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# The MHC influences NK and NKT cell functions associated with immune abnormalities and lifespan

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**Abstract**

The lifespans of H-2 congenic mice differ significantly. The B10.AKM (H-$2^m$) strain has a median survival time (MST) of 15 months, whereas the B10.BR (H-$2^k$) strain has an MST of 24 months. It was previously shown that B10.AKM mice at 13–15 months of age have immunological function comparable to those of B10.BR mice at 22–26 months of age. These functions include: a low proliferative response, reduced levels of intracellular calcium release $[Ca^{2+}]_i$, and an increase in the frequency of memory helper T-cells ($CD4^+$ $CD44^{hi}CD45RB^{lo}$). In this report similar deficiencies were demonstrated in B10.AKM mice at 2–4 months of age and show that activated spleen $NK1.1^+CD4^+$ T (NKT) cells from young B10.AKM mice produce a significantly higher level of IL-4 but a lower level of IFN-$\gamma$ as compared to NKT cells from B10.BR mice of the same age. Also, the cytotoxic activity of natural killer (NK) cells from spleens of young (2–4 months) as well as adult (12–16 months) B10.AKM mice is significantly lower ($P < 0.01$) than that of NK cells from B10.BR mice. These findings suggest that the NKT activity in young B10.AKM mice is a factor for the early onset of immune dysfunction leading to a shorter lifespan. 

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## 1. Introduction

Lifespan is a complex multigenic trait (Finch and Tanzi, 1997). Recent studies in mice have revealed that several genes affect lifespan in concert with the animals' environment (Jazwinski, 1996). Chromosome 17 carries the major histocompatibility complex (MHC) region H-2. Because this region encodes the immune response (Ir) genes that control immune function, and immune function declines with age, senescence may be affected by genes in the H-2 region. Studies of congenic strains, which differ in the H-2 region, show that differences in H-2 haplotypes are associated with differences in lifespan (Smith and Walford, 1977; Gelman et al., 1990; Salazar et al., 1995). Specifically, congenic B10.AKM ($H\text{-}2^m$) mice differ significantly from B10.BR ($H\text{-}2^k$) mice in lifespan.

T-cells from aged mice are less responsive to mitogens or antigens than are those from young mice (Weber et al., 1997). The age-related decline in responsiveness to mitogens or antigen is accompanied by the decline of intracellular calcium release ($[Ca^{2+}]_i$) by T-cells in response to mitogenic or antigenic stimuli (Grossmann et al., 1990; Philosophe and Miller, 1990; Weber et al., 1997) and a decline in the proportion of T-cells expressing IL-2 receptors (Vie and Miller, 1986). Also, such T-cells produce less IL-2 and more IL-4 upon stimulation (Engwerda et al., 1996).

Memory T-cells and T-cells from aged mice share several characteristics. For example, antigen-induced memory T-cells produce a low level of IL-2 and a high level of IL-4 (Ernst et al., 1990; Philosophe and Miller, 1990; Nagelkerken et al., 1991). Other characteristics shared by these cells include an increase in the frequency of $CD4^+$ T-cells, an increase in the expression of CD44 antigen ($CD44^{hi}$) with a concomitant decrease in the expression of CD45RB antigen ($CD45RB^{lo}$), and a decrease in the expression of CD62L antigen ($CD62L^{lo}$) (Lee et al., 1990; Flurkey et al., 1992; Linton et al., 1996). A decrease in the expression of CD45RA antigen on $CD4^+$ T-cells has also been associated with the antigen-induced memory phenotype. Although the populations defined by these surface markers overlap, several studies in vitro have shown that $CD4^+$ T-cells of the phenotypes $CD44^{hi}$ or $CD45RB^{hi}$ are functionally distinct from cells of the phenotypes $CD44^{lo}$ or $CD45RB^{lo}$. Naive $CD4^+$ T-cells are characterized by the phenotype $CD44^{lo}CD45RB^{hi}$ or by $CD44^{lo}CD45RA^{hi}$ and memory cells are characterized by the phenotype $CD44^{hi}CD45RB^{lo}$ or by $CD44^{hi}CD62^{lo}$ (Dobber et al., 1994; Linton et al., 1996; Swain et al., 1996). After antigen stimulation, naive T-cells differentiate into memory cells of the $CD44^{hi}CD45RB^{lo}$ or $CD44^{hi}CD45RA^{lo}$ phenotypes.

Spleen cells and thymocytes contain a subset of T-cells that express the natural killer (NK) cell surface antigen NK1.1 and CD4 ($NK1.1^+CD4^+$ T) (Vicari and Zlotnik, 1996). Interestingly, $NK1.1^+CD4^+$ T (NKT) cells also bear the $CD44^{hi}CD45RB^{lo}CD62L^{lo}$ phenotype and produce high levels of IL-4 upon stimulation (Yoshimoto and Paul, 1994). Upon activation, NKT cells also produce

IFN-γ (Chen and Paul, 1997). NKT cells from aged mice produce a significantly higher level of IL-4 than do cells from young mice (Murakami and Paul, 1998). A reduction in NK cell activity is associated with age (Albright and Albright, 1985; Mikael et al., 1994). This age-related decline in endogenous NK cell activity increases the susceptibility of mice to infectious agents (Albright and Albright, 1998).

To determine whether the shorter lifespan of B10.AKM mice was associated with changes in NKT and NK cell function at a young age, the production of IL-4 and IFN-γ by NKT cells, and the cytotoxic function of NK cells in young (2–4 mo) and adult (12–16 mo) B10.AKM and B10.BR mice were compared. It was observed that the young B10.AKM mice have both reduced NK cytotoxic activity and increased activation-induced IL-4 production by NKT cells. This suggests that the shorter lifespan of B10.AKM mice may be associated with an early defect in NKT and NK cell function.

