Transgenic Mouse Models for Immunosenescence

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Abstract: Ageing-dependent dysfunctions develop for each of various cell types in living organisms. Amongst them, immunosenescence, an ageing-dependent deterioration of the immune system, seriously affects the health condition of an aged individual. The central problem in immunosenescence is a decrease in the ability of T-cells to respond to antigens for proliferation and cytokine production, accompanied by accumulation of T-cells with a memory cell phenotype ($CD44^{high}CD45RB^{low}$) replacing those with a naïve cell phenotype ($CD44^{low}CD45RB^{high}$). Recently, a transgenic mouse model and a genetically modified mouse model have been reported to display promoted immunosenescence. One is a mouse line transgenic to human CD2 promoter/enhancer-guided rabbit protein kinase C(PKC)α, and the other is a mouse line in which the p53 gene is deficient ($p53^{-/-}$). Both of these mouse lines display accelerated accumulation of memory T-cell replacing naïve T-cells during ageing, accompanying progressively diminishing responsiveness to sheep red blood cell antigens for cytokine production. T-cells activate PKCα when they receive either an antigenic or stress stimulus. Repetitions of antigenic and stress stimuli that recurrently activate PKCα are probably mimicked by continuously elevated PKCα activity in the PKCα transgenic mice. Activated PKC-α probably counteracts the apoptosis-inducing signal, which prevents activation-induced cell death of T-cells and causes accumulation of memory T-cells as the descendants of activated T-cells that have survived. On the other hand, p53 is known to mediate the signaling for apoptosis induction that follows DNA damage due to oxidative stress. The apoptotic signal pathway fails to work well in $p53^{-/-}$ mice. During ageing of these mice, T-cells must encounter a number of antigenic and stress stimuli for activation, and activation of T-cells that is not followed by cell-death causes accumulation of memory T-cells. Results of experiments using the two transgenic mouse models for immunosenescence introduced here support the view that immunosenescence develops by chronic exposure to antigenic and stress stimuli, which is promoted by a defect in the mechanism for efficient elimination of activated T-cells.

INTRODUCTION

Ageing-dependent dysfunctions develop for each of various cell types in living organisms. The cells in the immune system dynamically use genes in the cells, which are frequently replicated and transcribed in response to antigenic and stress stimuli, during the whole life of an individual. The dynamics of these cells influences the fate of other types of cells with which the former cells interact. For this reason, immunosenescence, an ageing-dependent deterioration of the immune system seriously affects the health condition of an aged individual.

Immunoscenescence develops at the levels of humoral immunity, cellular immunity and innate immunity, the former two of which are primarily caused by changes in the T-cell compartment [1-4]. According to Pawelec *et al.*[5], the factors contributing to T-cell immunosenescence may include 1) stem cell defects, 2) thymus involution,3) defects in antigen-presenting cells, 4) ageing of resting immune cells, e) disrupted activation pathways in immune cells, and 5) replicative senescence of clonally expanding cells. However, we do not know yet which is the leading mechanism for T- cell immunosenescence. Results of recent experiments have suggested that the central problem in immunosenescence is a decrease in the ability of T-cells to respond to antigens for proliferation and cytokine production, accompanied by accumulation of T-cells with a memory cell phenotype ($CD44^{high}CD45RB^{low}$) replacing those with a naive cell phenotype ($CD44^{low}CD45RB^{high}$) [4,6,7]. Naive T-cells, but not memory T-cells, are able to respond to fresh antigens that the cells encounter for the first time. Reduction of the number of naïve T-cells thereby cause immunodeficiency. If such a change in the proportion of T-cell subpopulations is crucial for immunosenescence, identification of the causal agents for this change is important for fully understanding the mechanisms of immunosenescence.

Recently, we have reported a transgenic mouse model and a genetically modified mouse model in which age-dependent changes in proportions of T-cell subpopulations are apparently promoted. One is a mouse line that is transgenic for human CD2 promoter/enhancer-guided rabbit protein kinase C(PKC)α [8], and the other is a mouse line in which the p53 gene is deficient ($p53^{-/-}$) [9]. Both of these mouse lines display accelerated accumulation of memory phenotype $CD4^{+}$ T-cells replacing naïve T-cells bearing a $CD44^{low}CD45RB^{high}$ phenotype during ageing, accompany-

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ing progressively diminishing responsiveness to CD3-specific (αCD3) mAb for DNA synthesis and to sheep red blood cell antigen for antibody and cytokine production. T-cells are known activate PKCα when they receive either an antigenic or stress stimulus. The PKCα that is recurrently activated by repeated antigenic, and stress stimuli is probably mimicked by constitutively activated PKCα in PKCα transgenic mice.

