

AGING AND ANTIMICROBIAL IMMUNITY
Impaired Production of Mediator T Cells as a Basis for the
Decreased Resistance of Senescent Mice to Listeriosis*

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There is evidence that aging of mammals is accompanied by a profound decrease in an individual's capacity to resist infections caused by microbial pathogens (1-6). It is generally inferred that decreased resistance in aged mammals is a reflection of their waning immunocompetence. This notion derives support from the findings that are primarily concerned with age-related changes in the antibody response to sheep erythrocytes and in the ability of lymphocytes from aged donors to proliferate in vitro in response to stimulation with polyclonal mitogens (4-6). To date, however, very few studies have attempted to analyze the immunological basis for age-associated changes in resistance to infectious agents (7, 8).

We are using infection of mice with *Listeria monocytogenes* as a model to investigate the effect of aging on antimicrobial immunity. This model offers two advantages: first, murine listeriosis is an acute infection that runs its course for <2 wk. This makes it unlikely that the immunological status of the host will change because of aging during the course of infection. Second, the immunobiology of murine listeriosis has been extensively studied in the past in several laboratories (9-14). This has resulted in the knowledge that acquired immunity to *Listeria* infection ultimately depends for its expression on the generation of an activated macrophage system with increased microbicidal capacity. However, the activation of macrophages is mediated by an acquired population of specifically sensitized, thymus-derived lymphocytes (T cells), some of the properties of which have already been determined (11-14). Thus, murine listeriosis provides opportunity to study the effect of aging on functional activity of two major classes of protective host cells.

The results presented in this paper show that the response of aged mice to a sublethal immunizing infection with *Listeria* is characterized by a smaller than normal increase in spleen weight, spleen cellularity, and the number of splenic T cells. It will also be shown that aged mice responding to listeriosis generate greatly reduced numbers of protective T cells in their spleens at the time of a peak response. The impaired ability of aged mice to produce protective T cells appears to be one of the important contributing factors for the diminished capacity of these mice to resist listeriosis.

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Materials and Methods

Mice. AB6F₁ (A/Tru × C57BL/6) mice of either sex were used. This is a long-lived hybrid with a median life expectancy of about 28 mo (15). All mice were bred and aged under specific pathogen-free conditions in the Trudeau Institute Animal Breeding Facility. Young mice used were 8–12 wk old, whereas senescent mice were used at the age of 24–28 mo. There were at least five mice in each group.

Bacteria. *L. monocytogenes* strain EGD, was used. A large log-phase culture (2×10^8 bacteria/ml) was grown in Trypticase soy broth and frozen at -70°C in 1-ml portions. For each experiment, a vial was thawed and diluted in saline to contain required numbers of bacteria in a volume of 0.2 ml for intravenous infusion. *Listeria* was enumerated by homogenizing spleens and livers in saline and plating 10-fold serial dilutions of whole organ homogenates on Trypticase soy agar.

Adoptive Transfer of Anti-Listeria Immunity. Spleen cell suspensions from normal and *Listeria*-infected donors were prepared as described previously (16). Cells were washed, counted, and resuspended at a desired density. Normal young (8- to 12-wk-old) recipients were infused with immune or normal spleen cells and challenged 1 h later with 5×10^4 *Listeria* intravenously. The organisms were enumerated at stated times in the organs of recipient mice. Protection was expressed as the \log_{10} difference between the number of *Listeria* in the spleens of recipients of immune spleen cells and normal control 48 h after the challenge infection.

Immunofluorescence. The number of immunoglobulin (Ig)⁺ and Thy-1⁺ lymphocytes in the spleens were examined by immunofluorescence. There were at least five spleens in each group. Spleen cells were washed three times and resuspended at 5×10^7 cells/ml in RPMI 1640 containing 2% fetal calf serum (RPMI-FCS).¹ 0.1 ml of cell suspension was added to 1 ml of diluted antibody solution (see below) and allowed to stand on ice for 30 min. This was followed by three washes with RPMI-FCS, the final pellet was resuspended on 0.25 ml RPMI-FCS, and examined with a Zeiss fluorescent microscope (Carl Zeiss, Inc., New York). A total of 300 cells per cell suspension were counted to determine the percent fluorescent cells.

