENTS

1. ABSTRACT

The Rel/NF kappa B family of eukaryotic transcription factors are critical in immune and inflammatory processes regulating the expression of a wide variety of cytokines including IL-1, IL-2, IL-6, TNF-alpha and GM-CSF. Its ubiquitous distribution, rapid induction and regulation, the complexity of its subunits and its apparent involvement in several diseases has made this transcription factor a subject of intense study in normal cellular growth and cancer. Emerging studies have implicated a role for this transcription factor in the normal processes of aging. As significant declines in immune function is a natural concomitant to advancing age, the regulation of transcription factor NF kappa B appears to play a pivotal role in immune dysregulation during senescence, contributing to down regulation of both IL-2 and IL-2 receptor-alpha expression. Our studies have contributed to understanding the regulation of lowered NF kappa B induction in T cells during aging in humans and mice. Since we have shown that the lowered induction of NF kappa B in activated T cells from the elderly can be attributed to impaired degradation of the inhibitor I kappa B-alpha due to lowered proteasomal activity, we suspect that a similar alteration in proteasomal activity may be operative in age-dependent failure of immune function including the inability to initiate DNA synthesis following activation, skewing of T cell repertoire, lowered cytolytic activity and accumulation of aberrant proteins. Understanding the regulation of the proteasome pathway during immune senescence may provide new avenues for therapeutic intervention for immune based geriatric diseases.

2. INTRODUCTION: AGING AND IMMUNE FUNCTION

One of the extensively studied and well documented physiologic declines in the aged is immune senescence (1-5). Aging results in a significant decline in immune function which has been directly or indirectly linked to increased susceptibility to infections, autoimmunity, and cancer (2,4,6-11). Previous studies have clearly established that neither changes in numbers of lymphoid cells nor changes in cellular environment contribute to age-related immune dysfunction, thus suggesting that the underlying cause for this dysfunction may be found in regulatory changes within the immune apparatus (5,12,13).

Age-associated decreases in both humoral and cell mediated immune responses have been well documented (14-17). The two main cellular components of the immune system mediating specific immune responses, i.e. T lymphocytes and B lymphocytes, exhibit functional deficits with advancing age (17-25). Although phagocytic activity has been reported to decrease with age (26), preliminary studies on antigen presentation by both macrophage and dendritic cells appear not to be affected with advancing age (27-29). Total immunoglobulin production is unchanged with age, while serum immunoglobulin components and specificities, show age-related alterations. In older humans, serum concentrations of IgG and IgA increase, whereas the concentration of IgM is either unchanged or decreases with age (30-33). While antibody responses against foreign antigens such as tetanus toxoid decrease with age (4,14,17,18), the production of antibodies directed towards self antigens such as thyroglobulin, nuclear proteins, and DNA has been documented to increase in aging humans (18,30,33,34), and these observations have often been the bases for theories of declining self/non-self regulation in the elderly. Other tests of B cell function such as proliferation for the large part demonstrate lowered responses after treatment with mitogens and antigens (2,19,35), however, scattered reports show either age-related increase or no change in B cell proliferative

responses (2, 36). Although B lymphocyte function appears to be clearly affected with advancing age, it still remains to be unequivocally established whether this is independent of the actions of the regulatory T cells and/or their secreted cytokines.

2.1 T lymphocyte function during immune senescence

In contrast to studies dealing with B cells, most of the reports on age-related immune dysfunction point to a significant decline in T cell function. In fact, decline in T cell function has been proposed to be the central defect in immune senescence (2,4,5). The organ responsible for T cell maturation and function, the thymus, undergoes considerable involution with age. It has been suggested that the manifestations of immune senescence are all related to the involution of the thymus, however the precise role of thymic involution in immune senescence is unclear since this occurs as early as 20 years of age in humans while T cell deficits are not observed until the fourth or fifth decade of one's life (5,11,37). It cannot, however, be ruled out that the involution of the thymus may be a forerunner to immune deficits observed in the elderly.

Aging results in a few changes in the phenotypic profiles of T lymphocytes. It has been observed that the changes that accompany aging in the T cell compartment is that of a shift toward a greater number of memory (CD44hi/CD45RO^{+}) and fewer naive (CD44lo/CD45RA^{+}) T cells (38-41). The increase in ratio of memory to naive T cells is thought to be a reflection of the decreased supply of naive cells to the periphery accompanied by the continual antigen-driven conversion of naive to memory cells (2,42). Memory cells are thought to differ from naive cells in terms of activation requirements and cytokine production (43-45). It has been proposed that many age-related alterations in T cells are attributable to the increase in memory cells based on the poor activation capacity of memory cells (38,39,46-48), however this argues against some studies which have indicated that memory cells have similar or even enhanced activation capabilities compared to naive cells (43, 49-54). Furthermore, recent studies have provided evidence that CD45RA^{+} and CD45RO^{+} positive cells can interconvert thus, suggesting that the previous findings may not be absolute and may be meaningful only when discussed in the context of activating antigen (55,56). Therefore, the consequence of age-related increase in memory (CD45RO^{+}) T cell subsets remains to be fully delineated. Although changes in the numbers of CD4 and CD8 T cell subsets have been demonstrated in humans, these changes are not consistent from study to study and are too small to account for the significant changes seen in the functional abilities of T cells (2).