Activated PKCα possibly counteracts the apoptosis-inducing signal, which prevents activation-induced T-cell death and causes accumulation of memory T-cells as descendants of activated T-cells [8].

On the other hand, p53 is known to mediate the signaling for DNA repair, cell cycle arrest and programmed cell death (apoptosis), phenomena that follow DNA damage due to various types of oxidative stress [10]. The p53-mediated apoptotic signal pathway thus fails to work in $p53^{-/-}$ mice. During ageing of p53-/- mice, T-cells encounter a number of antigenic and stress stimuli for activation, and activated T-cells that fail to receive subsequent cell-death signals become memory T-cells.

Here, we briefly review the results obtained using these two genetically modified mouse models of accelerated immunosenescence and, based on the results obtained in these models, we discuss our view that immunosenescence develops by chronic exposure to antigenic and stress stimuli, which is promoted by an imbalance in signaling for cell survival or cell death of activated T-cells.

LESSONS FROM PROTEIN KINASE Cα-TRANSGENIC MICE

T-cells carry three categories of protein kinase C (PKC). i.e., classical (α, β1,β2, γ), novel (δ, ε, η, θ, μ) and atypical (z, i, l) PKCs [11, 12]. Originally for identifying the role of PKCα in activation of T-cells to proliferate and to produce cytokines in response to antigens, we have established a transgenic mouse line that expresses an excess amount of PKCα. The established mouse line carries multicopies of rabbit PKCα cDNA under the control of the regulatory element of human CD2 (Fig. **1**), and expresses rabbit PKCα mRNA at a high level in the thymus and at a low level in the spleen. The expression levels of overall and membrane-associated activated PKCα proteins in young (< 3-mo-old) PKCα transgenic mice are, however, only minimally higher than those in non-transgenic control mice. Correspondingly, there is no detectable change in the overall and subpopulation sizes of T lymphocytes in the thymus and spleen of the transgenic mice, and the responses of transgenic thymic T-cells to α CD3 mAb and concanavalin A (Con A) for proliferation and cytokine (IL-2) production *in vitro* are only minimally higher than those of control cells.

Interestingly, however, upon stimulation with α CD3 mAb or phorbol 12-myristate 14-acetate (PMA) *in vitro* for 5 hrs or more, the expression level of PKC-α protein quickly increases in transgenic thymic T lymphocytes, whereas the mRNA level is invariant [14], The transgenic T-cells thus carry rapidly increasing levels of membrane-associated activated PKCα after stimulation (Fig. **2**). These results

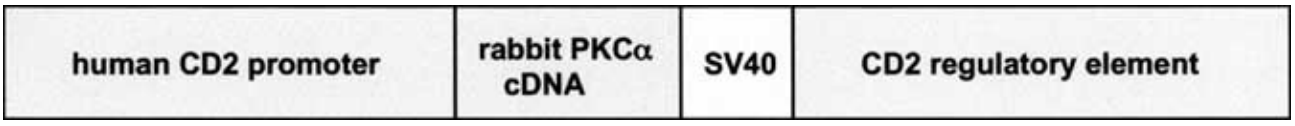

Fig. (1). Construction of the DNA fragment for producing PKCα-transgenic mice.

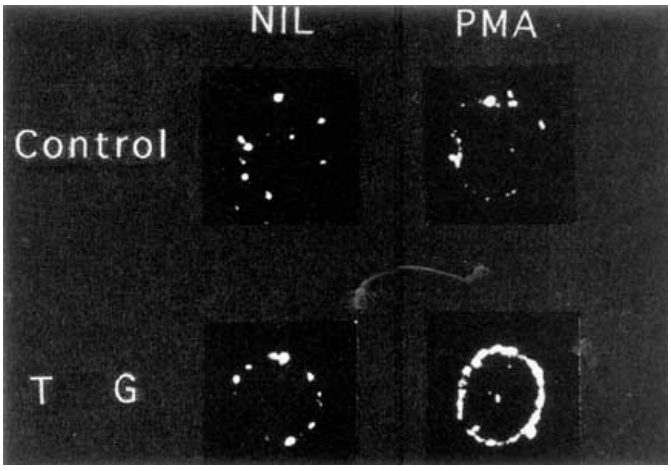

Fig. (2). Demonstration of membrane-translocated PKCα in thymic T-lymphocytes by confocus microscopy. Cells from PKCα -transgenic (TG) and non-transgenic control mice were examined for membrane-translocated PKCα, after incubation in the presence or absence of PMA for 1 h (reproduced from Ref. 14 with permission).

suggest that there is a mechanism to suppress the protein level of the transgenic PKC-α at the posttranscriptional stage, either by suppressing the translation of PKCα mRNA to PKCα protein or by accelerating the degradation of PKCα protein. Whatever the underlying mechanism is, these results suggest that use of modified genes for protein expression is normally regulated and that such regulation may be cancelled by exogenous stimuli to cells.

