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Accelerated Development and Aging of the Immune System in p53-Deficient Mice

Kozo Ohkusu-Tsukada,*† Teruyo Tsukada,*‡ and Ken-ichi Isobe2*

Development and aging of the immune system lead to an accumulation of memory T cells over the long term. The predominance of T cells of the memory phenotype in the T cell population induces an age-related decline in protective immune responses. We found that development and aging of the immune system were accelerated in p53-deficient (p53−/−) mice; the accumulation of memory T cells was spontaneously accelerated, and a strong T cell-dependent Ab response and Th2 cytokine expression (IL-4, IL-6, and IL-10) were induced by Ag stimulation in young p53−/− mice in the developmental stage. The high T cell proliferative response in the young mice rapidly progressed to a depressed proliferative response in adult mice. It was suggested that the loss of regulation of the cell cycle, DNA repair, and apoptosis by p53 deficiency potentially leads to immunosenescence with the accumulation of memory T cells. The Journal of Immunology, 1999, 163: 1966–1972.

It is believed that near-senescence fibroblasts exhibit a prolonged proliferative life span and that fully senescent fibroblasts are in the viable G1-arrested state with a limited proliferative lifespan (1). The p53 gene has been shown to be a critical regulator of the cell cycle. Enhanced expression of the p53 protein induces DNA repair, cell cycle arrest (G1 or G2), or programmed cell death (apoptosis) (2). Recently, the expression and transcriptional activity of p53 were reported to be increased in senescent fibroblasts (3, 4), while the induction of p53 expression has been reported to be impaired in activated T cells in certain elderly people (5). Accordingly, T cells derived from elderly humans are considered to be different from senescent fibroblasts cultured in vitro with respect to p53 expression.

It has been reported that the immune responses declines with aging in mammals (6–10). In particular, much of the age-related decline in protective immune responses is thought to be induced by changes in the T cell composition. The accumulation of memory-phenotype (CD44high CD45RBlow) CD4+ T cells in the place of naive-phenotype (CD44low CD45RBhigh) CD4+ T cells (7, 8), while the induction of p53 expression has been linked to the reduced proliferative response (9) and decreased production of IL-2 by T cells (10) with aging. Some cytokines such as IL-4 and IL-10, which are preferentially secreted by the memory T cells, are reported to be produced in greater concentrations by T cells from aged mice (8, 11). Reports concerning other cytokines typically produced by memory T cells, such as IFN-γ, are still inconsistent (12).

In contrast, the incidence of malignant tumors, which are a major cause of death, frequently increases with aging. The decline in the activity of the immune system and the increased risk for almost all forms of tumors with aging suggests that the immune system provides, at least in part, protection against tumors. Although p53-deficient (p53−/−) mice develop different types of tumors, the most frequently observed tumor is malignant lymphoma, which occurs in >60% of these animals. The high incidence of lymphoma was originally ascribed to the genetic background of the mice, which is 75% in C57BL/6 mice and 25% in 129/Sv mice (13). It is known, for example, that the incidence of lymphoma in normal C57BL/6 mice is relatively high, but the average age of onset is about 27 mo (14). Presumably, the increased incidence of lymphoma in p53−/− mice is also potentially related to the age-related changes in the internal milieu of the animal, in addition to its relationship to the genetic background of the mice. In short, the effects of aging are potentially accelerated by p53 deficiency.

For analysis of naive/memory CD4+ T cells, spleen cells suspended in PBS were stained with a mixture of PE-conjugated CD44-specific mAb (PharMingen, San Diego, CA), and Tricolor-conjugated CD4-specific mAb (Caltag, San Francisco, CA). For analysis of T cells/B cells, samples were stained with a mixture of PE-conjugated B220-specific mAb (Caltag) and FITC-conjugated Thy-1.2-specific mAb (Caltag). For analysis of NK cells/NK T cells, samples were stained with a mixture of NK1.1-specific mAb (PharMingen) and FITC-conjugated Thy-1.2-specific mAb (Caltag). Three-color or two-color analysis was conducted by flow cytometry, and the type was confirmed by microscopic examination of Giemsa-stained sections.