T cell function in elderly humans is characterized by decreases in classic tests of T cell mediated immunity such as Mixed Lymphocyte Reactions (MLR) and Delayed Type Hypersensitivity responses (DTH) (2,4,13,15,57). Cytotoxic T cell (CTL) generation and activity has also been reported to decline with age (2,58,59). A hallmark feature of age-related T cell dysfunction is the inability of T cells from the elderly to proliferate at a level comparable to that

seen in young individuals after activation with antigen or mitogens such as Phytohemagglutinin (PHA), Concanavalin A (Con A), or anti-CD3 (20-23). Diminished proliferative capacity of lymphocytes from elderly humans is also evident in in vitro responses to recall antigens, i.e. antigens to which they have been previously sensitized, such as Varicella Zoster and Mycobacterium Tuberculosis (4,14,15,24). The decrease in mitogen-induced proliferative responses have been demonstrated to be not attributable to alterations in cell number, viability, number or binding affinity of cell surface receptors on T cells (12,13,25,60,61), but rather to a decline in their ability to generate second messengers (2,20,62-65). Numerous studies have demonstrated significant age-related decrease in both IL-2 production and IL-2R-alpha expression following T cell activation (20,24,61,66-68). These defects are also evident at the level of gene expression as mRNA for IL-2 and IL-2-R-alpha is also decreased with age following mitogenic stimulation (61,66). The effect of age on mitogen induced generation of cytokines other than IL-2 has been studied less extensively and information is not consistent, however some studies have reported increases in IFN-gamma production by T cells, while others have reported increases in IL-10 and IL-6 in T cells and mouse spleen cells (69, 70-72). Taken together these studies appear to suggest an underlying defect in the generation of second messengers following T cell receptor mediated activation.

The cascade of early biochemical events that follows TCR ligation ultimately trigger the transcription of genes including c-fos, c-myc, IL-2 and IL-2R-alpha whose protein products are essential for T cell differentiation and effector function (73,74,75). Activation of transcription factors is an important mechanism for the transmission of extracellular signals from the cytoplasm to the nucleus. Few newly emerging studies including our own have implicated a decrease in the induction of transcription factors for the resulting decline in signaling and proliferation observed in the aged (76-79). These studies indicate that in addition to defects occurring immediately following receptor ligation, defects also occur in downstream signaling events of which the induction of transcription factors may be vital to the events resulting in aberrant nuclear signaling. T cell activation results in the activation of several important transcription factors including Activator Protein-1 (AP-1), Nuclear Factor of Activated T cells (NFAT), and Nuclear Factor Kappa B (NF kappa B) which play key roles in mediating transcription of genes involved in the generation of T cell immune responses (73-75). NFAT is a transcriptional activator that is a specific target for signals from the antigen receptor and binds to sites in the IL-2 promoter. This complex is largely restricted to T lymphocytes and is thought to be responsible for the T cell-specific inducibility of IL-2 (73,80). Binding sites for NFAT have also been observed in the regulatory regions of genes for other cytokines such as IL-3, IL-4, GM-CSF, and TNF-alpha indicating that NFAT may regulate the expression of cytokine genes other than IL-2 (81-84).

3. TRANSCRIPTION FACTOR NF KAPPA B

NF kappa B is induced in response to many different noxious agents. It is activated by a myriad of agents including cytokines like IL-1 and TNF-alpha, bacterial LPS, Viral infection and certain viral proteins such as HTLV-1 Tax, LMP1 of EBV, antigen receptor cross linking on T and B lymphocytes, Calcium ionophores, phorbol esters, UV radiation and others. The genes regulated by NF kappa B family of transcription factors are just as diverse as the activators and include those involved in immune function, inflammatory response, cell adhesion, cell growth, and cell death. First characterized in mature B and plasma cells as a nuclear protein that binds specifically to a 10-bp sequence in the kappa intronic enhancer , NF kappa B has now been demonstrated in virtually all cells. In most cells with the exception of mature B cells, macrophages and some neurons, NF kappa B remains dormant in the cytoplasm bound to its inhibitor, I kappa B. Treatment with various agents leads to the dissociation of the inhibitor and the translocation of the free NF kappa B to the nucleus.

NF kappa B was originally identified as a B lymphocyte nuclear factor binding to a site in the immunoglobulin kappa light chain enhancer (85). It is now recognized as a pleiotropic transcription factor binding to many cellular and viral gene promoters. NF kappa B is recognized for its central role in immunological processes via the expression of a wide variety of immune response genes (86-89). Recent studies have provided evidence for the involvement of NF kappa B in growth control of certain tumors (90-94). NF kappa B is maintained in the cytoplasm of the cell by the inhibitor kappa B (I kappa B) which tightly regulates the nuclear expression and biological function of NF kappa B (95-97). The speed of induction and its ubiquitous expression makes NF kappa B an ideal regulator of rapid-response genes (87,98).

NF kappa B family of transcription factors are homo- and heterodimeric complexes formed from combinations of members of the Rel family of proteins (86-89). The Rel family of proteins belong to the v-rel oncogene found in the Reticuloendotheliosis Virus Strain T (RevT) and are characterized by having a common Rel homology domain (RHD) which consists of 300 amino acids in length. Specific sites within the RHD are responsible for DNA binding to kappa B sites, dimerization with other Rel family proteins, and interaction with I kappa B. The C-terminal portion of the RHD contains a group of positively charged amino acids that function as the nuclear localization signal (NLS) (86-89, 99,100). There are five mammalian members of the Rel family of proteins. These include c-Rel, NF kappa B1 (p105/p50), NF kappa B2 (p100/p52), RelA (p65), and RelB (86-89). NFATp(nuclear factor of activated T cells) is considered to be related to the Rel family of proteins as it has a region of 430 amino acids that shares 17% amino acid identity to RelA (p65). There also appears to be some functional similarities between p65 and NFATp since both these proteins can interact with c-fos and c-jun (101-103).