Ig-bearing cells were assayed by direct immunofluorescence using fluorescein thiocyanate-conjugated (FTC) IgG fraction of goat anti-mouse IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) at dilutions of 1:10, 1:20, and 1:40. The antiserum was supplied with immunoelectrophoretic data that attested its specificity for mouse IgG. Thy-1⁺ were assessed by an indirect immunofluorescence. Rabbit anti-mouse brain-associated theta antiserum (MBATA) prepared according to published method (17) was a gift from Dr. George Spitalny of this Institute. The serum was absorbed extensively with mouse erythrocytes, liver tissue, and spleen cells from athymic nude mice and was used at dilutions 1:40, 1:60, and 1:80. The cells were stained with FTC goat anti-rabbit Ig (N. L. Cappel Laboratories, Inc.).

The specificities of above reagents were established by the findings that FTC rabbit anti-mouse Ig did not stain AB6F₁ thymocytes, and MBATA stained >95% thymocytes, but <1% of spleen cells from athymic nude mice.

T Cell Enrichment. A positive cell selection method of panning (18) was used. Falcon 150 × 25-mm plastic bacteriological petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) were coated with rabbit anti-mouse Ig (N. L. Cappel Laboratories, Inc.). 20 ml of 0.05 M Tris, pH 9.5, containing 2 μg/ml of antibody was evenly poured on the plates. After 45 min at room temperature, the buffer was decanted, and the dishes were washed three times with RPMI 1640 and once with RPMI-FCS. 20 ml of spleen cell suspension at a density of 10^7 cells/ml was poured on each antibody-coated dish. The plates were allowed to stand on a level surface at 4°C for 1 h. The nonadherent cells were collected gently, washed twice, and resuspended at a desired concentration for functional testing. T cell enrichment was ascertained by immunofluorescence in which it was found that >95% of T-enriched cells were stained with MBATA, whereas <5% of cells were stained with FTC anti-mouse Ig.

Results

Evidence for an Impaired Ability of Aged Mice to Acquire Anti-Microbial Immunity. In a previous publication (1), it was shown that differences in the growth kinetics of *Listeria*

¹ Abbreviations used in this paper: FCS, fetal calf serum; FTC, fluorescein thiocyanate-conjugated; MBATA, rabbit anti-mouse brain associated theta antiserum.

in the organs of mice of different ages were dependent upon the size of intravenous inoculum. In confirmation of this, it can be seen in Fig. 1 that upon intravenous infection with a small (2×10^3) dose of *Listeria*, both young and old mice similarly inactivated completely the bacterial load in their spleens and livers within 8 d of infection. On the other hand, even though bacterial growth in the spleens and livers of young and old mice inoculated intravenously with a large (5×10^4) dose of *Listeria* was identical over the first 4 d of infection, larger numbers of *Listeria* were present in the organs of old mice at days 6, 8, and 11 than were present in the organs of young mice. These results were interpreted to be the reflection of diminished ability of old mice to acquire anti-bacterial immunity.

It was of interest to determine whether differences also existed in the capacity of