Flow cytometry

For analysis of naive/memory CD4+ T cells, spleen cells suspended in PBS were stained with a mixture of FITC-conjugated CD44-specific mAb (Sigma, St. Louis, MO), PE-conjugated CD45RB-specific mAb (PharMingen, San Diego, CA), and Tricolor-conjugated CD4-specific mAb (Caltag, San Francisco, CA). For analysis of T cells/B cells, samples were stained with a mixture of PE-conjugated B220-specific mAb (Caltag) and FITC-conjugated Thy-1.2-specific mAb (Caltag). For analysis of NK cells/NK T cells, samples were stained with a mixture of PE-conjugated NK1.1-specific mAb (PharMingen) and FITC-conjugated Thy-1.2-specific mAb (Caltag). Three-color or two-color analysis was conducted by flow cytometry (FACSCaliber, Nippon Becton Dickinson, Tokyo, Japan).

Materials and Methods

Mice

CBA/N background, p53−/− (15) and p53+/+ male mice were purchased from Oriental Cobo Industrial (Tokyo, Japan). p53−/− mice between 5 and 20 wk old and p53+/− mice between 5 and 60 wk old were used. These animals were bred under pathogen-free conditions, and any mice obviously suffering from lymphoma or tumors were eliminated from the study. Lymphoma in the spleen was recognized by the size vs granularity of the cells, determined by the forward scatter (FS) vs side scatter (SS) field in flow cytometry, and the type was confirmed by microscopic examination of Giemsa-stained sections.

Abbreviations used in this paper: p53−/− mice, p53-deficient mice; p53+/+ mice, wild-type mice; PFC, plaque-forming cells; FS, forward scatter; SS, side scatter.

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Cellular culture

T cell enriched spleen cells, prepared by passing the cells through a nylon wool column, were cultured at a density of $1 \times 10^6$ cells/200 ml in RPMI 1640 medium supplemented with 5% FCS in 96-well microplates. For stimulation, CD3-specific mAb (1:1000, KT3) (Bio Source International-Tago Products, Camarillo, CA) or Con A (5 μg/ml) (Sigma) or PMA (10 nM) (Sigma) was added to the culture. For the proliferation assay, after 48 h incubation at 37°C under 5% CO₂, the cells in each well were labeled with 18.5 kBq of [3 H]thymidine for 4 h and harvested onto filter mats.

RT-PCR

Total cellular RNA from the spleen was extracted by the acid guanidium thiocyanate phenol-chloroform method. RNA was retrotranscribed after annealing with 1 μg/20 μl oligo(dT) primers (Life Technologies, Rockville, MD) in the presence of 100 U of reverse transcriptase (Superscript II RT; Life Technologies), 10 mM DTT (Life Technologies), and 500 nM of each deoxynucleotide, and 200 nM each of the two (forward and reverse) primers. The sequences of the primers for amplification of the cytokine genes are as follows: IFN-γ forward, 5'-TGAACGCTACACACTGCATCTTGG-3'; IFN-γ reverse, 5'-CGACTCCTTTTCCGCTTCCTGAG-3'; IL-2 forward, 5'-AACAGGCACCCACTTCA-3'; IL-2 reverse, 5'-TTGAGATGATGCTTTGACA-3'; IL-4 forward, 5'-TAGTTGTCATCCTGCTCTT-3'; IL-4 reverse, 5'-CTACGAGTAATCCATTTGC-3'; IL-6 forward, 5'-GAACACGATGATCTTTGACA-3'; IL-6 reverse, 5'-CTTAGCCACTCCTTCCTGAC-3'; GATA3 forward, 5'-GAAGGCATCCAGACCCGAAAC-3'; GATA3 reverse, 5'-ACCCATGGCGGTGACCATGC-3'; GAPDH forward, 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3'; GAPDH reverse, 5'-CATGTAGGCCATGAGGTCCACCAC-3'.

Samples were amplified in 32 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 50 s, and extension at 72°C for 80 s). The PCR products were visualized after electrophoresis through 2% agarose gels by staining with ethidum bromide.