Theoretically, five members of the Rel family proteins can form almost any possible combination of homo or heterodimers although only certain combinations have been detected in vivo (89).The classic and most well studied NF kappa B molecule is a heterodimer of p50/p65 subunits. This heterodimer is the most abundant complex and is found in virtually all cell types. Heterodimers of p50/p65 are rapidly translocated to the nucleus following cellular activation and bind the consensus sequence 5'GGGRNNYYCC3'. The transactivation function in vivo is mediated by RelA (p65), RelB, and c-Rel which contain transactivation domains in their C-terminal domains, while the p50 and p52 subunits primarily serve as DNA binding subunits (86,104-108). Transcriptionally active complexes are usually heterodimers consisting of p50 or p52 in combination with one of the transactivating subunits (p65, c-Rel, or RelB), while homodimers of p50 correlate with transcriptional repression (86,109,110). Each of the heterodimers exhibit unique properties including cell type specificity, DNA binding site preference, differential interactions with I kappa B isoforms, differential activation requirements, and kinetics of activation, thus being capable of regulating gene expression in a uniquely specific manner (86,104,111-115). For example, in pre-B cells the active NF kappa B complexes are primarily dimers of p50/p65 while the constitutive form of NF kappa B in mature B cells are c-Rel/p50 dimers (114). While p50/p65 dimers rapidly appear in the nucleus following stimulation, dimers of p50/c-Rel exhibit a more delayed response and accumulate in the nucleus more slowly (115).

3.1 Inhibitor of kappa B, I kappa B

The rapid inducibility of NF kappa B can be attributed to the fact that it preexists in the cytoplasm of cells in an inactive form complexed to I kappa B, thus requiring no new protein synthesis (96-98). I kappa B proteins regulate the cellular location, DNA binding, and transcriptional properties of NF kappa B/Rel family of proteins. The current mammalian I kappa B family of proteins includes I kappa B-alpha, I kappa B-beta, I kappa B-gamma, I kappa B-delta, (generated from alternative splicing of the NF kappa B1 gene, NF kappa B1, NF kappa B2), I kappa B-epsilon and the predominantly nuclear protein Bcl-3 (86,87,116). All I kappa B proteins have in common a conserved domain containing six to eight repeats of the erythrocyte protein ankyrin (117,118). I kappa B binds to the NF kappa B dimer and masks its nuclear localization signal thereby sequestering NF kappa B in the cytoplasm of the cell (95). The NF kappa B/I kappa B complex itself cannot bind to DNA, however disassociation of I- kappa B from NF kappa B which can be achieved in vivo with various activating agents or in vitro with agents such as deoxycholate will produce an NF kappa B dimer which is capable of translocating to the nucleus and binding DNA (96,119).

I kappa B-alpha is the most extensively studied and most abundant I kappa B family member and unlike I kappa B-beta, is primarily involved in regulating the rapid and transient activation of NF kappa B (116,120). I kappa B-alpha is a 37 kd protein which can be structurally divided

into a 70 amino acid N terminus, a central section of 205 amino acids composed of 6 ankyrin repeats, and an acidic 42 amino acid C- terminus that contains a PEST (pro-glu-ser-thr) sequence, a motif correlated with rapid protein turnover. I kappa B-alpha performs several critical functions including cytoplasmic retention of NF kappa B in resting cells, release of NF kappa B in response to activating signals, and inhibition of DNA binding by NF-kappa B (87,121). I kappa B-alpha binds to specific Rel subunits and masks the NLS of all dimers containing any of the transactivating subunits (RelA, c-Rel, RelB), especially those containing RelA, thereby retaining these complexes in the cytoplasm (87). Studies of stoichiometry have shown that one dimer of NF kappa B is bound to one I kappa B-alpha molecule (118,121). Thus the classic cytoplasmic NF KAPPA B complex contains a p50/p65 dimer bound to one I kappa B-alpha. I kappa B-alpha can have differential affinity for the various NF kappa B dimers. For example, I kappa B-alpha binds a RelB/p50 heterodimer more efficiently than a RelB/p52 heterodimer. Complexes with the highest affinity for I kappa B-alpha are thought to be mainly cytoplasmic and represent the inducible pool, while those complexes with low affinity for I kappa B-alpha are nuclear and provide constitutive activity.

Removing DNA bound dimers in the absence of an activating signal may also be a role of I kappa B-alpha since I kappa B-alpha not only prevents DNA binding of strongly activating complexes but can also dissociate bound complexes from DNA (122). The underlying mechanism for the inhibition of DNA binding mediated via I kappa B-alpha is not clearly understood, however, it has been shown to require the C-terminal region of I kappa B-alpha (121). Recent studies have shown that exogenously introduced, over expressed, or newly synthesized I kappa B-alpha can be found not only in the cytoplasm, but in the nucleus as well (123-125). In addition, recent studies of I kappa B-alpha knockout mice have demonstrated that TNF-alpha treatment of embryonic fibroblasts from these mice results in a prolonged and sustained nuclear induction of NF kappa B indicating that I-kappa B-alpha plays a role in the termination of an NF kappa B response (126). Thus, it is likely that newly synthesized I kappa B-alpha may enter the nucleus and regulate NF kappa B activity, resulting in a transient response.

