LOSS OF SUPPRESSOR T CELLS IN ADULT NZB/NZW MICE

BY R. S. KRAKAUER, T. A. WALDMANN, AND W. STROBER

(From the Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

The genetically determined autoimmunity in NZB/NZW F1 mice is similar to that observed in human systemic lupus erythematosus, and these mice, therefore, provide an animal model for the study of the latter disease. The immunological abnormalities of NZB and the hybrid NZB/NZW mice include defects in the induction and maintenance of tolerance to foreign host antigens (1-3), in the development of antibodies with specificity for nucleic acids (4, 5), erythrocytes (5, 6), and lymphoid cells (7, 8), in the development of immune complex glomerulonephritis (5), and Coombs' positive hemolytic anemia (5, 6). Current notions as to the pathogenesis of autoimmunity in NZB/NZW mice center on the possibility that these animals lose suppressor cell activity as they age, thereby allowing the development of forbidden clones with self antigen specificity (3, 5, 6, 9, 10). The data supporting these concepts are, as yet, indirect. In brief, they indicate that various circulating and cellular immune responses are increased in older NZB/NZW mice and that this enhancement is reduced by the injection of thymus-derived cells of young NZB/NZW mice (11-14). The possibility remains that this evidence in favor of loss of T-cell suppressors could also be explained by assuming that NZB/NZW animals acquire progressively increasing helper T-cell function as they age which can be diluted by the addition of young NZB/NZW T cells to the system under study. Alternatively, the B cells from the NZB/NZW animals might be unresponsive to the negative signals produced by suppressor T cells. Finally, the view that there is a reduction in suppressor cells in NZB/NZW mice has recently been challenged by Roder et al. (15) who have reported that the number of suppressor cells in the spleens of NZB/NZW mice increase rather than decrease as the animals age.

To approach the question of T-cell suppressor function in NZB/NZW mice more directly, we have taken advantage of the observations of Dutton (16, 17) and Rich and Pierce (18, 19) that splenic T cells can be activated by Concanavalin A (Con A) to become cells which suppress the immunological functions of other cells. Furthermore, Rich and Pierce (20, 21) have shown that Con A-activated cells produce a soluble immune response suppressor (SIRS) which is suppressive to a range of immune responses in vitro. On the basis of these studies it becomes possible to measure T-cell suppressor function by studying the ability of Con A-activated cells and their supernate (SIRS), derived from a variety of sources, to inhibit defined immune responses in vitro. In the present

1 Abbreviations used in this paper: Con A, concanavalin A; PWM, pokeweed mitogen; SIRS, soluble immune response repressor.
investigation, we have utilized this system to ask two questions concerning NZB/NZW mice: (a) whether these animals have normal numbers of precursors of suppressor T cells that can be activated to become suppressors by Con A, and (b) whether the B cells from these animals can respond to negative suppressor signals from Con A-activated normal cells.

Materials and Methods

Animals. Female NZB/NZW (NZB female × NZW male matings), BALB/c, and C57BL/6 mice were obtained from colonies maintained at the National Institutes of Health, Bethesda, Maryland.

In Vitro Biosynthesis of IgM. To study the terminal differentiation of splenic B cells into IgM-producing plasma cells, exhaustively washed cells were cultured in vitro in the presence of the B-cell mitogen, pokeweed mitogen (PWM). The IgM produced and secreted by such cells during 7 days of culture was then measured by a sensitive radioimmunoassay technique specific for IgM. In this technique, splenocytes were aseptically obtained from the appropriate animals, washed four times by centrifugation through fetal calf serum, and cultured in the presence of PWM at a concentration of 2 × 10^6 splenocytes/ml; the culture was carried out at 37°C in 5% CO_2 in loosely capped culture plates containing RPMI 1640 medium (Media Unit, National Institutes of Health, Bethesda, Md.) supplemented with glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (total culture volume: 1.1 ml). PWM (Grand Island Biological Co., Grand Island, N. Y.) was used at a concentration of 10 μg/ml. At the termination of the culture, the tubes were centrifuged and the amount of IgM synthesized and secreted into the culture medium was determined by double antibody radioimmunoassay of IgM. Techniques for producing the antisera and defining the specificity and accuracy of the radioimmunoassay test were essentially identical to those previously described for a double antibody radioimmunoassay of IgE (22). In these assays, rabbits immunized with purified IgM derived from the serum of mice bearing the MOPC-104E tumor were the source of anti-IgM, radiolabeled mouse IgM (MOPC-104E) served as the labeled ligand, and whole mouse serum containing defined amounts of mouse IgM was used as the standard. The IgM was purified from the serum by a combination of block electrophoresis and chromatography with Sephadex G-200. Rabbits were immunized with the purified IgM, and the antisera were made specific by absorption with Sepharose bound to mouse IgG. The antiserum used was shown to be monospecific by Ouchterlony analysis and by the demonstration that it bound radiolabeled mouse IgM but not mouse IgG, IgG2, and IgA.