We have found that normal as well as transgenic PKCα expression levels in mice gradually increase along with ageing from 3 to 16 months old (Fig. **3**). This suggests that the regulatory mechanism for preventing transgenic and normal PKCα genes from overexpression at the protein level

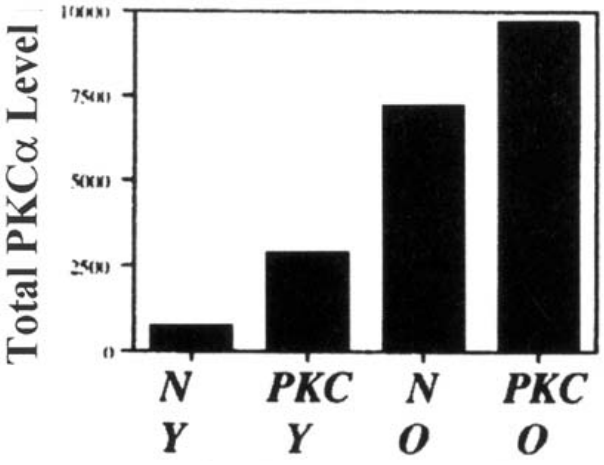

Fig. (3). Promotion of ageing-associated increase in PKCα levels in T-cells of PKCα - transgenic mice. PKC-α levels in T-cells from young (Y: 3-mo-old) and old (O: 16-mo-old) PKCα-transgenic (PKC) and normal control (N) mice were determined by Western blotting (reproduced from Ref. 8 with permission).

is gradually destroyed during ageing. In parallel to the age-dependent change in the PKCα level, there seems a shift of the phenotype of T-cells from naive type to memory type. (Fig. **4**) shows how the rates of memory phenotype ($CD44^{high}CD45RB^{low}$) of CD^{4+} T-cells change in comparison to naïve phenotype ($CD44^{low}CD45RB^{high}$) during ageing in normal control and PKCα transgeinic mice. The rate of memory phenotype T-cells gradually increases along with ageing in non-trangenic control mice [4, 6, 7]. In PKCα transgenic mice, this age-dependent increase in the rate of memory phenotype is accelerated.

In association with the accelerated accumulation of memory phenotype T-cells, the ability of T-cells from older (9-or 16-mos-old) PKCα transgenic mice to respond to αCD3 mAb and PMA for DNA synthesis *in vitro* is greatly reduced compared with that of normal age-matched control mouse T-cells (Fig. **5**). Correspondingly, the level of antibody (plaque-forming cell) response of 12-mos-old PKCα transgenic mice to injection of sheep blood cells is much lower than that of age-matched control non-transgenic mice, and the response of the former mice accompanies abnormally biased cytokine production for increased IFN-γ and IL-12 and diminished IL-10 and IL-4. A biased IFN-γ production by memory T-cells has also been reported elsewhere [15]. These results suggest that an ever-increasing PKCα level along with ageing accelerates the ageing of T-cells.

Antigenic stimulation of young (3-mos-old) normal non-transgenic mice by injection of sheep red blood cells induces a temporal increase in the rate of memory phenotype of $CD4^{+}$ T-cells. Interestingly, the antigenic stimulus-induced increase in memory phenotype T-cells is more striking and more prolonged in PKC-α transgenic mice than in normal control mice. This partially proves that by antigenic stimulus the memory phenotype T-cells are more extensively produced and more slowly eliminated in PKCα transgenic mice than in control mice.

Taken together, the results suggest that immuno-senescence is promoted in PKCα transgenic mice, possibly through an imbalance between survival and death of activated T-cells, which leads to accumulation of memory T-cells replacing naive T-cells and thereby reduces the ability of T-cells to respond to fresh antigenic stimuli. In these transgenic mice, T-cell lymphoma develops with ageing (Yokoyama *et al.*, unpublished observation), although it is not clear whether this is due to the impairment of immune surveillance of neoplastic cells or to the evasion of T-cells from activation-induced death for neoplastic change.