4. SIGNALS AND SIGNALING PATHWAYS FOR NF KAPPA B

Activation of NF kappa B results from the degradation of I kappa B-alpha and subsequent translocation of the active NF kappa B dimer to the nucleus (86,87,116), figure 1. NF kappa B can be activated by a wide variety of physiological and non-physiological stimuli; these include cytokines, mitogens, viruses and viral products, oxidative stress, and chemical agents such as phosphatase inhibitors and ceramide. In T cells, almost any stimuli capable of activation results in NF kappa B induction including PHA, PMA, TNF-alpha, IL-1, IL-2, antigen receptor ligation, crosslinking of surface molecules such as CD2, CD3, CD28, and TCR, and infection with a wide variety of viruses such as HIV-1, HTLV, and HSV

(86-89).Most signals tested so far target I kappa B-alpha as evidenced by its degradation in response to these activating stimuli, however the upstream signaling pathways preceding I kappa B-alpha degradation are very divergent and may be specific for a given activator (86,87, 127-130). I kappa B inactivation, without proteolytic degradation, has also been reported to occur as a consequence of tyrosine phosphorylation (131). Much research has been directed at elucidating the signal transduction pathways involved in regulating NF kappa B activation. Early studies implicated PKC as playing a central role in NF kappa B activation in response to many activators since phorbol esters, potent activators of PKC, were NF kappa B inducers and purified PKC released NF kappa B from I kappa B-alpha when incubated with cytosolic extracts (128-130,132,133). Furthermore, the activation of NF kappa B following stimulation of T cells with anti-CD3 has been demonstrated to be dependent upon PKC (134). Recent studies have revealed the existence of PKC independent pathways of NF kappa B activation including activation mediated by TNF-alpha, one of the most potent physiologic inducers of NF kappa B (135). The activation of NF kappa B by TNF-alpha has been shown to occur via the sphingomyelin pathway. In this pathway, phosphatidyl choline specific phospholipase C (PC-PLC) is activated which results in the generation of DAG which in turn leads to the activation of an acidic sphingomyelinase that hydrolyzes sphingomyelin to produce ceramide, in turn activating a ceramide dependent serine/threonine (ser/thr) kinase. Exogenous addition of PC-PLC, DAG, acidic sphingomyelinase, or ceramide can all activate NF kappa B in permeabilized cells (87,136). Although TNF-alpha mediated activation of NF kappa B has been shown to be independent of classical PKC activation, studies have demonstrated an important role for the nonclassical PKC isozyme PKC- zeta) (137-139). A dominant negative inhibitor of PKC-zeta inhibits NF kappa B activation by TNF-alpha, while a constitutively active mutant activates NF kappa B in NIH 3T3 cells (138).

Given the convergence of all signaling pathways at or before the I kappa B-alpha target, existence of a common upstream effector has been postulated. Most of the studies have pointed to reactive oxygen intermediates (ROI's) as the common and critical effector molecule for various activating signals. This notion has been supported by the observations that most inducers of NF kappa B lead to the generation of ROI's and that activation of NF kappa B by many stimuli can be inhibited by antioxidants such as N-acetyl cysteine. Furthermore, treatment of cells with H_2O_2 can also activate NF kappa B (86,87,140). Recently published studies, however, have provided evidence against the role of oxidants in the induction of NF kappa B, leaving no proof of a direct functional role for ROI's in mediating the induction of NF kappa B (141-142).

4.1 Modification and Degradation of I kappa B
Regardless of the upstream signaling events initiated by various activators the ultimate target is I kappa B-alpha. Previous studies demonstrated that modification of I kappa B-alpha rather than NF kappa B was important in the activation of NF kappa B (97,119). I kappa B-alpha was post-
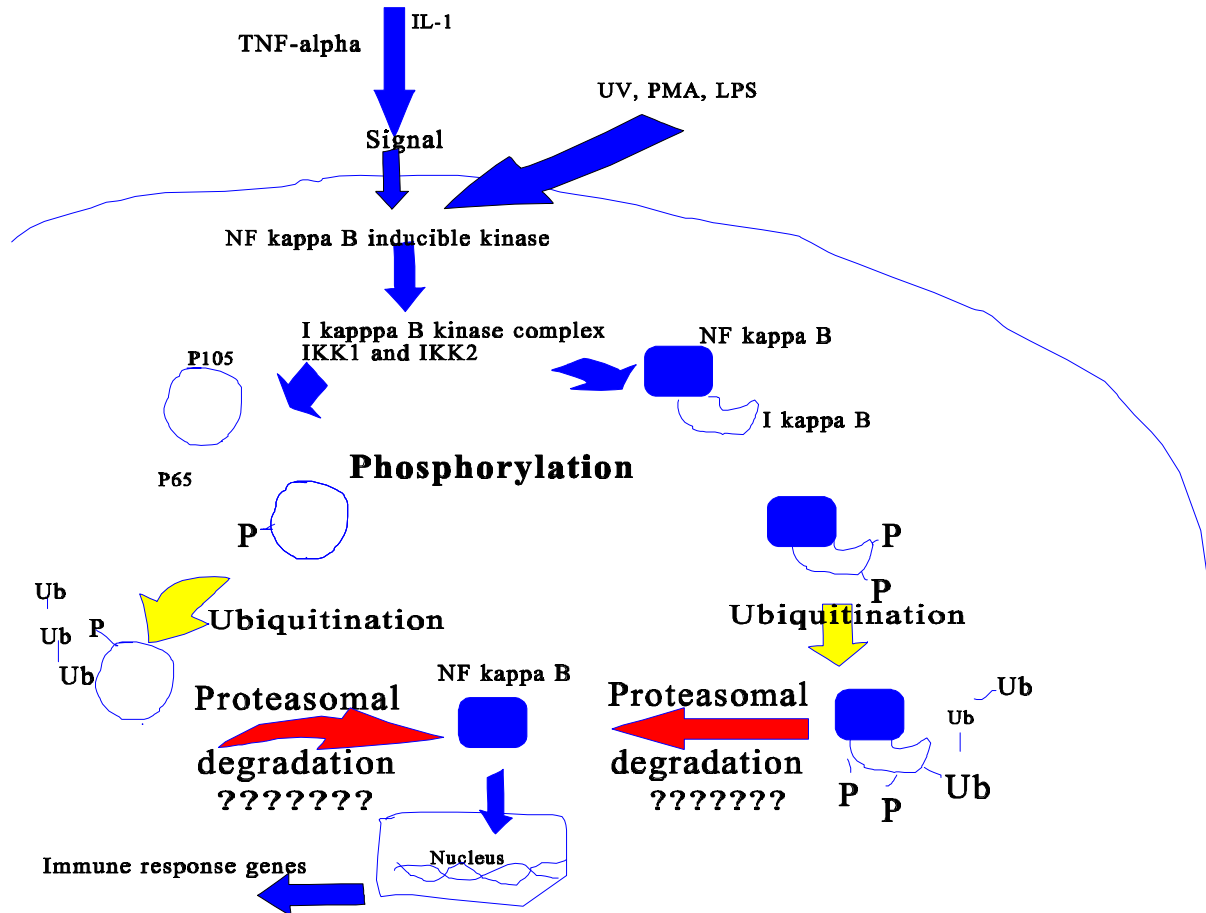
Figure.1. Following treatment with a variety of activators upstream signal/s activate the NF kappa B inducible kinase (NIK) which in turn phosphorylates at least IKK1 and perhaps IKK2 (I kappa B kinase-alpha and I kappa B kinase-beta) in the I kappa B-kinase complex. Other signals such as PMA, UV or LPS etc., may directly activate the kinase complex or may first activate NIK. This kinase complex may also phosphorylate the p105/p65 complex, as well. IKK1/IKK2 then phosphorylates the NF kappa B-I kappa B-alpha complex on I kappa B-alpha series 32/36, which is followed by ubiquitination, proteasomal degradation and the nuclear translocation of NF kappa B. In the nucleus, NF kappa B induces the transcription of several immune response genes. ???? indicates possible defect in the proteasomal degradation pathway that likely results in the lowered nuclear translocation of NF kappa B in activated T cells from the elderly.