## 2. Materials and methods

### *2.1. Mice and lifespan studies*

Congenic B10.AKM (H-$2^m$) and B10.BR (H-$2^k$) mice 4–6 weeks old were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained in a conventional environment. For lifespan studies, 45 mice each were used for the strains B10.AKM and B10.BR. Forty mice of each strain were used in functional studies. Male and female mice were separated at weaning and housed, in groups of six to eight mice per polycarbonate cage, in the same room at the Redstone Animal Facility of Dana-Farber Cancer Institute. They received a diet of pelleted chow and bottled water, ad libitum. The room was maintained at 25°C with an alternating 12 h cycle of light and dark.

Surveillance mice (DBA/two females) were kept in the room at all times. Once a month, three or four of these mice were tested for infection by endoparasitic, ectoparasitic bacterial and viral pathogens (Charles River Labs, Wilmington, MA). No infection was detected during the 33-month course of the lifespan study. No viruses were detected in the sera, and no tumor lesions were found when young B10.AKM and B10.BR mice were killed for studies of immune function. However, adult (12–16 months) B10.AKM mice occasionally showed loss of hair and presence of skin lesions. No gross pathologic abnormalities were observed upon necropsy in any of the mice studied. Spleens were macroscopically normal, spleens with ischemic or necrotic areas were excluded from the study.

### *2.2. Monoclonal antibodies and reagents*

Hamster IgG anti-mouse CD3ε (clone 145-2C11), biotin-conjugated hamster anti-mouse CD3ε, PE-conjugated anti-CD44 and FITC-conjugated rat anti-CD4 were purchased from Caltag (Burlingame, CA). Biotin-conjugated rat anti-mouse

CD45RB, rat anti-mouse CD45RA, anti-mouse NK1.1 (PK136), and goat anti-hamster IgG were obtained from Pharmingen (San Diego, CA). FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG were purchased from Sigma (St. Louis, MO). Streptavidin-conjugated Cy5 was purchased from Coulter-Immunotech (Miami, FL). RPMI-1640 medium was purchased from GIBCO (Grand Island, NY). Penicillin, streptomycin, sodium pyruvate, 2-mercaptoethanol (2-ME) and fetal calf serum (FCS) were all purchased from Sigma. Nylon wool was from Robbins Scientific (Sunnyvale, CA). Indo-1 was purchased from Molecular Probes (Eugene, OR).

### 2.3. Cell preparation

Mice were killed by $CO_2$ asphyxiation or by cervical dislocation, according to a protocol approved by the institutional animal care committee. Fig. 1 diagrams the cell isolation procedures used. Spleens were removed aseptically and transferred to dishes containing RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM 2-ME (referred to as culture medium). To prepare single-cell suspensions, spleens were gently pressed through a sterile stainless steel wire mesh with a rubber stopper or

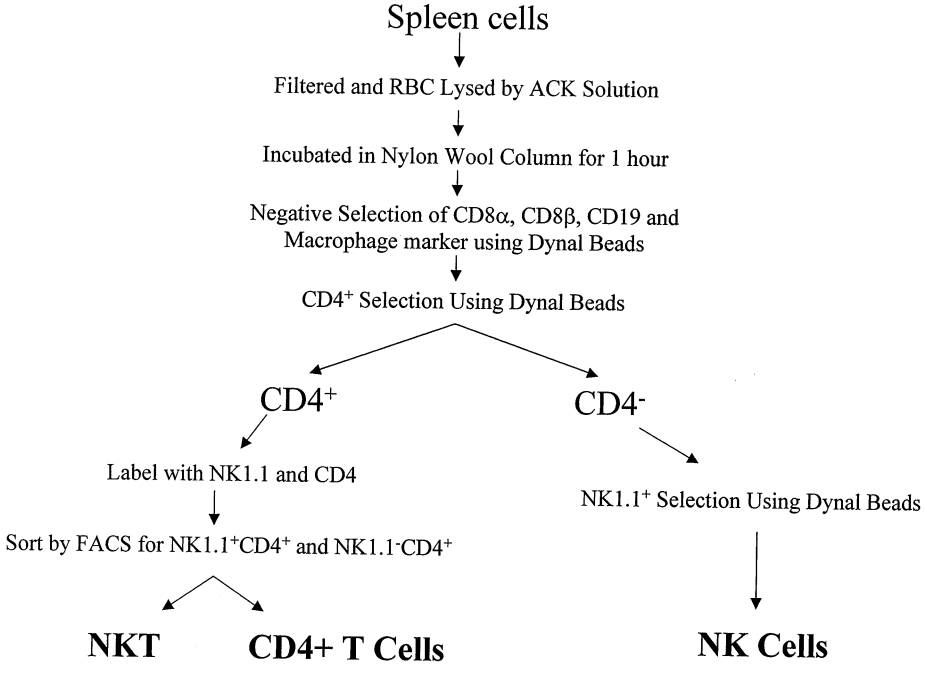


Fig. 1. Isolation of splenic natural killer (NK) and $NK1.1^+CD4^+$ T (NKT) cells. Young (2–4 months) and adult (12–16 months) B10.BR and B10.AKM mice were used for the isolation of NK and NKT cells. Details of the protocol are described in Section 2.

gently meshed between two glass slides. The cells were then washed in culture medium and spun at $400 \times g$ for 10 min. To lyse erythrocytes, 4.5 ml of sterile de-ionized water was added to the pellets for 15 s, followed by the addition of $10 \times$ PBS (0.5 ml). Cells were then kept on ice for 10 min before centrifugation at $400 \times g$ for 10 min. The cell suspension was filtered through a cell strainer (Falcon cell strainer # 2340, Becton Dickinson, Mountain View, CA), washed twice and viability was determined by trypan blue exclusion. To remove $CD8^+$ T-cells, B-cells and monocytes/macrophages, the spleen cells were passed through a nylon wool column and collected the non-adherent cells. The non-adherent cells were passed through a Collect™ plus mouse CD4 immunocolumn for the enrichment of $CD4^+$ cells (Collect CD4 column, Cytovax Biotechnology, Edmonton, Alberta, Canada). Cells were used from this stage of the isolation procedure for memory cell phenotyping. Further isolation procedures, used for other experiments, are described below.