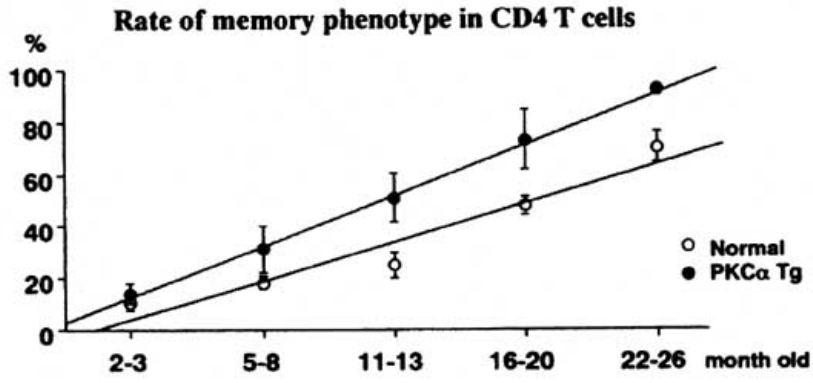

Fig. (4). Promotion of ageing-associated accunmulation of memory phenotype $CD4^{+}$ T-cells in PKCα-transgenic mice (reproduced from Ref. 8 with permission).

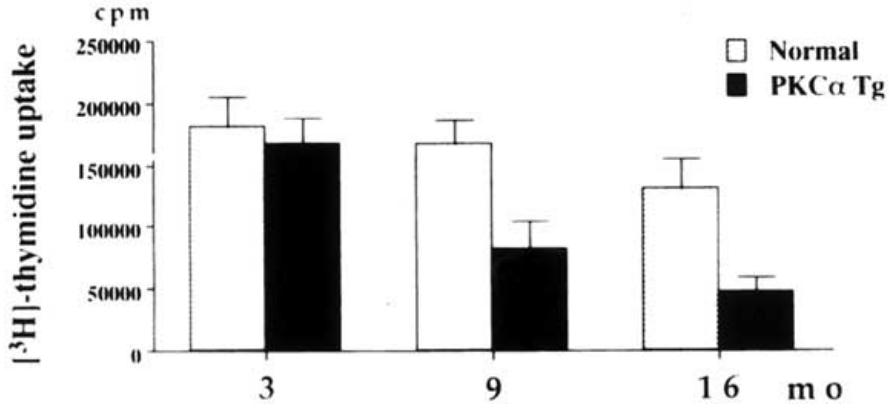

Fig. (5). Promotion of ageing-associated T-cells dysfunction in PKCα -transgenic mice. The levels of peak growth response of T-cells from normal and PKCα -transgenic mice of indicated ages, attained on day 2 after stimulation with α CD3 mAb and PMA *in vitro*, are shown (reproduced from Ref. 8 with permission).

LESSONS FROM P53-DEFICICIENT MICE

p53 is a key molecule mediating signal transduction triggered by DNA-damaging stress stimuli, such as ultraviolet irradiation, X-ray and anti-cancer drugs, which works to repair damaged DNA or causes apoptotic cell death [10]. In a number of tumors, p53 is mutated and inactive, allowing the cells to escape from death for neoplastic growth. Correspondingly, lymphomas frequently develop in mice in which p53 is deficient ($p53^{-/-}$) [9, 16]. Ohkusu *et al.* have recently analyzed phenotypic changes in CD4+ T-cells along with ageing in $p53_{-/-}$mice before lymphoma development. In these $p52^{-/-}$ mice, the progress of phenotypic change in CD4+ T-cells from naive ($CD44^{low}$ $CD45RB^{high}$) to memory ($CD44^{high}CD45RB^{low}$) along with ageing is promoted (Fig. **6**). Young $p53^{-/-}$ mice show augmented responses to injection of sheep red blood cells for Th2-dominant cytokine production, inducing high levels of IL-4 and IL-10 and low levels of IFN-γ and IL-12 production. In contrast, T-cells from aged $p53^{-/-}$ animals show diminished responses to αCD3 mAb stimulation *in vitro* for DNA synthesis. It is speculated that a defect in p53-mediated signal delivery for cell death causes an accumulation of activated or memory T-cells replacing naive T-cells [17].

ANTIGENIC AND STRESS STIMULI INDUCING BOTH T-CELL ACTIVATION AND T-CELL DEATH

It is likely that organisms repeatedly receive antigenic stimuli throughout life from the environment through infection of microorganisms or uptake of allergens, such as foods, drugs and pollens. The general signal transduction cascade has been frequently described in recent review articles in the field of immunology [18-20] and is outlined in the following. The antigenic stimuli trigger the T-cell