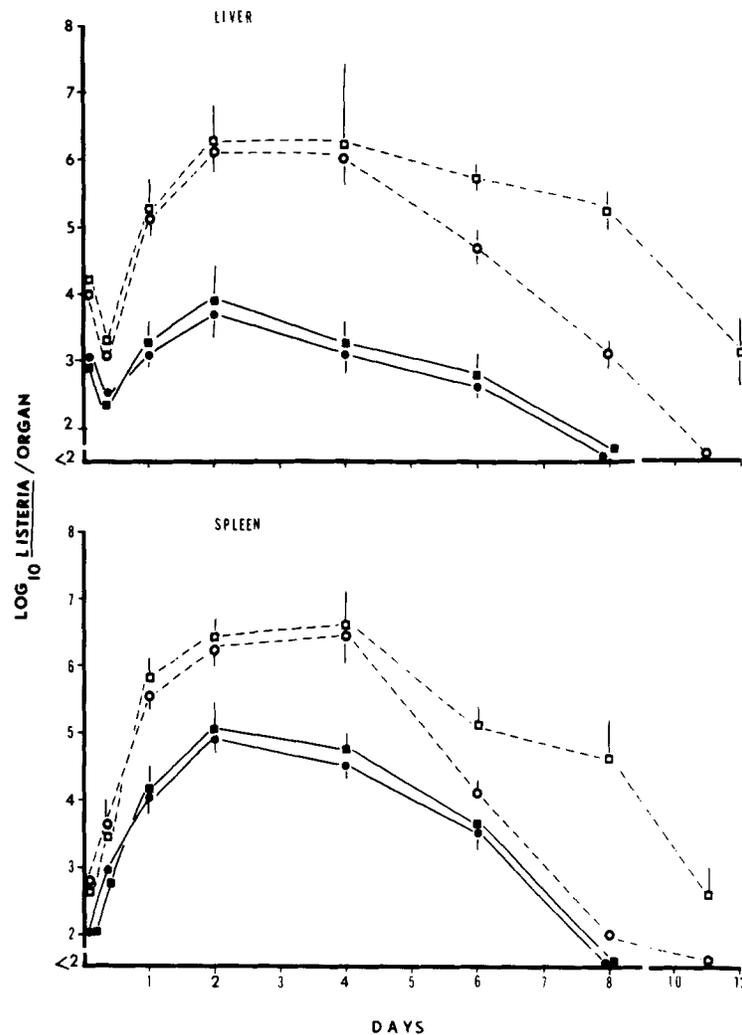


FIG. 1. Bacterial growth kinetics in the spleens and livers of young and senescent mice intravenously infected with either 2×10^3 (●, young mice; ■, old mice) or 5×10^4 (○, young mice; □, old mice) *Listeria*.

young and old mice to resist a homologous secondary challenge infection. This was examined by comparing the growth of an intravenous challenge infection of 5×10^4 *Listeria* in the organs of young and old day-6 *Listeria*-immune mice with the growth in the spleens and livers of age- and sex-matched control mice. This inoculum of *Listeria* grew log linearly in the organs of control mice of either age group (Fig. 2). Even so, day 6 immune mice of both age groups contained larger numbers of *Listeria* in their organs initially (because of the residual number of bacteria present in these organs from the primary infection, see Fig. 1); these mice displayed a high level of immunity to a challenge infection. It is notable that young mice displayed a more rapid and eventually a larger level of specific immunity than old mice.

Evidence for an Impaired Production of Anti-Listeria-protective Cells in Aged Mice. It is well documented (11-13, 19-21) that specific anti-*Listeria* immunity is exclusively mediated by specifically sensitized T lymphocytes that are found in maximum numbers in the spleen of mice at day 6 of an immunizing infection. It was reasonable to propose, therefore, that the impaired ability of aged mice to resist a secondary infection could be a result of the generation of a smaller number of mediator T lymphocytes. This possibility was investigated by comparing the ability of 2×10^8 spleen cells from young and old mice at day 6 of an immunizing infection to immunize adoptively normal recipients. Fig. 3 shows that *Listeria* grew unrestrictedly in a log linear fashion in the organs of control mice and mice that were infused with normal cells from young or old donors. In contrast, mice infused with day 6 immune cells displayed a high level of immunity by limiting the growth of challenge inoculum. It is apparent that the protection transferred with immune cells from young donors was of a larger magnitude than that transferred with immune cells from aged donors.

Comparison of the Kinetics of the Production of Anti-Listeria Mediator Lymphocytes in Young and Old Mice, and Its Relationship with Spleen Cellularity and Spleen Weight. Fig. 4 shows the time-course of the production of protective T cells in the spleens of young and old mice given an intravenous immunizing inoculum of *Listeria*. This was measured by determining the capacity of 10^8 spleen cells taken at progressive stages of infection, to

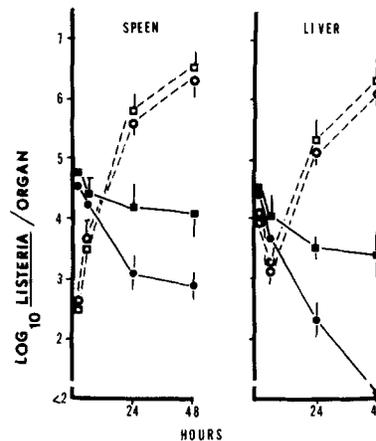


FIG. 2. Evidence that specific immunity acquired by aged mice in response to a sublethal infection with *Listeria* is of smaller magnitude than that acquired by young mice. Shown are the kinetics of growth of a standard *Listeria* challenge in the organs of normal (○, young; □, old) and day 6 (●, young; ■, old) *Listeria*-immune mice of two age groups.