translationally modified in response to activating signals which ultimately led to its degradation (116,132). This induced degradation was usually rapid and in some cell types completed by 10 minutes. *In vivo* analysis of NF kappa B activation has shown that one of the first modifications of I kappa B-alpha is that of phosphorylation (128,131). Most NF kappa B activating stimuli result in hyperphosphorylation (86,87,143,144), which occurs on serine 32 and serine 36 of the I kappa B-alpha molecule (145,146). Phosphorylation of these residues are critical since mutations of these sites block phosphorylation by various activators and prevent degradation of I kappa B-alpha which in turn inhibits nuclear NF kappa B induction (145,146). Other agents such as the chymotrypsin inhibitor tosylphenylchloromethylketone (TPCK) which prevent the phosphorylation of I kappa B-alpha also prevent degradation and nuclear translocation of NF kappa B (130,147).

Phosphorylation of I kappa B-alpha is required for an additional modification of I kappa B-alpha, i.e. ubiquitination. The addition of ubiquitin, a 7kd protein occurs at lysine-21 and lysine-22 in the amino terminal region of the I kappa B-alpha molecule (148,149). Mutations in I kappa B-alpha that block phosphorylation have been shown to block ubiquitination in vitro (150). Mutations of lysine 21 and 22 block ubiquitination and degradation of I kappa B-alpha but not phosphorylation, demonstrating that phosphorylation precedes ubiquitination and ubiquitination of lysines are required for degradation of I kappa B-alpha (148,149). The addition of ubiquitin is proposed to target I kappa B-alpha for degradation by the 26S proteasome. It has been demonstrated that covalent attachment of polyubiquitin chains to I kappa B-alpha is

Table 1. Observed immune defects following knock out of Rel subunits and I kappa B-alpha genes in mice.

GENE KNOCK OUT	OBSERVED DEFECTS (IMMUNOLOGICAL)
c-Rel	Proliferative defects in T and B cells, 2-3 fold lower IL-3, IL-15, GM-CSF, TNF and IFN, T cell dependent antibody affected...lower antigen specific IgG1[164]
RelB	No Thymic dendritic cells, Poor stimulators in MLR, Poor Cell mediated Immune response as seen in DTH [166,167].
RelA	Embyronic lethality due to apoptosis in the liver [169].
I kappa B-alpha	Normal development, but die in about 7 days due to wide spread dermatitis [126].
P50/P105	Cannot clear pathogenic organisms, T cell function and immunity impaired, B cell response impaired [168].

Numbers in parenthesis refer to the citation.

required for proteasome mediated degradation of the protein following cellular activation (150-152). Thus, the signal dependent phosphorylation and ubiquitination of I kappa B-alpha targets the cytoplasmic inhibitor to the ubiquitin-proteasome pathway. Degradation is mediated by the 26S proteasome complex which is a multicatalytic protease that degrades multi-ubiquitinated proteins in an ATP-dependent manner. The 26S proteasome complex is involved in the turnover of short-lived and abnormal proteins and is also involved in processing of peptides for antigen presentation by Class I MHC (153). Proteasome activity has been demonstrated to be essential for the degradation of I-kappa B-alpha as pretreatment of cells with peptide aldehyde inhibitors of the proteasome such as N-acetyl leucinal-leucinal-norleucinal (LLnL) or lactacystin block I kappa B-alpha degradation and NF kappa B induction. Furthermore, in the presence of proteasomal inhibitors the phosphorylated form of I kappa B-alpha accumulates (143,144,154).