Results

The recognition of nonlymphomatous cells in p53⁻⁻ spleens from the FS vs SS field in flow cytometry

Because the G₂-gated lymphomatous cells are larger in size and granularity than the normal G₁-gated normal lymphocytes (Fig. 1967 The Journal of Immunology by guest on June 7, 2022 http://www.jimmunol.org/ Downloaded from
FIGURE 2. Acceleration of the accumulation of (CD44<sup>hi</sup> CD45RB<sup>lo</sup>) memory phenotype CD4<sup>+</sup> T cells in young p53<sup>−/−</sup> mice. Splenic cells from p53<sup>+/+</sup> and p53<sup>−/−</sup> mice of the indicated ages were stained for CD4, CD44, and CD45RB with three colors. The population of CD4<sup>+</sup> T cells was gated for analysis of CD44 and CD45RB expression levels. The representative flow cytometry quadrants with percentages of the individual subpopulations (a) and the percentages of CD44<sup>hi</sup> CD45RB<sup>lo</sup> memory phenotype CD4<sup>+</sup> T cells in individual mice of the indicated ages (b) are shown. The following data indicated as the mean ± SD for the individual age groups are p53<sup>+/+</sup>: 5–6 wk, 10.54 ± 2.55; 10–12 wk, 17.81 ± 5.10; 18–20 wk, 31.50 ± 3.12; 30 wk, 41.88 ± 5.54; 60 wk, 56.40 ± 6.96; p53<sup>−/−</sup>: 5–6 wk, 22.18 ± 6.09; 10–12 wk, 50.97 ± 7.72; 18–20 wk, 58.42 ± 8.98. Adult p53<sup>−/−</sup> mice with lymphoma or malignant tumors at the start of the study were not included in the study. The difference in the values between p53<sup>+/+</sup> and p53<sup>−/−</sup> mice, 10–20 wk old, is statistically significant as determined by the Student’s t test (p < 0.0001).

a, Changes in the population of NK cells and NK T cells in p53<sup>+/+</sup> and p53<sup>−/−</sup> mice on day 0, 5, and 15 after injection of SRBC. Splenic cells from each of the mice stained with specific mAb for NK1.1 and Thy-1.2 were analyzed by flow cytometry.
FIGURE 3. Increase in the percentage of memory phenotype CD4⁺ T cells but not NK T cells in young p53⁻/⁻ mice (5–6 wk) in response to Ag (SRBC) stimulation. a, The population of CD4⁺ T cells as determined by the CD44 and CD45RB expression levels in splenic lymphocytes of p53⁺/+ and p53⁻/⁻ mice was analyzed by flow cytometry in the control mice and the mice administered an injection of SRBC after 4 days. The representative flow cytometry
FIGURE 4. High levels of production of Th2-type cytokines in young p53−/− mice (5–6 wk) in response to Ag (SRBC) stimulation. The expression levels of IFN-γ, IL-4, IL-6, IL-10, GATA-3, and GAPDH mRNA were measured by RT-PCR in the spleens of p53+/+ (lane 1) and p53−/− (lane 2) as control mice, and the spleens of p53+/+ (lane 3) and p53−/− (lane 4) mice administered an injection of SRBC after 4 days. A representative of three experiments is shown.

FIGURE 5. Depressed proliferative response of T cells following TCR stimulation in adult p53−/− mice. The peak response achieved 2 days (48 h) after stimulation with Con A (5 μg/ml) (a) or CD3-specific mAb (1:1000) (b) is shown. Open bars denote data of p53−/− mice; closed bars denote those of p53+/+ mice. Each bar represents the mean ± SD of the data for three mice. The difference in the T cell proliferative responses between p53−/− and p53+/+ mice, 12–13 wk old, is statistically significant (p < .001).

CD45RBlow CD4+ memory phenotype cells (16), we examined whether the increased population of memory phenotype cells contained any NK T cells. Although the memory phenotype cells were determined not to be NK T cells, a substantial number of cells (NK1.1+ Thy-1−) appeared in the spleen of p53−/− mice after day 5 of SRBC immunization; furthermore, the number of NK cells in p53−/− mice was comparatively higher after day 15 of SRBC immunization (Fig. 3b). Then, we performed a comparative analysis of cytokine production in the two SRBC-injected groups of mice. High Th2-type cytokine (IL-4, IL-6, IL-10) production by splenic T cells from p53−/− mice was noted (Fig. 4). The expression level of the transcriptional factor GATA-3 necessary for expression of the gene for Th2-type cytokines (17) was also significantly increased in p53−/− mice (Fig. 4).

