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Short Report

Age-related changes in cell surface and senescence markers in the spleen of DBA/2 mice: A flow cytometric analysis

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Abstract

In murine studies of immune senescence cell preparations from whole organs, e.g. splenocytes, are frequently reported. However, age-related changes in spleen cell types have been poorly defined throughout the spectrum of adult age or across murine strains. The aim of this study was to use flow cytometry to more fully characterize cell types within the spleen of DBA/2 mice, a strain available from the National Institute of Aging (NIA), across the entire adult age spectrum. In advanced age, B cells comprise a greater percentage of the total splenocyte population, and there is a decline in the percentage of $\gamma\delta^+$T cells in the spleen. The percentage of memory $CD4^+$T cells increases in middle age with a corresponding decrease in the percentage of naïve $CD4^+$T cells. Expression of the co-stimulatory molecule CD28 on splenic $CD4^+$ and $CD8^+$T cells increases with age, but CD28 expression on peripheral blood T cells does not change. The senescence marker $p16^{INK4a}$ increases in B cells and $CD8^+$T cells within the spleen and can be measured by flow cytometry.

Keywords: Aging; Murine spleen; Lyphocyte sub-populations; Flow cytometry; CD28; γδ T cells; $p16^{ink4a}$

1. Introduction

With advanced age there is a waning of the immune system, termed 'immune senescence', which contributes to the morbidity and mortality associated with infectious diseases of older adults (High, 2004). Immune senescence is difficult to study in humans because of the complexity in separating the effects of aging from co-existent illness, the duration required to study humans over decades, and the difficulty controlling experimental conditions in humans (Miller, 1996; Castle, 2000). Thus, murine models are widely used to examine immune senescence (High, in press), and splenocytes are commonly used, but a thorough description of the cellular composition of the spleen throughout the age spectrum has not been reported for most strains of mice available from the aging colony of the NIA. Species-specific data are essential as differences in immune response by strain are common (Vannier et al., 2004; High, in press).

In this study we attempt to more fully characterize the cell types within the spleen and blood across the adult age spectrum in DBA/2 mice. We determined proportions of the major cell types and subtypes including: $CD4^+$ and $CD8^+$T cells, $\gamma\delta^+$T cells, naïve and memory T cells, immature and mature B cells, macrophages, and plasmacytoid and myeloid DCs. Finally, we examined a marker of cellular senescence, $p16^{INK4a}$, in B and T cells of the spleen to define the magnitude of age-related accumulation and assess the inter-individual variability of $p16^{INK4a}$ accumulation.

2. Materials and methods

2.1. Animals

Young adult (2 mos), mature (6 mos), middle aged (12 mos), and old (18 mos) nulliparous female DBA/2 mice were purchased from Harlan Laboratories (2 mos) (Indianapolis, IN) or the NIA (Bethesda, MD), (6, 12 and 18 mos), and were housed under standard conditions (i.e. not specific pathogen free (SPF)) in the Wake Forest University School of Medicine Animal Resource Center (N.B. DBA/2 mice shipped from the NIA are certified for health and pathogen exposure by Harlan Laboratories). Mice were anesthesized by CO_2 inhalation

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and sacrificed via cervical dislocation in accordance with approved animal care and use committee protocols.

2.2. Antibodies

PE-anti-Mac-3, FITC-anti-CD11b, PE-anti-CD45R/B220, APC-anti-CD19, PE-anti-CD28, PE-anti-CD3 molecular complex, APC-anti-CD11c, APC-CD8a-(Ly-2), APC-anti-CD4 (L3T4), PE-anti-CD62L (L-selectin, LECAM-1, Ly-22), FITC-anti-CD45RB, FITC-anti-γδ, PE-anti-IgM, FITC-anti-IgD, and purified anti-mouse CD16/CD32 (FcγIII/II Receptor) monoclonal antibodies were purchased from BD Biosciences (San Jose, CA). FITC-anti-p16 antibody was purchased from eBioscience (San Diego, CA). Isotype control antibodies were FITC-rat IgG_{2a}, FITC-anti-hamster IgG, APC-rat IgG_{2a}, and PE-rat IgG_{2a} obtained from BD Biosciences (San Jose, CA).

2.3. Immunoflourescence staining

One, two, or three color staining was performed on isolated splenocytes. One million cells in 100 μl PBS were first blocked with 1 μg anti-mouse CD16/CD32 (Fc Block) and then cells were stained with the appropriate concentration of antibody. Cells were washed twice and resuspended in PBS 2% FCS for flow cytometry. Cells stained for $p16^{INK4a}$ were first permeabilized with BD FACS permeabilization solution 2 (BD Biosciences, San Jose, CA), washed, and then stained according to the protocol above. All samples were run in duplicate.