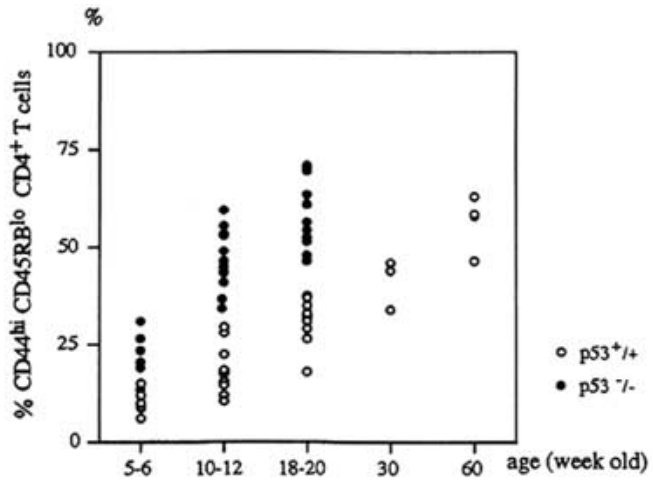

Fig. (6). Acceleration of the accumlation of memory phenotype $CD4^+$ T-cells in young $p53^{-/-}$ mice (reproduced from Ref. 9 with permission).

receptor (TCR)-mediated signal transduction cascade for T-cell activation. Activated CD4+ T-cells come to express cell-death receptors, such as Fas, and are subjected to attack by cytotoxic T-cells (CTL) that express FasL. The death or survival of T-cells is decided by mutually counteracting signals triggered through TCR and co-stimulatory molecules.

Ligand (antigen plus MHC on antigen-presenting cells)-mediated crosslinkage of TCR triggers the signal transduction cascade, first causing activation of Src family tyrosine kinases (Lck/Fyn) and then activation of Syk family tyrosine kinases (ZAP70). This induces phosphorylation of adaptor proteins, such as LAT, in membrane microdomains termed rafts [21], which bind several signal transducing elements to be phosphorylated and activated by ZAP70. These signal-transducing molecules include PLC-γ, which cleaves PIP2 to release DG and IP3/IP4. DG activates classical PKC, including PKC-α in the presence of Ca^{2+}, whose intracellular level is increased by IP3/IP4-mediated recruitment from the endoplasmic reticulum and from outside the cells. The LAT-mediated signal transducing pathway also includes activation of Ras following chain reaction of adaptor proteins Shc, Erb and Sos, which leads to activation of MAK family kinases. MAK family kinases are also activated by activated PKC-α. These signal cascades end in activation of transcription factors, such as AP-1 and NF-AT, which accelerates transcription of cytokine and cytokine receptor genes.

Cell survival/activation-promoting signals are also provided through co-stimulatory molecules, such as CD28, that binds CD80/CD86 on antigen-presenting cells. This signaling cascade includes activation of PI-3 kinase, which activates Akt (protein kinase B) for cell survival. Akt may phosphoryrate Bad to inactivate its cell-death promoting activity and/or may activate NF-κB transduce a cell survival signal. The cell-survival signal is also transduced by a number of cytokines, such as IL-3 again through activation of PI-3 kinase and Akt. TNFR family molecules, such as CD40L, which is activated by CD40 on antigen-presenting cells, also deliver a cell-survival signal to T-cells.

On the other hand, the signals delivered through TCR and co-stimulatory molecules induce the expression of cell-death receptors, such as Fas on T-cells, which are stimulated by FasL or other specific ligands [22-24], This signal first induces activation of caspase 8, which in turn activates caspase 3 for DNA fragmentation and Bid for activation of a mitochondrial cell-death pathway. Cross-linkage of TCR may also trigger a cell-death receptor-independent, mitochondria-dependent pathway for cell death induction, which includes release of cytochrome c and ATP for activation of Apaf-1, caspase 9 and caspase 3 successively. The signal cascade upstream of the mitochondrial event has not yet been clarified, but it is thought to include a redox-linked mechanism potentially initiated by superoxide production following receptor cross-linkage. Superoxide or other reactive oxygen species (ROS) might injure mitochondria, resulting in the release of cytochrome *c* and ATP directly or indirectly through activation of ASK1-JNK cascade [25]. Cell death-inducing signals either through cell-death receptors or mitochondria are subjected to a number of cell- death inhibiting and promoting signals, the balance of which finely decides either survival or death of T-cells dynamically. Some of the activated T-cells that escaped apoptotic cell death become memory cells bearing the phenotype of $CD44^{high}CD45RB^{low}$. PKCα has been suggested to play an important role in activation of T-cells and possibly in blocking Fas-mediated cell-death-inducing signals [8].

T-cells may also receive antigen-nonspecific signals in association with or independently of specific antigenic stimuli that recruit various types of cell surface receptors and their ligands for activation, possibly guided by TCR cross-linkage with antigen [26-29]. Biologically active microbial products, represented by bacterial lipopolysaccharide (LPS), and chemical and physical agents such as ROS that can be physiological metabolites of inflammatory cells and T-cells, oxidative metabolic products of sugar and lipids, drugs, heavy metals from polluted environments, X-ray and UV light might all affect T-cells either in association with or independently of the TCR-mediated signaling. Recent evidence has suggested that these biologically active agents trigger signals for inducing activation or death of T lymphocytes.