Enhanced cytokine production and depressed T cell proliferative response following TCR stimulation in adult p53−/− mice

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Enhanced cytokine production and depressed T cell proliferative response following TCR stimulation in adult p53−/− mice

The T cell proliferative response to Con A (5 μg/ml) in the spleen of young p53−/− mice (5–6 wk) was 1.43 times stronger than that in p53+/+ mice of the same age (Fig. 5a), and the response to anti-CD3 mAb (1:1000) in p53−/− mice was 1.25 times stronger than that in p53+/+ mice of the same age (5–6 wk) (Fig. 5b). These differences were not significant. However, the T cell proliferative responses in adult p53−/− mice (17–18 wk) were weaker than those in p53+/+ mice of the same age; the response in p53−/− mice decreased 3-fold stronger responses than p53+/+ mice of the same age (PFC/spleen; p53+/+, 22.18 ± 2.55% (5–6 wk) to 31.5 ± 3.12% (18–20 wk), that in p53−/− mice increased dramatically from 22.18 ± 6.09% (5–6 wk) to 58.42 ± 8.98% (18–20 wk). The percentage of memory T cells in adult (18–20 wk) p53−/− mice was similar to that in elderly (60 wk) p53+/+ mice (Fig. 2b).

Increase in the percentage of memory phenotype cells and strong Th2-type cytokine and Ab production responses to Ag stimulation in young p53−/− mice

Next, we examined the T cell-dependent Ab responses to SRBC. Young p53−/− mice (5–6 wk) showed ~3-fold stronger responses than p53+/+ mice of the same age (PFC/spleen; p53+/+, 72,680 ± 21,472; p53−/−, 209,330 ± 68,962). Although the percentage of memory T cells was greatly increased in both p53+/+ mice and in p53−/− mice following Ag (SRBC) stimulation, the increase was more marked in p53−/− mice (Fig. 3a). In contrast, because NK T cells (NK1.1+ Thy-1+) also have a similar percentage of CD44high CD45RBlow CD4+ memory phenotype cells (16), we examined whether the increased population of memory phenotype cells contained any NK T cells. Although the memory phenotype cells were determined not to be NK T cells, a substantial number of cells (NK1.1+ Thy-1−) appeared in the spleen of p53−/− mice after day 5 of SRBC immunization; furthermore, the number of NK cells in p53−/− mice was comparatively higher after day 15 of SRBC immunization (Fig. 3b). Then, we performed a comparative analysis of cytokine production in the two SRBC-injected groups of mice. High Th2-type cytokine (IL-4, IL-6, IL-10) production by splenic T cells from p53−/− mice was noted (Fig. 4). The expression level of the transcriptional factor GATA-3 necessary for expression of the gene for Th2-type cytokines (17) was also significantly increased in p53−/− mice (Fig. 4).

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mice to Con A was 0.39 times and to anti-CD3 mAb was 0.42 times that in p53<sup>+/+</sup> mice. In contrast, analysis of cytokine production in adult p53<sup>−/−</sup> mice (17–18 wk), measured by RT-PCR (Fig. 6), revealed that the production of IFN-γ, IL-4, IL-6, and IL-10 in response to anti-CD3 mAb stimulation in vitro was significantly lower than that in p53<sup>+/+</sup> mice of the same age; however, the IL-2 expression levels were almost the same in both p53<sup>−/−</sup> mice and p53<sup>+/+</sup> mice of the same age; how- ever, the IL-2 expression levels were almost the same in both p53<sup>−/−</sup> mice and p53<sup>+/+</sup> mice of the same age. The response was remarkably up-regulated by combined stimulation with anti-CD3 mAb and PMA (10 nM), an activator of protein kinase C. IL-10, which had been considered to be an immunosuppressive cytokine for the anergic state (18), was frequently secreted in adult p53<sup>−/−</sup> mice.