Although many kinases have been reported to phosphorylate I kappa B-alpha in vitro and cause activation of NF kappa B, a kinase that specifically phosphorylates I kappa B-alpha on ser 32 and 36 has remained elusive, until now (89). Maniatis et. al., demonstrated a kinase complex that specifically phosphorylates I kappa B-alpha on ser 32 and ser 36 (155). In vitro reconstitution experiments demonstrated that the activity of this kinase required both ubiquitin and ubiquitin conjugating enzymes suggesting that ubiquitination of the I kappa B -kinase is required for its activation. Recent studies by three groups have identified I kappa-B kinase, which comprises of a previously identified serine/threonine kinase known as CHUK (conserved helix-loop-helix kinase) and a second kinase closely related to CHUK. These kinases have now been named as IKK1 and IKK2. These two kinases appear to occur in a high molecular mass complex (700kDa) (156-158). It appears that IKK1 and 2 act in concert. Activity of IKK1 is inducible, and is autophosphorylated. Several studies are now underway to determine the kinetics of activation by these two kinases.

4.2. Autoregulation of NF kappa B and I kappa B

Studies demonstrating that the degradation of I kappa B-alpha correlated with nuclear appearance of NF kappa B also demonstrated rapid induction of I kappa B-alpha mRNA within 20 minutes of stimulation and restoration of I kappa B-alpha levels within an hour (87,127,128). Transfection of cells with transactivating NF kappa B subunits resulted in the production of high levels of I kappa B-alpha mRNA, indicating a regulatory pathway (127,128). Analysis of the I kappa B-alpha promoter has shown that it contains multiple NF kappa B binding sites and that these sites are functional in the upregulation of gene expression in response to inducers that activate NF kappa B (158,159). Thus, transactivating NF kappa B dimers can induce their own inhibitor, I kappa B-alpha, which then binds to cytoplasmic dimers to restore the inhibited state and reestablish cytoplasmic pools of NF kappa B/I kappa B complexes. The I kappa B-beta gene, however, is not upregulated by NF kappa B (125,130). The accumulation of newly synthesized I kappa B-alpha can also repress NF kappa B activity by entering the nucleus to inhibit previously activated NF kappa B once the stimulating agent is removed (129). This model is supported by the observation that I kappa B-alpha deficient cells exhibit high nuclear levels of NF kappa B for long times following induction with TNF-alpha (130). This built in feed back inhibition may assure a transient response once the initiating event fades preventing dysregulation of genes whose functions may be harmful if expression goes unchecked (87).

NF kappa B dimers are regulated not only at the level of cytoplasmic retention but also at the level of synthesis. The genes encoding p105, p100, and c-Rel all contain kappa B binding sites in their promoter regions and stimulation of cells leads to increased synthesis of these proteins. Synthesis of p65, however, the most potent activator of transcription, is not upregulated by NF kappa B dimers (87,159,160).

4.3. Role of NF kappa B in immune response

NF kappa B is well recognized for its critical role in regulating immune response genes. Extensive research has established a clear role for NF kappa B in the inducible regulation of a wide variety of genes involved in immune function and inflammatory responses including GM-CSF, IL-6, IL-8, IL-2, IL-2R-alpha, IFN-beta, cellular adhesion molecules such as VCAM-1, IFN-beta, and Class I MHC (86-88). In terms of T cell function NF kappa B plays a vital role in the regulation of both IL-2 and IL-2R-alpha genes. Mutations in the NF kappa B binding site in an IL-2 promoter inhibits promoter activation of the transcription unit (161). Furthermore, TNF-alpha mediated IL-2R-alpha expression following T cell activation is highly dependent upon NF kappa -B (162,163).

Gene knockout of Rel subunit and I kappa B-alpha genes in mice has been accomplished and has confirmed the important role of these proteins in immune function. There appears to be no redundancy within the Rel family proteins as data from knockout mice show that loss of a particular Rel protein cannot be compensated by another Rel protein (164-167). Table 1 summarizes the effect of subunit specific gene knockout on immune function.

Absence of the c-rel gene has a large impact on immune function. Both mature B and T cells exhibit proliferative defects in response to various activating agents. The defect in T cell proliferation correlated with a lack of IL-2 production as IL-2 levels in c-rel deficient T cells were 50 fold lower than that observed in wild type T cells. Production of other cytokines in response to stimulation was also affected as evidenced by 2-3 fold lower amounts of IL-3, IL-5, GM-CSF, TNF-alpha, IFN-gamma when compared to wild type T cells. Additionally, c-Rel deficient mice showed impaired T cell dependent humoral responses when antigenically challenged. Antigen-specific IgG1 levels were decreased by 50-100 fold (164,165). RelB knockout mice develop normally but also show defects in immune function. Loss of the RelB gene results in an absence of thymic dendritic cells indicating that RelB plays an important role in the development of dendritic cells. Furthermore, antigen presenting cells from the spleen of RelB -/- mice showed extremely poor stimulating capacity in mixed lymphocyte cultures providing evidence that RelB is important for antigen presenting cell function. Cell mediated immunity was also impaired in these mice as evidenced by poor delayed type hypersensitivity responses (166,167).