We and others have shown that exposure of T-cells to protein cysteine thiol (SH group)-reactive heavy metals, such as Hg, Au and As, causes activation of Src family protein tyrosine kinase Lck and downstream signal molecules for promotion of cell proliferation in association with or bypassing TCR ligand-binding-mediated signal [30,31]. SH group-reactive chemicals cross-link cell surface proteins, including GPI-anchoring Thy-I, on murine T-lymphocytes. This causes extensive clustering of membrane rafts on which GPI-anchoring Thy-I, transmembrane CD4 and intracellular Lck are located together, and this clustering of membrane rafts is immediately followed by activation of Lck. Chemical cell surface receptor cross-linkage and accompanying membrane raft clustering-linked activation of Lck induce activations of MAP family kinases and PLC-γ, which cause intracellular Ca^{2+} level increase and PKCα activation, ultimately leading to promotion of DNA synthesis and cytokine production [32-33]. The chemically induced cross-linkage of cell surface receptors and clustering of rafts also trigger the signal transduction for cell death, which involves raft structure-dependent superoxide production followed by mitochondrial membrane potential decrease and caspase activation possible through the ASK1/JNK activation signaling pathway [31].

Oxidative stress also promotes production of carbonyl compounds from sugars and lipids, such as glyoxal, methylglyoxal, 4-hydroxynonenal and acrolein. These compounds also react with signal transducing cell surface molecules by Schiff-base formation or Michael addition for cell activation or cell death induction in a way similar but not identical to that triggered by SH-group-reactive chemicals [26,34-38].

Another pathway for cell death induction, which is triggered by environmental oxidative agents, such as X-ray, UV light and anti-cancer drugs, starts at damage to DNA. The DNA damage induces activation of p53-medicated signal pathways for repair of damaged DNA or DNA fragmentation through a mitochondria-dependent pathway.

It is therefore likely that, in addition to antigen/ligand-mediated signaling for cell activation or cell death, oxidative stress-linked chemical reactions that structurally modify cell surface receptors and intracellular signal-transducing molecules affect the dynamics of activation/survival or death of T-cells during ageing.

A HYPOTHETICAL VIEW ON THE MECHANISM OF T-CELL IMMUNOSENESCENCE

Based on the results obtained in the two genetically modified animal models for promotion of immunosenescence, we here discuss the possible mechanism of immunosenescence for deteriorating T-cell immunity along with ageing.

In the former animal model, PKC-α is overexpressed at the protein level and activated in a manner of gradual progression due to ageing-dependent loss of regulation of the transgenic gene. This probably mimics the physiological process of ageing in which antigenic and non-antigenic stress stimuli repetitively attack naive T-cells for destroying the regulation of PKCα expression and activation, and thereby provide cell-survival signals that inhibit cell death signals in a population of activated/memory T-cells (Fig. **7A**). In the latter animal model in which p53 is deficient, the normally working mechanism to protect animals from attack by DNA-damaging agents, such as UV, X-ray and anti-cancer drugs, through elimination of damaged cells is missing, and absence of this mechanism may allow fully activated T-cells to survive as memory cells replacing naïve cells (Fig. **7B**).