The purity of the isolated cells was determined by immunostaining with anti-CD3 or anti-NK1.1 antibody. In brief, $1 \times 10^6$ cells were incubated on ice with the appropriate FITC or PE-conjugated antibody, washed in PBS, and fixed with 1% paraformaldehyde prior to flow cytometric analysis. Isotype controls were included in all assays.

### 2.4. Flow cytometry

Aliquots of isolated cells were stained for CD4, CD44, CD45RA, CD45RB, or NK1.1 antigens using antibodies conjugated with FITC, phycoerythrin, or biotin. Biotinylated antibodies were stained with streptavidin-conjugated with Cy5. The antibody-stained cells were analyzed with an Elite V or EPICS-XL flow cytometer (Coulter, Miami, FL).

### 2.5. Proliferation assay

Nylon wool non-adherent cells ($2 \times 10^6$/ml) were dispensed, in triplicate, into U-bottom, 96-well microtiter plates (Linbro) and cultured in the presence of anti-CD3ε (2C11) antibody in 200 μl of culture medium for 3 days at 37°C. [$^3$H]Thymidine (1 μCi) in a 25 μl volume was added to each well. After 16 h further incubation, the cells were harvested, and incorporated [$^3$H]thymidine was measured by scintillation counting in a Beta Plate counter (Wallace, Gaithersburg, MD).

### 2.6. Measurement of calcium flux by flow cytometry

Intracellular calcium release ($[Ca^{2+}]_i$) was determined by an assay based on changes in the fluorescence emission of the $Ca^{2+}$-sensitive fluorescent dye Indo-1 (Chused et al., 1987). In brief, $2–5 \times 10^6$ nylon wool non-adherent cells were washed in 2% FCS medium (2% FCS, 25 mM Hepes in RPMI-1640) and incubated with 5 μM of the acetoxymethyl ester of Indo-1 in 2% FCS medium containing 0.5% DMSO for 30 min at 37°C. Cells were washed twice, resuspended at a final

concentration of $10^6$ cells/ml in 2% FCS medium, and kept in the dark on ice. $[Ca^{2+}]_i$ was determined using a Coulter Elite V flow cytometer as previously described (Weber et al., 1997). Hamster anti-CD3ε antibody followed by cross-linking with goat anti-hamster IgG was used for cell stimulation. Cells were pre-incubated with hamster anti-CD3ε (1 μg) for 15 min at 4°C. Flow cytometry was conducted at room temperature using cells at $1 \times 10^6$ cells/ml. After the cytometer had been allowed to run for 2 min to produce a steady base line, 1 μg (5 μl) of goat anti-hamster IgG was added to the cells. Spectral histograms were generated at various time points. Cellular response was analyzed in terms of relative levels of $[Ca^{2+}]_i$, the mean violet/blue ratio and the percentage of responding cells. Because at least 10 000 cells were analyzed at each time point, the error of measurement was less than 1%.

### *2.7. Studies of NK and NKT cells*

#### *2.7.1. Isolation of NKT and NK cells*

Non-adherent spleen cells were obtained by passing through a nylon wool column (Fig. 1). Non-adherent cells were then depleted of $CD8^+$ T-cells, B-cells and monocytes, and $CD4^+$ T-cells were isolated. In some experiments where NKT isolation was required, a magnetic bead method was used to isolate NKT cells (Dynal, NY). An aliquot was removed from each isolated cell preparation for surface phenotyping. Cells were then incubated in the presence of biotinylated CD4 antibody at 2 μg/ml in PBS containing 0.2% bovine serum albumin (BSA) for 30 min on ice. Streptavidin-conjugated Dynabeads M-280 were used to isolate the $CD4^+$ and $CD4^-$ cells according to the manufacturer's protocol. Briefly, after three washes, the cells were incubated, in a 1:4 ratio with Dynal beads, for 30 min on ice, and the cells bound to beads were removed with a magnetic particle concentrator. The cells bound to the beads were subjected to two rounds of washing to remove $CD4^-$ cells, and the magnetic beads were removed from the $CD4^+$ cells according to the manufacturer's protocol.

$CD4^-$ cells were then incubated in the presence of NK1.1 antibody (2 μg/ml in PBS/0.2% BSA) for 30 min on ice. Anti-mouse IgG2a-conjugated Dynabeads M-450 were used to isolate the $NK1.1^+$ $CD4^-$ (NK) cells and to remove magnetic beads by the same procedures described above. The $CD4^+$ cells were separated further into $CD4^+NK1.1^+$ (NKT) and $CD4^+NK1.1^-$ ($CD4^+$T) sub-populations using standard procedures by FACS sort (Elite, Coulter, Miami, FL) after staining with saturating amounts of FITC-conjugated CD4 and PE-conjugated NK1.1 antibodies. In the authors' hands, this method provided highly enriched ($>90\%$) NK, NKT and $CD4^+$ T-cell sub-populations. These cell preparations were used in the studies of $CD3^-$ or NK1.1-induced IL-4 and IFN-γ production.

#### *2.7.2. In vitro activation of NKT cells*

Ninety-six well flat-bottom plates (H-B2, Dynex,VA) were coated with hamster anti-mouse CD3ε monoclonal antibody (2C11) and anti-mouse NK1.1 antibody (PK 136) at 10 μg/ml overnight at 4°C, and washed three times with PBS with 0.1%

BSA (PBS/BSA) before use. NKT cells were isolated from B10.BR and B10.AKM mice, aged 2–4 and 12–16 months. NKT cells ($10^4$) were cultured in 200 μl per well in the presence or absence of 10 U/ml rIL-2. On day 4, supernatants were collected for cytokine determination, and cells were transferred to a 24-well plate and expanded for 3 days in the presence of 100 U/ml of IL-2. After 3 days, cells were collected, washed and plated at 40 000 cells in each well pre-coated with either anti-CD3 or anti-NK1.1 antibody and in the presence or absence of 10 U/ml rIL-2. Supernatants were removed, and cells were collected after 48 h incubation for RT-PCR analysis of cytokine mRNA.