Immune response defects are also observed in mice lacking the p50/p105 subunit. These mice cannot effectively clear the pathogens Listeria or Streptococcus. B cells from these mice are defective in their ability to proliferate and produce antibody in response to Lipopolysaccharide (LPS), while T cells proliferate poorly in response to TCR and CD28 stimulation. In addition, IgE levels were decreased by 40 fold suggesting that p50 plays an important role in heavy-chain class switching (168). Unlike c-Rel and RelB knockout mice, RelA knockout mice exhibit embryonic lethality due to widespread apoptosis within the liver, suggesting the importance of p65 in liver development. Embryonic fibroblasts from RelA deficient mice fail to induce mRNA for I kappa B-alpha and GM-CSF in response to TNF-alpha suggesting an essential role for RelA in the induction of these genes (169).

I kappa B-alpha knockout mice develop normally but exhibit widespread dermatitis and die about 7 days after birth. In splenocytes and thymocytes of these mice, NF kappa B was found to be constitutively activated. Treatment of I kappa B-alpha -/- embryonic fibroblasts resulted in a prolonged and sustained NF kappa B DNA binding activity in the nucleus, indicating the importance of the I kappa B-alpha isoform and not other I kappa B isoforms in the termination of an NF kappa B response (126).

5.0. NF KAPPA B AND IMMUNE SENESCENCE

Several studies have demonstrated transcription factor NF kappa B to be central in T cell activation through the regulation of IL-2 and IL-2R-alpha genes (161-163,170) both of which significantly decline with advancing age. Given its important role, any alteration in the induction and regulation of NF kappa B is likely to affect T cell function. Results from our laboratory demonstrate that NF kappa B induction is significantly decreased in activated T cells from the elderly regardless of the activator or signaling pathway used, indicating this to be an age-related phenomenon rather than an activation dependent event (76). Consistent with these results, Whisler *et al*. (78), have also shown NF kappa B to be decreased in T cells from three out of five elderly donors in response to PMA and ionomycin, anti-CD3, either alone or in combination with PMA or PHA. This is however, in contrast to those reported by Albright *et al*. (171) which demonstrate no age-related decrease in the induction of NF kappa B in T cells from aged mice following activation with anti-CD3. The decrease in nuclear induction of NF kappa B observed during aging is consistent with the observation of lowered IL-2R-alpha detected following activation in T cells from the elderly (20,24,61). As NF kappa B is a critical regulator of IL-2R-alpha expression, a decrease in the induction of NF kappa B is likely to negatively impact on IL-2R-alpha expression following activation.

Studies on other transcription factors provide evidence that physiological aging and in vitro cellular aging does in fact down regulate some transcription factors. Senescent fibroblasts, which have been studied as a cellular model of aging, exhibit decreases in the levels of transcription factors such as AP-1 and E2F, but not NF kappa B (79,172,173). A defect in the induction of heat shock transcription factor (HSF) was reported following heat shock treatment of hepatocytes from aging rats and this was postulated to contribute to the age-related decrease in the expression of heat shock protein 70 (HSP70) (174,175). In addition, studies on other transcription factors in immune cells have demonstrated decreased NFAT in activated T cells from both aging rats and humans (176,78) and the induction of AP-1 has been demonstrated to be defective following activation of T lymphocytes from both mice and humans (77,171). Thus, dysregulation of transcription factors appears to accompany aging in several cell types and may contribute to the age-related changes in cellular function.

Aging in mice and humans has been shown to be accompanied by a shift in the ratio of memory (CD45RO⁺) T cells to naive (CD45RA⁺) T cells, and this shift has often been postulated to underlie age-related immune hyporesponsiveness. Experiments designed to test the inducibility of NF kappa B in memory and naive T cell subsets, produced results which demonstrated that regardless of donor age, memory T cells were not altered in their responsiveness to TNF when compared to similarly treated naive T cells and that an underlying defect in the induction of NF kappa B existed in both memory and naive T cells from elderly donors (177). This suggests that the observed age-related decrease in the levels of NF kappa B cannot be attributed solely to the skewing of the T cell population towards the memory phenotype in the elderly. It was previously believed that activation induced conversion of T cells from the CD45RA⁺ to the CD45RO⁺ phenotype was unidirectional and irreversible thus defining CD45RA⁺ as naive and CD45RO⁺ as memory T cells. Recent studies, however,

have brought into question the validity of using CD45RO and CD45RA as markers to define naive and memory T cells. Using a polyclonal human T cell line, Rothstein et al. (55) showed that although initially down regulated after stimulation, CD45RA was reexpressed when cells returned to a resting state. Bell and Sparshott (56) transferred CD45R$^+$ (naive) or R-(memory) allotype marked T cells into athymic nude rats and demonstrated that both subsets could generate cells of the opposite phenotype suggesting that the CD45 markers can interconvert. It has therefore been suggested that the expression of CD45RO or CD45RA may actually reflect the activation state of the cell or may confer functionally distinct properties on T cells rather than being indicative of antigenic exposure (127). The conflict surrounding the identification of memory and naive T cells makes it difficult to assess their role in aging. Until another stable surface marker is identified for the isolation of naive and memory subsets, in humans, we may have to contend with identifying them as CD45RO$^+$ and CD45RA$^+$ subsets.

Different activators utilizing different signaling pathways resulted in lowered NF kappa B induction in the elderly which suggested a defect in a common mediator and/or event regulating the induction of NF kappa B. Experiments examining the constitutive levels of NF kappa B present in the cytoplasm of resting T cells from young and elderly donors demonstrated that differential levels of precursor NF kappa B available for induction could not account for the lowered amount of NF kappa B induced following T cell activation (76), similar to the results reported by Richardson et al. (175), who demonstrated that alterations in the constitutive levels of HSF was not responsible for age-related decline in the induction of HSF. Although mediating different functions, both HSF and NF kappa B require post translational events for their activation. This suggests that aging may affect post translational processes required for activation of transcription factors rather than affecting constitutive levels.