**Discussion**

In this study, we found remarkable age-related accumulation of memory phenotype (CD44<sup>high</sup> CD45RB<sup>low</sup>) CD4<sup>+</sup> T cells that were already detected in younger p53<sup>−/−</sup> mice (Fig. 2, a and b). p53 deficiency was thus presumed to accelerate immunosenes-cence. Some researchers report that age-related accumulation of memory T cells is related to the poor proliferative responses of T cells (6–9). The proliferative response of T cells to anti-CD3 mAb or Con A, which was previously reported as being depressed in aged mice (7, 9), was depressed in adult p53<sup>−/−</sup> mice (Fig. 5, a and b). Furthermore, our data conform, in part, to the report of Con A-induced T cell proliferation being suppressed following inhibition of the p53 gene expression by p53-specific antisense oligodeoxynucleotides (19). In these studies, it will be concluded that the observed immune changes in p53<sup>−/−</sup> mice are not secondary to lymphomatous development, because we carefully selected out the mice that had no lymphoma by flow cytometry (Fig. 1).

Development and aging lead to the accumulation of memory phenotype T cells, and some of the cytokines produced by the accumulated memory T cells potentially induce Th1/Th2 imbalance; accordingly, the predominance of memory phenotype cells in T cell populations is considered to induce much of the decline in the protective immune responses. Although age-related alterations in the production of cytokines (high IL-4, IL-10, IFN-γ, and low IL-2) have been reported in 24-mo-old mice (10–12), examination of cytokine production in p53<sup>−/−</sup> mice (17–18 wk) revealed a high level of expression of IL-4, IL-6, IL-10, and IFN-γ following TCR stimulation, but the expression of IL-2 in p53<sup>−/−</sup> mice did not change as much as that in p53<sup>+/+</sup> mice (Fig. 6). Considering the reports of the promoters of IL-2, IL-4, IL-6, and other cytokines being repressed by p53 (20, 21), it is suggested that the negative regulation of the transcription of various cytokines is lost in the presence of p53 deficiency. Furthermore, after Ag (SRBC) stimulation, the stronger Ab production and Th2-type cytokine production in p53<sup>−/−</sup> mice might be caused by the absence of the negative regulation of cytokines transcription (Fig. 4). After day 5 of Ag stimulation, an increase in the number of NK cells, largely of splenic origin, was seen in p53<sup>−/−</sup> mice (Fig. 3b). Studies on NK cells in senescence have shown that whereas the number of NK cells increases, NK cell activity decreases with aging (22). In a recent report, the expression of Ly-49A receptors, which down-regulate NK cell activation after physical interaction with target cell MHC class I molecules (23), increases with aging (24). Age-related down-regulation of NK cell activity is probably induced by the increase in expression of Ly-49A receptors with aging. NK clones from p53<sup>−/−</sup> mice did not express Ly-49A receptors on the cell surface, and NK cell activity was independent of MHC class I molecule expression (25). Therefore, the mechanisms underlying the alterations in the number/activity of NK cells in p53<sup>−/−</sup> mice may be different from those underlying NK cell alterations with aging.

Much attention has been focused on the etiology of oxidative damage in cells and organisms with aging (26, 27); a recent report strongly suggested a relationship between oxidative stress and aging. It was indicated that an increase in superoxide levels leads to oxygen hypersensitivity and premature aging in mev-1 (kn1) mutant nematodes (28). Evidence has also been presented for p53 being required for cellular apoptosis in response to oxidative stress by hydrogen peroxide (29). Chromosomal damage is potentially accelerated by oxidative stress in p53<sup>−/−</sup> mice. Extensive evidence has been accumulated that suggests that T cell senescence is due to the acceleration of chromosomal damage and their limited proliferative lifespan. The most significant consequences of these impairments are dysregulation of DNA repair and apoptosis (30). The evidence that T cell senescence was greatly reduced in CD2-Fas transgenic mice was accounted for by the speculation that immunosenescence results from the increase in the number of memory T cells with aging (31). We suggest that the regulation of cytokine production and cell cycle is perturbed by p53 deficiency and that the cellular differentiation induced by the high level of expression of cytokines and the loss of cell cycle arrest cause the increase in the number of memory T cells in p53<sup>−/−</sup> mice. Additionally, the loss of both DNA repair and apoptosis by p53 deficiency in response to oxidative stress potentially leads to the accumulation of memory T cells, which escaped from cell death in
p53\(^{-/-}\) mice. The phenomenon of immunosenescence in p53\(^{-/-}\) mice might explain, in part, the mechanism of aging.

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