*2.7.3. Cytokine determination*

IL-4 and IFN-γ production were quantified after primary stimulation (3 days after CD3ε or NK1.1 activation) following standard ELISA procedures. Kits were purchased from BioSource International (Camarillo, CA). All samples were assayed in duplicate. Cytokines were quantitated based on the standard curves obtained with each ELISA assay. The sensitivities (minimum detectable dose) of these kits were: for IFN-γ, $< 1$ pg/ml, and, for IL-4, $< 5$ pg/ml.

*2.7.4. RT-PCR determination of IL-4 mRNA in activated cells*

To analyze expression of cytokines, mRNA was isolated from NKT cells after secondary stimulation (after 9 days of culture) as described above. RNA was isolated from cells using Ultraspec (Biotecx, TX). One μg of RNA was used to reverse transcribe using standard first strand reaction conditions: 5 mM $MgCl_2$, $1 \times$ PCR buffer II, 1 mM of each dNTP, 2000 U/ml RNAse inhibitor (Promega, Madison, WI), 2.5 U/ml MMLV reverse transcriptase (Gibco, BRL, Gaithersburg, MD) and random hexamer primers. Reaction tubes were placed in a thermal cycler, and incubation was carried out at 37°C for 30 min, followed by 5 min at 95°C. First strand reaction products were used for semi-quantitative amplification of cytokine mRNA using *Taq* polymerase for 30 cycles with the following primers at an annealing temperature of 58°C:

IL-4: 5′-GAATGTACCAGCAGCCATATC,
3′-CTCAGTACTACGAGTAATC-CA;
IFN-γ: 5′-AACGCTTACACACTGCATCTTGG,
3′-GACTTCAAAGAGTCTG-AGG;
β-actin: 5′-GATGACGATATCGCTGCGCTG,
3′-GTACGACCAGAGGCA-TACAGG.

Amplified PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV following ethidium bromide staining. No reverse transcriptase controls were used to check that no genomic DNA was being amplified.

*2.7.5. Determination of NK cell activity*

Non-adherent spleen NK cells were tested for their ability to kill the NK sensitive target YAC cells by a standard $^{51}Cr$ release assay. Briefly, freshly isolated spleen

cells were passed through a nylon wool column to remove monocytes and B cells. The non-adherent cells enriched for NK cells were mixed with the $^{51}$Cr-labeled YAC targets ($5 \times 10^3$ cells/well) at effector:target cell ratios ranging from 1:25 to 1:100 and incubated for 4 h at 37°C. Supernatants (100 μl) were collected from each well. $^{51}$Cr released into the supernatant was determined in a gamma counter. Specific cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = (\text{EXP}_{\text{cpm}} - \text{SR}_{\text{cpm}})/(\text{TC}_{\text{cpm}} - \text{SR}_{\text{cpm}}) \times 100,$$

where SR is the spontaneous release and TC is the total $^{51}$Cr released by the target cells in presence of 0.1% NP-40 detergent.

### *2.8. Statistical analysis*

Percent survival was estimated by the product-limit method of Kaplan and Meier, and survival curves were compared using the log-rank test (Kaplan and Meier, 1958; Lindsey, 1997). Two-sided $P$ values less than 0.05 with a Bonferroni correction were considered statistically significant.

Data analysis was performed using SPSS version 7.5 (SPSS, Chicago, IL) and SAS version 6.12 (SAS Institute, Cary, NC). The statistical significance of the differences in NK activity between young and aged and between B10.AKM and B10.BR strains were tested using the unpaired Student's $t$-test.

## 3. Results

### *3.1. The lifespan of B10.AKM mice is significantly shorter than that of B10.BR mice*

The median survival time (MST), defined as the interval between birth and 50% survival, was $15 \pm 1$ months for the B10.AKM strain and $24 \pm 1$ months for the B10.BR strain (Fig. 2). Kaplan–Meier survival analysis showed that the MSTs of these two strains were significantly different (log-rank test, $P < 0.0001$). Although the maximum survival time ($t_{\max}$) for the B10.BR strain was significantly greater than that for the B10.AKM strain, the times of death for the first mice of each strain were not significantly different. This suggested that the mortality rate of the two strains differed significantly with age.

### *3.2. Young B10.AKM mice show a lower CD3-induced non-adherent spleen (T/NKT) cell activation response than young B10.BR mice*

The polyclonal proliferative response induced by anti-CD3 antibody was measured in non-adherent spleen cells (composed primarily of T, NKT and NK cells) from young (2–4 months) and adult (12–16 months) B10.BR and B10.AKM mice (Table 1). The cellular stimulation index (SI) was significantly lower in young B10.AKM mice than in young B10.BR mice ($24 \pm 6$ and $86 \pm 22$, respectively;

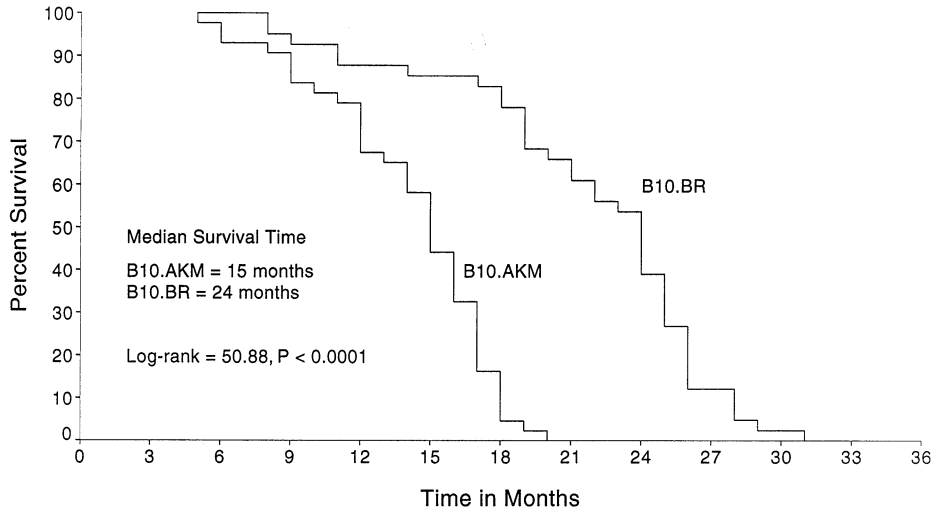


Fig. 2. Survival curves for the congenic B10.BR and B10.AKM mice. Survival curves were analyzed using the method of Kaplan–Meier and compared by the log-rank tests. The lifespan of the two strains differ significantly ($P < 0.0001$). The median survival time (MST) was 24 months for B10.BR and 15 months for B10.AKM mice.