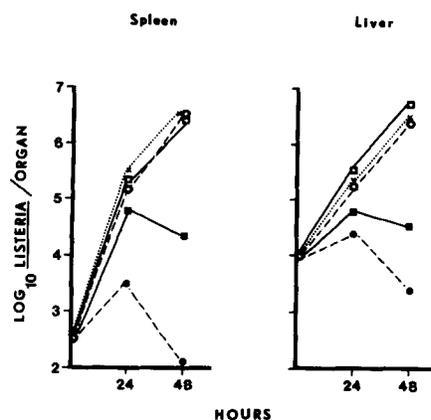


FIG. 3. Growth curves showing the inhibition of a *Listeria* challenge in the organs of young mice infused with 2×10^8 normal (open symbols) or day 6 *Listeria*-immune (closed symbols) spleen cells from young and senescent donors. X, no cells; circles, young donors; squares, old donors.

protect normal recipients against a standard (5×10^4) *Listeria* challenge. The production of mediator cells is shown in relation to changes in total spleen cellularity and spleen weight. Protective T cells were first produced in the spleen of immunized mice between days 2 and 4 of infection. They rapidly increased in numbers to peak on day 6, and started to decline thereafter (top panel). However, at the time of peak response (day 6), immune spleen cells from young donors conferred about 10,000-fold more protection than equivalent number of spleen cells from old donors. The production of protective T cells was obviously associated with a striking increase in spleen cellularity and spleen weight (lower panel). Once again, there were marked differences in the increase in cellularity and spleen weight between young and old mice. For example, the 2.4-fold increase in spleen cellularity and spleen weight seen in young mice at day 6 was much greater than the 1.3-fold increase in old mice.

Comparison of Cellular Composition of Spleen Associated with Peak Production of Listeria-committed Lymphocytes in Young and Old Mice. A comparison between the cellular composition of the spleens of normal mice and those of day 6-infected young and old mice is shown in Table I. The results presented are those obtained with a 1:20 dilution of FTC-goat anti-mouse IgG and a 1:40 dilution of MBATA. No differences in the percentage Ig⁺ and Thy-1⁺ staining cells were seen between young and old control mice. In fact, because of a higher spleen cellularity, spleens from aged control mice contained slightly high numbers of Ig⁺ and Thy-1⁺ staining cells than young mice. An infection-induced increase in cellularity was once again evidenced both by an increase in a number of nucleated cells. In confirmation of the results described above, young mice showed much higher increases in these activities. The increase in spleen cellularity was mainly found to be associated with an increase in the percent Ig⁺ staining cells. On the other hand, the percentage of Thy-1⁺ staining cells decreased by ~10%. However, a comparison of net changes in the actual cell numbers of Ig⁺ and Thy-1⁺ revealed that day 6-infected spleens of young mice contained ~3.5 and 2 times more, respectively, of these cells than age- and sex-matched normal controls. On the other hand, day 6-infected spleens from old mice showed about twofold increase in Ig⁺ staining cells and virtually no change in the numbers of Thy-1⁺ staining cells.