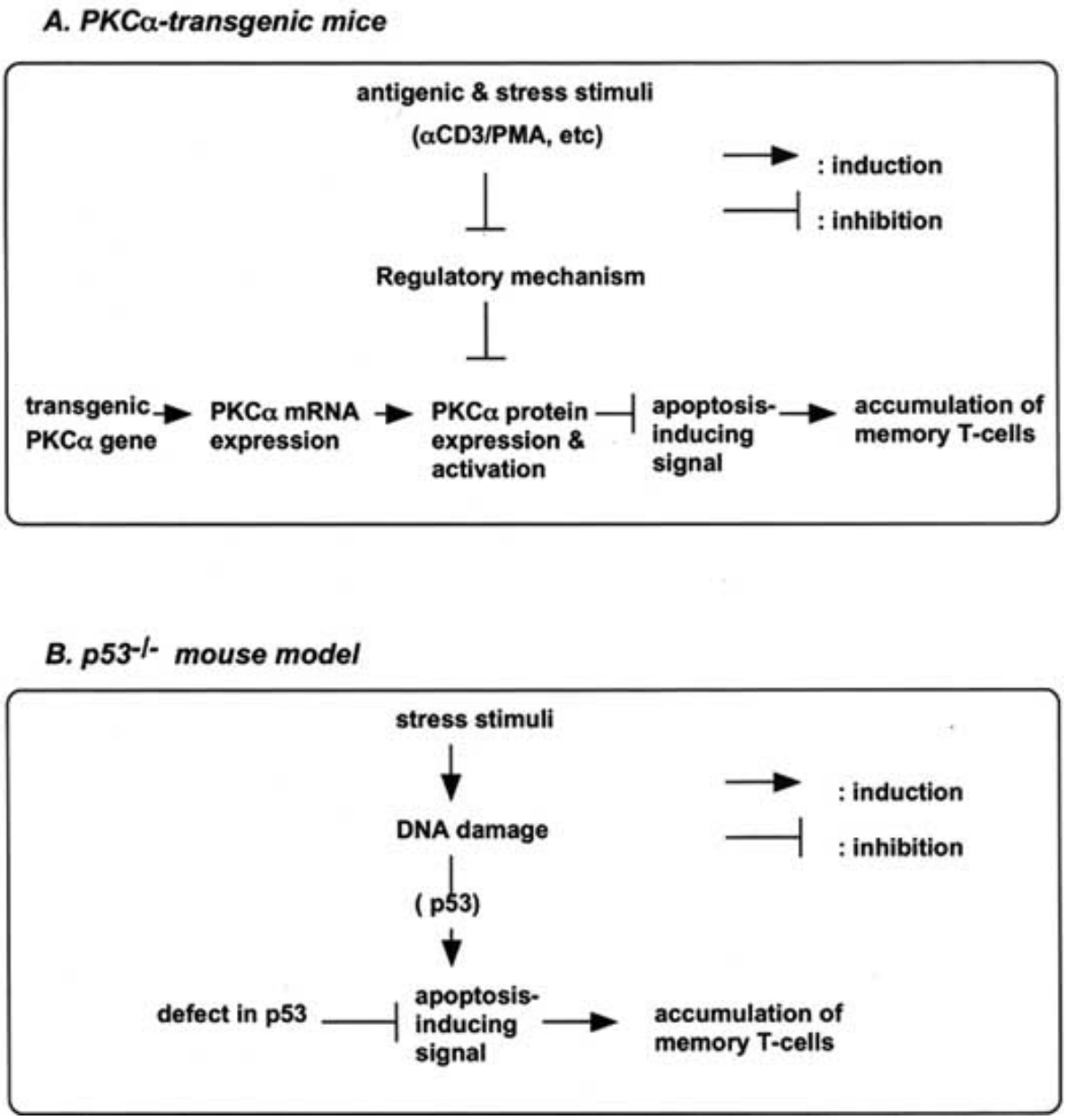

Fig. (7). Suggested mechanisms of accelerated accumulation of memory phenotype T-cells in PKCα –transgenic mice (A) and $p53^{-/-}$ mice (B).

A hypothetical view on the mechanism of T-cell immunosenescence is as follows (Fig. **8**). When T-cells are normally stimulated with antigen presented by antigen-presenting cells for proliferation and for activation to promote the production of cytokines, cytokine receptors and adhesion / co-stimulatory, the activated T-cells also acquire