Table 1
Proliferative response and calcium mobilization in non-adherent spleen cells from B10.BR and B10.AKM mice upon CD3 cross-linking

| Strain | Proliferative response (SI) | | | % Cells with CD3-induced $[Ca^{2+}]_i$ | | |
|---|---|---|---|---|---|---|
| | Young | Adult | % Change | Young | Adult | % Change |
| B10.BR | $86 \pm 22$[a] | $39 \pm 9$ | 53 | $18 \pm 1$[b] | $13 \pm 3$ | 29 |
| B10.AKM | $24 \pm 6$ | $14 \pm 3$ | 46 | $11 \pm 3$ | $6.1 \pm 1.6$ | 43 |

[a] Data represent the mean SI $\pm$ standard deviation (S.D.) of four independent experiments.
[b] Data represent the mean percentage $\pm$ S.D. of cells which released intracellular calcium upon cross-linking of antibody bound to cell surface CD3ε above the background levels recorded after anti-CD3ε binding but prior to crosslinking.

$P < 0.001$). Cells from B10.AKM and B10.BR adults had mean SI values of $14 \pm 3$ and $39 \pm 9$, respectively. The percentage decrease in cellular SI values between young and adult mice within a given strain, however, was similar for both strains (46% for B10.AKM and 53% for B10.BR). Note that young B10.AKM mice have a weaker proliferative response than adult B10.BR mice.

It was then determined whether the low level of CD3-induced cell proliferation in young B10.AKM mice was associated with a poor intracellular $Ca^{2+}$ signaling capacity. The percentages of cells responding to CD3ε cross-linking were determined (Table 1). Cells from both strains showed a short lag phase followed by a rapid increase in $[Ca^{2+}]_i$ after addition of the cross-linking agent. A significantly

lower percentage of cells from young B10.AKM mice released calcium upon CD3 cross-linking as compared to cells from young B10.BR mice ($P < 0.01$). There was a similar (approximately 50%) age-dependent decrease in the number of responding cells in both mouse strains. These studies suggest that splenic lymphocytes from young B10.AKM mice respond relatively poorly to CD3 cross-linking, as determined by $[Ca^{2+}]_i$ and cellular proliferation. The decrease in response was contributed primarily by $CD3^+$ T-cells as the phenotypic analysis of spleen cells showed no difference in the proportion of T, NK and NKT cells between the two strains of mice (data not presented).

### *3.3. High frequency of $CD4^+$ spleen cells with memory cell markers in young B10.AKM mice*

Both memory $CD4^+$ T and NKT cells express increased levels of CD44 ($CD44^{hi}$) and reduced levels of CD45RB ($CD45RB^{lo}$) simultaneously (Lee et al., 1990; Flurkey et al., 1992; Linton et al., 1996; Vicari and Zlotnik, 1996). To evaluate whether the frequency of memory cells in young B10.AKM mice is higher than in young B10.BR mice, the memory phenotypes of $CD4^+$ fraction of non-adherent spleen ($CD4^+$ T) cells from young and adult mice of the two strains were compared. The frequency of $CD4^+$ splenic lymphocytes expressing CD44 and CD45RB antigens in young and adult B10.BR and B10.AKM mice was compared. Non-adherent splenic preparations from the young B10.AKM mice had higher percentages of $CD44^{hi}CD45RB^{lo}$ cells than did similar preparations from young B10.BR mice (Table 2). In contrast, there was no difference in the percentages of $CD44^{hi}CD45RB^{hi}$ cells in these mouse strains. The percentage of cells with memory cell markers ($CD44^{hi}CD45RB^{lo}$) increased with age in both mouse strains though the frequency of B10.AKM was higher than in B10.BR strain. These results suggest that B10.AKM mice always have a higher percentage of splenic lymphocytes with the memory cell phenotype than do B10.BR mice of the same age. Most signifi-

Table 2
Distribution of CD44 and CD45RB antigens on mouse splenic T/$NK1.1^+CD4^+$ T (NKT) cells[a]

| Phenotype | Percent of total splenic T/NKT cells | | | |
|---|---|---|---|---|
| | B10.BR ($H\text{-}2^b$) | | B10.AKM ($H\text{-}2^m$) | |
| | Young (2–4 months) | Adult (12–16 months) | Young (2–4 months) | Adult (12–16 months) |
| $CD4^+$ $CD44^{hi}CD45RB^{hi}$ | 20.7 | 12.9 | 20.5 | 19.8 |
| $CD4^+$ $CD44^{hi}CD45RB^{lo}$ | 9.4 | 19.6 | 19.6 | 28.4 |

[a] lo, low MFI < 3.7; hi, high MFI > 20.