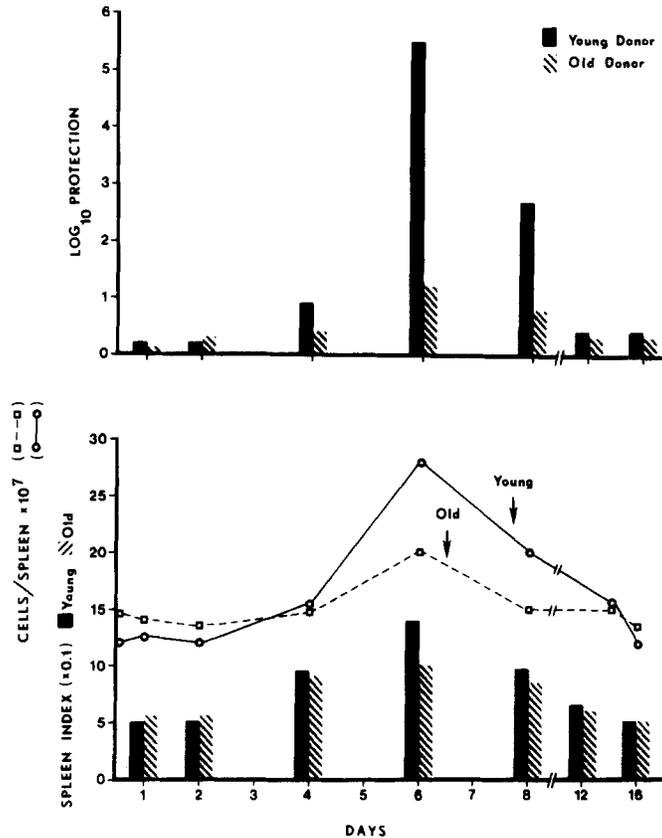


FIG. 4. Parameters of response to an intravenous immunizing infection of young and aged mice with *Listeria*. Shown are (a) production of cellular mediators of immunity in the spleens at progressive times of infection (top panel) in relation to (b) changes in the spleen cellularity and splenic index (lower panel). The relative number of mediator cells was determined by measuring the capacity (log protection) of 10^8 spleen cells from young or aged donors at the times indicated to adoptively immunize normal young recipients against a standard challenge infection. Spleen index = spleen weight in grams/body weight in grams $\times 100$.

Evidence that T Cell Enrichment Failed to Boost the Protective Capacity of Spleen Cells from Old Donors. The above results revealed that day 6 immune spleen cells from old donors contained about half the numbers of Thy-1⁺ staining cells as compared with day 6 immune spleen cells from young donors. Because the cells that mediate anti-*Listeria* immunity are T cells (11–13, 20), it could be envisaged that a comparison of protective capacity of mediator cells from young and old donors could be valid only if the numbers of Thy-1⁺ staining cells in both cell populations were similar. A comparison of protective capacity of day 6-immune spleen cells from young and old donors was made after T cell enrichment. As can be seen in Fig. 5, T enrichment of spleen cells resulted in an approximately twofold increase in their protective capacity in that 5×10^7 enriched spleen cells transferred about the same level of protection as 10^8 unfractionated spleen cells. This was true for both the age groups. However, T-enriched cells and unfractionated spleen cells from old immune donors transferred substantially lower levels of protection than equivalent numbers of cells from the young immune donors.

TABLE I
Comparison of Cellular Composition of the Spleen Associated with Peak Production of *Listeria*-committed Lymphocytes in Young and Old Mice

Group	Spleen index*	Cells/spleen $\times 10^6$	Percent cells		Number of cells ($\times 10^6$) bearing	
			Ig ⁺	Thy-1.2 ⁺	Ig	Thy-1.2
Normal						
Young	0.50 \pm 0.03	115 \pm 8	44 \pm 3	44 \pm 3	51 \pm 3	51 \pm 6
Old	0.48 \pm 0.03	150 \pm 9	41 \pm 8	43 \pm 9	62 \pm 12	65 \pm 14
Infected mice‡						
Young	1.70 \pm 0.08	320 \pm 32	54 \pm 4	35 \pm 6	173 \pm 13	112 \pm 19
Old	0.99 \pm 0.12	210 \pm 40	56 \pm 12	33 \pm 12	118 \pm 25	69 \pm 25

All results expressed as mean value \pm SD.

* Spleen index = spleen weight in grams/body weight in grams \times 100.

‡ Given 2×10^8 *Listeria* intravenously 6 d earlier.

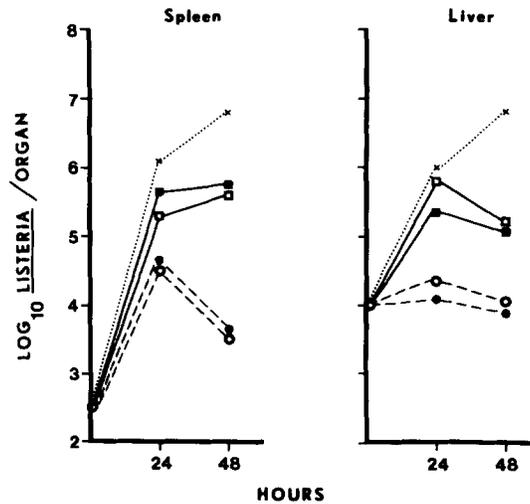


FIG. 5. Growth curves showing the inhibition of a *Listeria* challenge in the organs of young mice protected with unfractionated or T cell-enriched spleen cells from day 6 *Listeria*-immune young or aged donors. X, no cells; □, old donor 10^8 unfractionated cells; ■, old donor 5×10^7 T-enriched cells; ○, young donor 10^8 unfractionated cells; ●, young donor 5×10^7 T-enriched cells.

Discussion

Normal immune functions in mammals decline with advancing age. However, the onset, magnitude, and rate of decline vary with the species and the type of immune response (22, 23). The decrease in immunological vigor associated with senescence is frequently accompanied by an increased incidence of infections, autoimmune diseases, and cancer (23, 24), as is the case in immunodeficient newborns and immunosuppressed adults (25, 27). Whether the immunological dysfunction is the result or the cause of physiological aging, however, has yet to be determined.