Failure to detect any accumulation of NF kappa B binding activity in cytosols of activated T cells from the elderly suggested that modifications of NF kappa B necessary for nuclear translocation or processes involved in the nuclear translocation of NF kappa B per se remained intact with advancing age. Employing antibody supershift assays we demonstrated that p50 and p65 subunits were the primary components of the inducible NF kappa B heterodimer in both anti-CD3 and TNF-alpha activated T cells from both young and elderly donors (76). This result is consistent with several studies that identify p50 and p65 as being the primary inducible active NF kappa B dimer involved in the rapid and early activation response not only in T cells but also in many other cell types (86-89, 115). Although Pimentel-Muinos et al. have shown c-rel to be a component of induced NF kappa B in human T cells (115), c-Rel was not detected in our system. Pimental-Muinos et al. demonstrated c-rel to be a component of long term (7-40 hours) activated NF kappa B rather than being a part of the rapid and transiently induced NF kappa B response. In our studies, T cells were treated with activators for short periods of time (less than 4 hours) measuring the early and rapid phase of NF kappa B induction, rather than the sustained long term phase. From the studies of Pimental-Muinos et al it can be predicted that c-rel may be induced at later time points.

From our observation of lowered activation-induced degradation of I kappa B-alpha it became evident that defects in I kappa B-alpha degradation were central to the decreased induction of NF kappa B, and thus immune decline observed in the elderly (194). This result is consistent with reports of lowered I kappa B-alpha degradation in situations that induce lowered levels of NF-kappa B such as those observed following treatment with the anti inflammatory agents, salicylate or tepoxalin (178-181).

Further dissection of the underlying mechanisms for this lowered degradation of I kappa B-alpha led us to demonstrate a significant decline in proteasomal activity, specifically the chymotryptic activity (194). Alterations in proteasomal degradation have been demonstrated in other cell systems during aging (182,183). Ubiquitinated proteins, which are degraded by the proteasome, accumulate in the brains of aging mice and in neural tissues of patients with age-associated neurodegenerative diseases (183). As chymotrypsin-like activity is the most sensitive proteolytic activity in the proteasome, it is not surprising that it is this activity that appears to be most affected with advancing age (184). Although, alteration in proteasomal activity may not be an universal occurrence, this pathway may decline in certain tissues under particular conditions (reviewed in 195).

Information on the regulation of proteasomal activity in human T cells is limited, however, in other cell types and species enzymatic activity of the proteasome appears to be affected by several regulatory elements that either enhance or inhibit it. Rechsteiner et. al. (185) have demonstrated that a protein complex called the 11S regulator (REG) associates with the proteasome and can greatly enhance peptide cleavage activity. Furthermore, calcium appears to bind to the 11S regulator to inhibit its stimulatory activity. There are also several ATPases which have also been shown to associate with the proteasome and are required for proteasomal activity (153). Interferon-gamma treatment induces two subunits of the proteasome, LMP2 and LMP7, which modify the peptidase activities of the proteasome for enhanced proteolysis of peptides for antigen presentation by Class I MHC (186-188). Thus it is possible that the age-related decrease in proteasome activity may be due to alterations in any or all of these types of regulatory elements or events.

6. CONCLUSIONS AND PERSPECTIVES

Altered 26S proteasomal activity in T lymphocytes appears to contribute to age-related T cell dysfunction by inhibiting the induction of NF kappa B. This occurs via a mechanism in which decreased activity of the proteasome impairs the degradation of I kappa B-alpha which in turn results in lowered NF kappa B induction. Thus, altered activity of the proteasome correlates with altered NF kappa B induction. These results would predict that the degradation of other cellular proteins dependent on the chymotryptic activity of the proteasome would also be impaired during aging, including the precursors of NF kappa B, p105 and p100. Proteins such as the cell cycle inhibitor p27 kip and other cell cycle regulators such as cyclins A and B whose proteasome mediated degradation is important for cell cycle progression would be

affected during aging (189,190). Thus, decreased proteasomal activity may explain the age-related decline in T cell proliferation not only through the decreased induction of NF kappa B but also via impaired degradation of cell cycle regulators.

Decreased proteasomal activity in T cells from elderly individuals is a novel finding and has far reaching implications as the proteasome has now been recognized to play important roles in many cellular processes including antigen presentation, cell cycle regulation, and apoptosis (153,190-192). Therefore, decreased activity of the proteasome in the elderly may not only mediate lowered NF kappa B induction but dysregulation of other processes important in cellular function. Altered proteasome activity may affect the processing of self or viral peptides for presentation by MHC I thereby affecting immune responses. Whether, the decline in proteasomal activity is exclusive to any given subset of T cells or other cells of the immune system remains to be investigated. Earlier studies using *in vitro* cellular senescence models have demonstrated no age associated change in the 20S proteasome activity. Whether the decrease noted in T cells is exclusive to the immune system, needs further investigation.

Recently, proteasomal activity has been shown to be involved in apoptosis (191,192). An alteration therefore in the activity of the proteasome may interfere with the apoptosis of T cells during immune responses thus affecting homeostatic regulation of an ongoing immune response. The decrease in proteasomal activity with age in the T cell compartment needs rigorous future experimentation. As the proteasome plays a central role in the processes of the T cell, a defect in the proteasomal activity is likely to impact on several facets of cellular function ranging from simple protein turnover to complex events of mitosis. The accumulation of aberrant proteins and multi-ubiquitinated proteins during aging may be hypothesized to be due to a central defect in the proteasomal degradation.

7. ACKNOWLEDGMENT

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