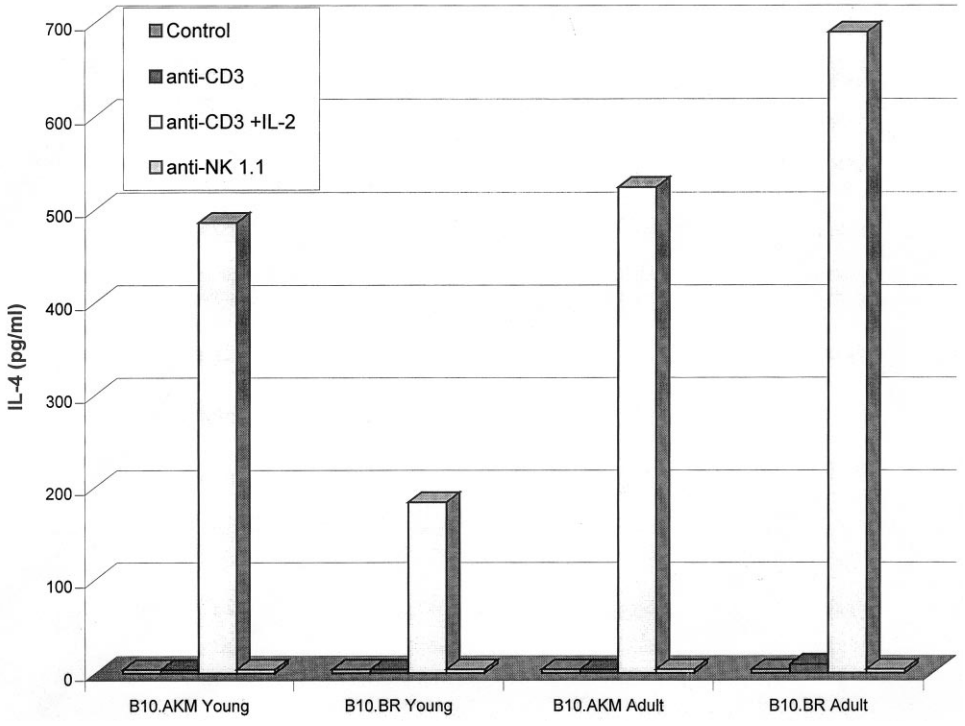


Fig. 3. Inducible IL-4 production by splenic NK1.1$^+$CD4$^+$ T (NKT) cells in response to activation by anti-CD3 or anti-NK1.1 antibodies. Splenic NKT cells from young (2–4 months) and adult (12–16 months) mice of B10.BR and B10.AKM strains were isolated as described in Fig. 1 and sorted by FACS. Supernatants were collected and tested for cytokine levels by ELISA after primary stimulation with anti-CD3 or -NK1.1 antibodies for 4 days in culture.

cantly, young B10.AKM mice appear to have as many of these cells as adult B10.BR mice.

### *3.4. NKT cells from young B10.AKM mice produce higher levels of IL-4 in response to activation by CD3 plus IL-2 than NKT cells from young B10.BR mice*

If the cellular functions of young B10.AKM mice were indeed comparable to those of adult B10.BR mice, NKT cells from young B10.AKM mice might produce higher levels of IL-4 and lower levels of IFN-$\gamma$ than NKT cells from B10.BR mice of the same age. Indeed, it was found that NKT cells from spleens of young B10.AKM mice produced about a 3-fold higher amount of IL-4 than did an equal number of NKT cells from B10.BR mice of the same age (Fig. 3). The study shows that NKT cells from young B10.BR mice stimulated with anti-CD3 and IL-2 produce a significantly higher level of IL-4 as well as IFN-$\gamma$ as compared to young B10.AKM mice. These results are consistent with the observations of Chen and Paul (1997). In both the strains, activation of NKT cells by anti-NK1.1 (PK136)

antibody does not induce IL-4 production, though there is a significant increase in the production of IFN-γ. Analysis of IFN-γ production in these two mouse strains showed that there was a decrease in the levels of IFN-γ produced by CD3 or NK1.1-activated NKT cells from young B10.AKM mice as compared to B10.BR mice of the same age (Fig. 4). Thus, the study confirmed the prediction that the young B10.AKM mice would produce significantly higher levels of IL-4 cytokine but lower amounts of IFN-γ than B10.BR mice of the same age.

### *3.5. Analysis of mRNA expression of cytokines IL-4 and IFN-γ*

To verify that the secreted cytokine pattern was also detectable at the mRNA level, cytokine gene expression following activation of NKT cells was determined. NKT cells were activated with anti-CD3 or anti-NK1.1 antibodies in the presence or absence of 10 U/ml of rIL-2, and IL-4 and IFN-γ mRNAs were determined by RT-PCR (Fig. 5). Detectable levels of both IL-4 and IFN-γ mRNA were present in activated NKT cells. Semi-quantitative analysis of IL-4 mRNA in activated NKT cells from young B10.AKM mice showed a 2-fold increase (based on scanning densitometry) over that of NKT cells from B10.BR mice of the same age after

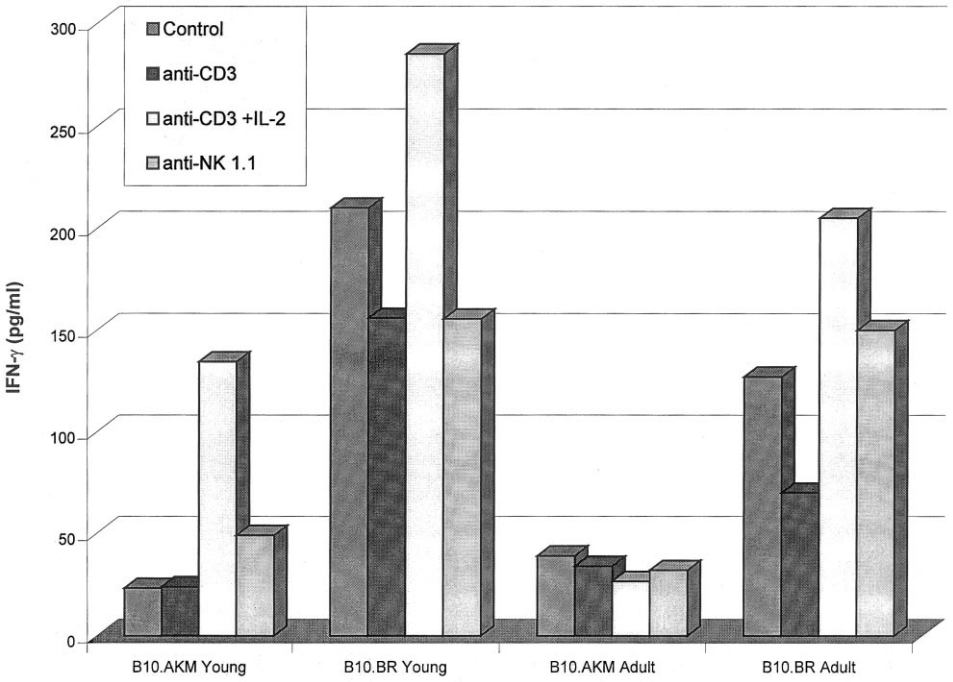


Fig. 4. Inducible IFN-γ production by splenic NK1.1$^+$CD4$^+$ T (NKT) cells in response to activation by anti-CD3 or anti-NK1.1 antibodies. Splenic NKT cells from young (2–4 months) and adult (12–16 months) mice of B10.BR and B10.AKM strains were isolated as described in Fig. 1 and sorted by FACS. IFN-γ was determined in supernatants by ELISA after primary stimulation as in Fig. 3.