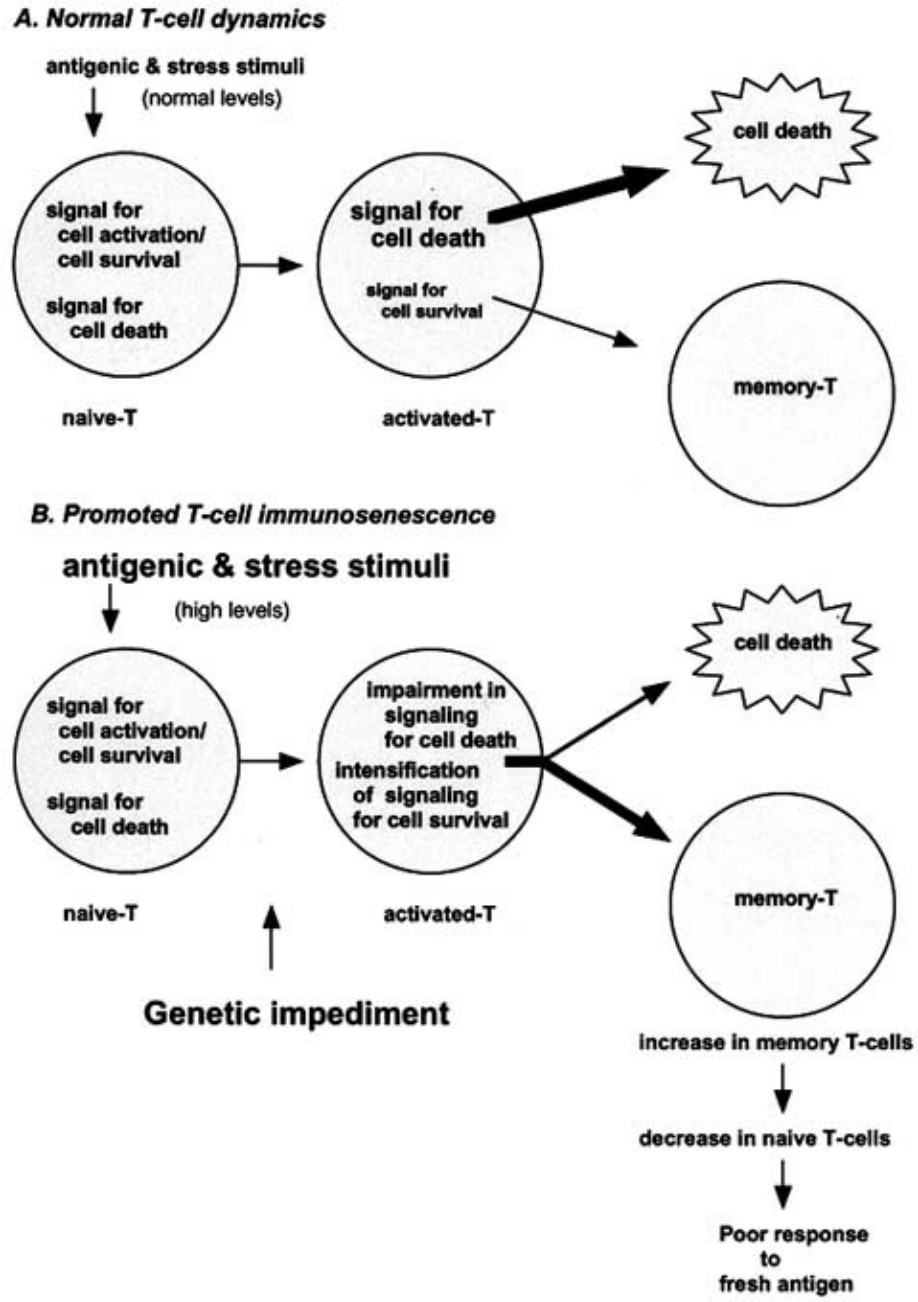

Fig. (8). A hypothetical view of the mechanism of T-cell immunosenescence. As illustrated in A, normal levels of antigenic and stress stimuli provide naïve T-cells with the signal for activation and survival followed by the signal for expression of cell-death receptors. The latter signal leads to death of the majority of activated T-cells, whereas a minority of activated T-cells that continuously receive the signal for cell survival become memory T-cells. As summarized in B, however, high levels of antigenic and stress stimuli, which induce activation of T-cells, may continuously deliver unbalanced signals to once-activated T-cells for intensifying the signal for cell survival and/or for impairing the signal for cell death, leading to accumulation of memory T-cells. Impairment in signaling for cell death and/or intensification of signaling for cell survival on once-activated T-cells may also occur due to some genetic impediments, represented by those in PKCα –transgenic and $p53^{-/-}$ mouse models for immunosenescence. An increase in memory T-cells is accompanied by a decrease in naïve T-cells, possibly through a yet- unknown regulatory mechanism, which ultimately causes poor responsiveness to freshly administered antigenic and stress stimuli.

expression of cell death receptors, such as FAS, which results in activation-induced death of the majority of activated T-cells. A small fraction of activated T-cells, however, survive as memory T-cells. Antigenic and non-antigenic stress stimuli to T-cells also induce cell-survival signals in addition to cell-death signals, and the balance of the two signals determines death or survival of individual activated T-cells. Any one of potentially multiple mechanisms to shift the balance biased for survival of once-activated T-cells may cause accumulation of activated/memory T-cells. Any high levels of antigenic or stress stimuli that break the balance between cell-survival and cell-death signals, which are mimicked by the presence of multi-copies of transgenic PKCα gene in the transgenic mouse model, could lead to accumulation of memory T-cells replacing naïve T-cells. Any mechanisms to impair normal cell death signaling represented by the p53 deficiency in $p53^{-/-}$ mice and functional defects in Fas/Fas-ligand in MRL-lpr/MRL-gld mice [39], seem also to allow activated/memory T-cells accumulation. It is thus speculated that a combination of various mechanisms linked to either cell survival promotion or cell death impairment, which affect T-cells during the normal process of ageing, might be involved in immunosenescence.

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