Results presented here show that mice, regardless of their age, were capable of acquiring anti-bacterial immunity against a small intravenous inoculum of *Listeria*. Mice of all age groups inoculated with a smaller *Listeria* dose completely inactivated

the bacterial load in their organs within 8–10 d. It is known that complete sterilization of tissues is dependent upon acquired T cell-dependent immunity (12, 20, 21), because the absence of T cells results in chronic and eventually fatal infection (28). Nevertheless, there were age-dependent differences in the capacity of mice to mount an immune response. The acquired immunity in the aged mice easily became overwhelmed upon infection with a larger dose of *Listeria*. This conclusion was reinforced by the demonstration that old mice possessed a much smaller capacity than young mice to resist a secondary challenge infection given at the time of peak immunity.

It is well established (12, 21) that cellular mediators of anti-*Listeria* immunity belong to an enlarged population of rapidly dividing T cells. These T cells are first produced in the spleens between days 2 and 4 of infection and reach peak production on day 6. The increased production of cellular mediators is coincidental with major increases in cell division, splenic T and B cell content, and spleen weight. Their production progressively decreases after 6 d of infection, presumably because of rapid elimination of infecting organisms from the tissues and consequent decline in the intensity of antigenic stimulus (21, 29). A measure of kinetics of protective T cell production revealed no significant differences between young and old mice. However, at the peak of immunity, spleen cells from young donors were capable of transferring at least 1,000-fold more protection than equivalent number of spleen cells from the old donors, even when enriched T cell populations were used to compensate for the fact that aged mice contained fewer T cells. The reduced capacity of mediator T cells from the aged mice to transfer protection was associated with markedly smaller increases in spleen cellularity, splenic T cell content, and spleen weight. This clearly implies that old mice produced fewer protective T cells. It should be emphasized that the interpretation of results obtained with adoptive immunization rests upon the assumption that the level of protection conferred upon recipient mice is directly proportional to the number of protective cells infused in the recipients. Even though the present technology does not allow the precise quantitation of protective cells present in any lymphoid cell populations, there is enough published evidence to favor such an assumption (30).

The decline in the capacity of aged mice to generate protective T cells in response to specific stimulus is clearly not the result of an age-related decline in T cell number, but appears to be caused instead by some intrinsic defect(s) in the ability of T cells to respond to antigenic stimulus. Such defects could result in a diminished entry of antigen-committed precursor cells in the differentiation process to become protective T cells and/or decrease in the proliferation rate for the aged cells after their differentiation. Using the bromodeoxyuridine incorporation system for the analysis of cellular kinetics of lymphocytes stimulated with polyclonal mitogen, it has been shown that lymphocytes from aged individuals enter the stimulated pool at approximately half the rate of that of lymphocytes from young individuals. Furthermore, lymphocytes from the aged individuals that enter the stimulated pool show a significantly longer cell cycle duration (31, 33). An age-related increase in cell generation time is also well documented for a number of *in vivo* and *in vitro* nonlymphocyte cellular systems (34–38), and appears to be a general feature of cellular aging.

The data presented here does not provide any direct evidence for either a decreased entry of T cells into a protective cells pool, or for their subsequent reduced proliferative

capacity. However, the fact that anti-*Listeria* protective cells belong to an enlarged population of actively dividing T cells coupled with the finding that aged mice displayed a much smaller increase in spleen cellularity, (most of which could be accounted for by increase in B cell content) at the time of peak immunity is certainly consistent with a decreased proliferative T cell response in these mice. Experiments are in progress to determine if this is indeed the case.

Summary

Immunity to infection of mice with the facultative, intracellular pathogen *Listeria monocytogenes* was employed as a model system to investigate the immunological basis for the age-associated decline in anti-microbial immunity. In response to a sublethal immunizing infection, aged (24-mo old or more) mice displayed a smaller increase in spleen weight, spleen cellularity, and splenic T cell content than young (3- to 4-mo-old) mice. Aged mice also generated a smaller number of anti-*Listeria* protective T cells at the time of a peak response, in that their spleen cells were 1,000-fold less protective than equivalent numbers of spleen cells from the young donors, even when enriched T cell populations were employed. These results suggest that the impaired ability of aged mice to produce protective T cells is mainly responsible for decreased resistance of these mice to infection with *Listeria*.

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