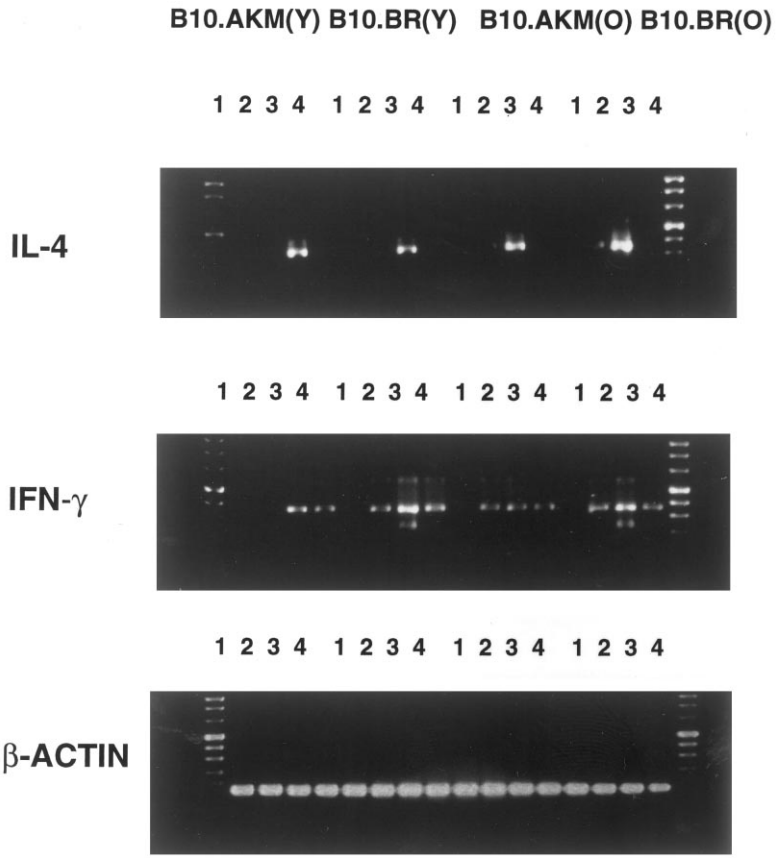


Fig. 5. Expression of cytokine mRNA by splenic NK1.1$^+$CD4$^+$ T (NKT) cells following activation by anti-CD3 and NK1.1 antibodies. Activated NK1.1$^+$CD4$^+$ T-cells, isolated from spleens of B10.BR and B10.AKM mice, were cultured in the presence or absence of 10 U/ml of rIL-2, re-activated with anti-CD3 or anti-NK1.1 antibodies (secondary stimulation, as described in Section 2) and subjected to RT-PCR for determination of IL-4 and IFN-γ expression. 1, control; 2, anti-CD3; 3, anti-CD3 + IL-2; 4, anti-NK1.1.

activation by α-CD3 and in the presence of IL-2. IL-4 gene expression in activated NKT cells from the adult B10.BR mice was somewhat higher than in cells from adult B10.AKM mice. There was a smaller difference in the IFN-γ gene expression in B10.AKM and B10.BR mice, with lower levels of IFN-γ gene expression detected in B10.AKM mice. These results clearly show that the cytokine production pattern by NKT cells from young B10.AKM mice was similar to that found in NKT cells from adult B10.BR mice.

### 3.6. NK activity is significantly lower in B10.AKM than in B10.BR mice

In aging mice, the cytotoxic activity of NK cells from spleens is significantly reduced (Albright and Albright, 1985; Mikael et al., 1994; Albright and Albright, 1998). It was predicted that, if young B10.AKM mice show early immune senescence and behave immunologically like adult B10.BR mice, NK cell activity of B10.AKM mice would be lower than that of B10.BR mice. The NK cell activity of young (2–4 months) and adult (12–16 months) B10.AKM, and B10.BR mice was compared. Nylon wool non-adherent spleen cells were used as the source of NK cells. Fig. 6 shows the cytotoxic activity of NK cells from young and adult B10.AKM and B10.BR mice against the YAC cell line. The NK cell cytotoxic activity of cells from the B10.AKM strain was lower than that of cells from the B10.BR strain in both young and adult mice. Furthermore, the NK cell cytotoxic activity of young B10.AKM mice was the same as of the 15-month-old B10.BR mice (percent specific cytotoxicity, $25 \pm 3$ and $26 \pm 5\%$, respectively, at an E:T ratio of 100). This suggested that NK cell activity in young B10.AKM mice is similar to adult B10.BR mice.