2.4. Blood cell processing

Peripheral blood was collected via cardiac puncture with a heparinized syringe. FITC-anti-CD8a and PE-anti-CD28 (BD Pharmingen, San Jose, CA) were added to 75 μl whole blood, and incubated in the dark for 20 min at room temperature. FACS Lysing Solution (Becton Dickinson, Franklin Lakes, NJ) was then added to the blood, each tube vortexed briefly and incubated in the dark for 15 min at room temperature. Cells were washed twice with PBS and flow cytometry performed.

2.5. Flow cytometry analysis

Flow cytometry analysis was performed on a Becton–Dickinson FACS Calibur flow cytometer. Data were analyzed using Win MDI software (http://www.facs.Scripps.edu). Single color immunofluorescence frequency distribution histograms allowed measurement of fluorescence intensity. The proportion of cells stained by one or more antibodies was then determined from the histograms. For $p16^{INK4a}$ levels, fluorescence was analyzed by gating on $CD4^+$, $CD8^+$, or $CD19^+$ cells and then determining the mean fluorescence intensity.

2.6. Statistical analysis

The mean and standard deviation was calculated for each cell marker and age group with log-transformation when appropriate for statistical analyses performed using ANOVA for multiple groups and two-tailed pairwise *t*-tests to determine significant group differences. A value of $p<0.05$ was considered significant.

3. Results

3.1. Splenocyte composition in female DBA/2 mice across the age spectrum

Flow cytometric analysis demonstrated that the spleen is primarily made up of $CD19^+$ cells, $CD3^+$, and $CD11b^+$ cells (52–60, 22 and 15%, respectively); pDCs (≈0.5%) and mDCs (≈1.1%) comprise a small fraction while other cell types including NK cells, neutrophils, and monocytes (≈8%) comprise the rest of the spleen (Fig. 1). The percentage of $CD19^+$ splenocytes (B cells) trended upward with advancing age ($p<0.05$), and a significant difference was noted between 2 and 18 months old mice ($p<0.05$). As expected for a peripheral organ, the state of B cell maturity within the spleen did not change across the age spectrum (Data not shown).

3.2. T cell subsets within the spleen across the age spectrum

We examined the different T cell subsets within $CD3^+$ splenocytes; roughly 15% are $\gamma\delta^+$, 60% are $CD4^+$, and 22% are $CD8^+$ (Fig. 2A). There was an overall trend toward a lower percentage of $\gamma\delta^+$T cells in the spleen of DBA/2 mice with increasing age ($p=0.03$), and a significant difference between 2 and 18 months-old mice ($p<0.02$).

Long recognized changes in immune cell populations with advanced age include an increase in the proportion of memory T cells, with a corresponding decrease in naïve T cells (Miller, 1996; Castle, 2000). This pattern was confirmed and extended in this study to include splenocytes of DBA/2 mice (Fig. 2B). The proportion of memory $CD4^+$T cells increases from 46.0±

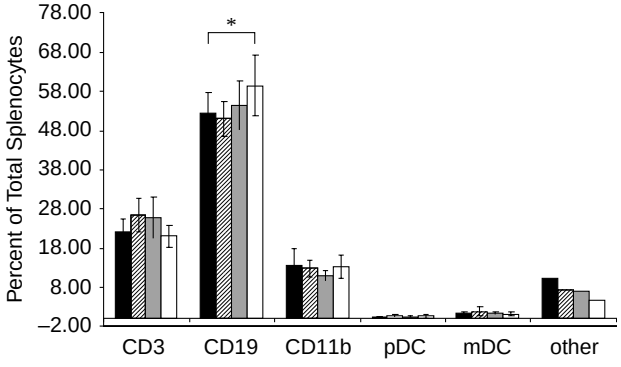

Fig. 1. Age-related changes in cell composition of the spleen in DBA/2 mice across the age spectrum. The percentage of T cells (CD3), B cells (CD19), macrophages (CD11b), pDCs ($CD11c^+/B220^+/CD11b^-$), and mDCs ($CD11c^+/B220^-/CD11b^+$) of total splenocytes was determined in 2 months (black bars), 6 months (grid bars), 12 months (gray bars), and 18 months (white bars) -old mice by flow cytometry. Data are expressed as mean±standard deviation. Overall trend of increasing percentage of B cells with increasing age $p<0.05$. * $p<0.05$. $n=4$–6 animals per group.