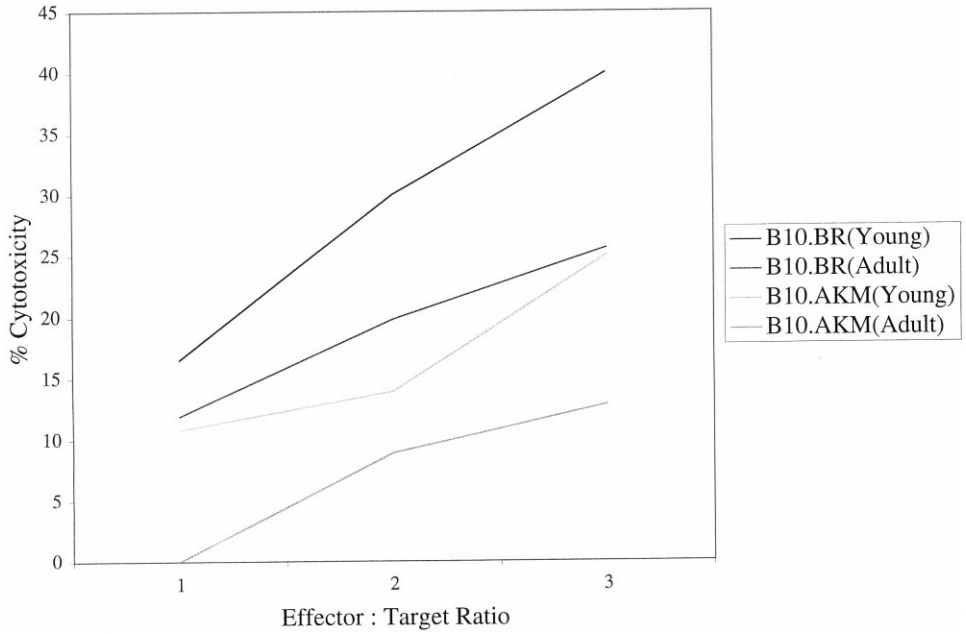


Fig. 6. Cytotoxic activity of spleen cells. Cytotoxic activity of spleen cells from young and adult mice of both strains was determined against YAC cells in a 4-h $^{51}Cr$ release assay. These results are representative of four independent experiments. Effector:target ratios were 1, 1:25; 2, 1:50; and 3, 1:100.

## 4. Discussion

Lifespan is influenced by genes, including several within the MHC (Smith and Walford, 1977; Jazwinski, 1996; Finch and Tanzi, 1997). The B10.AKM mouse strain has a median lifespan about half that of the B10.BR strain. Earlier, it was reported that adult B10.AKM mice (13–15 months) are deficient in the proliferative capacity of their T-cells, the frequency of intracellular calcium release by T-cells, and have increased memory T-cells. These immune parameters of B10.AKM mice at 13–15 months were comparable to those of B10.BR mice at 24–26 months of age (Weber et al., 1997). In the present study, the studies have been extended to compare immune functions earlier in the life of B10.AKM and B10.BR mice. It was found that young (2–4 months) B10.AKM mice have a decreased T-cell proliferative capacity, decreased $[Ca^{2+}]_i$ capacity in their spleen cells, and an increased number of cells of the memory phenotype ($CD44^{hi}CD45RB^{lo}$) as compared with young B10.BR mice. Since the percent of NKT cells is unaffected by age (data not presented), there is a shift in the T-cell population from naive to memory phenotype in young mice of the B10.AKM strain. Young B10.AKM mice also showed an increase in the production of IL-4 by NKT cells upon CD3 plus IL-2 stimulation as compared with young B10.BR mice.

A greater number of $CD4^+$ T-cells acquire the memory phenotype as an animal ages, while the proportion of naive cells decreases (Swain et al., 1996). This shift toward memory cells may not be entirely antigen-driven. A portion of the cells with memory phenotype may arise from some intrinsic change associated with senescence. Age-associated memory cells share some characteristics with antigen-associated memory cells, such as lower proliferative capacity and lower IL-2 secretion (Flurkey et al., 1992; Linton et al., 1996). It is possible that an altered cytokine environment explains both the decreased proliferative responses and the accumulation of cells with a memory phenotype.

Various reports have suggested a relation between age-associated decreases in immunocompetence and concurrent changes in the expression of different cytokines (Wiedmeier et al., 1994; Engwerda et al., 1996; Spencer et al., 1996). This seems to be a physiologically relevant phenomenon, since decreased immunocompetence is associated with both a reduced ability to produce important cytokines and an altered balance in their production. Increased IL-4 production has been observed from T-cells in aging mice (Engwerda et al., 1996). While it has also been reported NKT cells can be directly induced to produce IL-4, increased age-associated production of IL-4 in mice was shown not to be due to an increased number of NKT cells (Poynter et al., 1997).

The results suggest that, while the number of NKT cells present in splenocytes from B10.BR and B10.AKM remained unchanged (data not presented), there was an increased ability of these cells in young AKM mice to produce IL-4 following activation. It is possible that increased production of IL-4 by NKT cells may accelerate memory cell development in young B10.AKM mice. There have been reports that memory T-cells produce more IL-4 and IFN-$\gamma$ than naive T-cells, which predominantly produce IL-2 (Dobber et al., 1994). Also, IL-4 production

from $CD4^+$ T-cells, induced by anti-CD3 alone or with anti-CD28 mAb, has been shown to be higher in adult as compared to young mice (Poynter et al., 1997). Moreover, this was shown to be due, at least in part, to an increased half-life of IL-4 mRNA in older mice, suggesting that mRNA stability may have a role in regulating cytokine patterns during aging (Pioli et al., 1998).

In a previous study, it was reported that a T-cell proliferative defect in B10.AKM mice can be corrected by NAC (Weber et al., 1997). The role of redox status in immune regulation has come under considerable inquiry (Furukawa et al., 1987; Smyth, 1991; Staal et al., 1994). It is believed that the B10.AKM mouse strain may possess such a redox defect in a genetic region linked to the MHC. Cytotoxic responses of NK cells are regulated by the intracellular oxidation–reduction environment (Furuke and Bloom, 1998). Earlier results showing correction of T-cell proliferative capacity with NAC suggests that an altered redox level in the NK compartment may also exist resulting in the lower NK cytotoxic activity observed in the young B10.AKM mice.

It was speculated that redox changes in NK and NKT cells is due to altered levels of free radicals. This may affect cytotoxic function of NK cells and cytokine production by NKT cells. It is not clear whether the observed increase in IL-4 production at an early age affects the development of T-cells and NK cells in young B10.AKM mice. Further studies are needed to establish the mechanism of increased IL-4 production by NKT cells and its relationship to aging. In summary, it was concluded that NK and NKT function of B10.AKM mice is quantitatively different from B10.BR mice of the same age group. This may be a factor in the early onset of immune dysfunction and short lifespan of B10.AKM mice.

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