Preparation of Purified T- and B-Cell Populations from Mouse Spleens. Purified T cells were prepared by the use of anti-Fab Sephadex G-200 immunoabsorbents using a modification of the method of Chess et al. (23). The T-cell enriched populations obtained were in the nonadherent fraction, whereas the B-cell enriched populations were in the adherent fraction that was released from the column by the addition of mouse IgG. The T-cell populations thus obtained did not undergo proliferation in response to lipopolysaccharide but did proliferate in response to phytohemagglutinin P and Con A. On the other hand, B-cell enriched fractions were responsive to lipopolysaccharide but not to Con A; in this case the response to phytohemagglutinin P was reduced by 80%.

B-cell enriched populations were also obtained by treatment of whole spleen cell populations with mouse anti-θ (Litton Biometrics Laboratory Products, Kensington, Md.) serum and complement. Again, the resultant cells were responsive to lipopolysaccharide but not to Con A.

Production of Con A-Activated Cell Populations and SIRS. Con A-activated suppressor cells were obtained by a modification of the methods of Dutton (16, 17) and Rich and Pierce (18, 19). Specifically, mouse splenocytes at a concentration of 2 × 10^6/ml were cultured in 1-ml aliquots in the presence of tritiated 6 × crystallized Con A, 2 μg/ml (Elscint Inc., Palisades Park, N. J.) for 24 h. At the end of this period the cells were washed three times in balanced salt solution to remove the Con A. From the amount of remaining tritium counts in the washed cell suspension, it was determined that 98.5% of all Con A was washed out by this procedure. In separate studies it was found that this amount of residual Con A was not mitogenic and could not suppress IgM biosynthesis.

SIRS was produced by taking the supernate of the cells cultured for 24 h in 2% fetal calf serum in the presence of 2 μg/ml of tritiated Con A and extensively absorbing it with Sephadex G-75.
Again, Con A removal was estimated from the amount of tritium counts remaining in the supernate after absorption, and again it was found that 98.5% of all Con A had been removed by the absorption procedure. Here too it was shown that residual Con A in the supernate derived from activated cells is not mitogenic and is incapable of producing suppression of IgM biosynthesis in vitro.

Results

PWM-Induced IgM Biosynthesis by Spleen Cells. Spleen cells from normal BALB/c mice cultured in the presence of PWM synthesized 860±120 ng of IgM/2 x 10^6 cells in culture during a 7-day period. Similar synthetic values were obtained from cells derived from C57BL/6 and from NZB/NZW mice of various ages (Fig. 1). The synthesis of IgM by spleen cells of adult NZB/NZW mice (18 wk) was slightly greater (P = 0.5) than that of young NZB/NZW mice (4 wk) (1,050±140 ng vs. 770±130 ng/2 x 10^6 cells). An analysis of the time course of synthesis and release of immunoglobulin molecules by spleen cells in the presence of PWM indicated there was little synthesis and release of immunoglobulin during the first 3 or 4 days of culture. However, after this time the concentration of IgM in the culture medium increased rapidly with most of the synthesis occurring on the 6th and 7th days of culture. The IgM synthetic process could be at least 90% inhibited by irradiation of the cells with 2,000 R or by the addition of puromycin (5 x 10^-4 M), actinomycin D (20 µg/ml), or mitomycin C (25 µg/ml) to the culture medium. In addition, relatively little IgM was produced when the cells were cultured in the absence of PWM (mean: 120±80 ng/2 x 10^6 cells in culture). These studies indicate that the IgM measured in the supernatant fluid is the result of the differentiation of B cells into Ig synthesizing and secreting plasma cells rather than the result of release of IgM from pre-existing plasma cells in the spleen cell population.

Effect of Con A on PWM-Induced IgM Biosynthesis by Mouse Splenocytes. The addition of 2 µg/ml of Con A to BALB/c splenocyte cultures with PWM caused a 94% suppression of IgM synthesis by these cultures (Fig. 1). The addition of Con A caused a comparable suppression of PWM-induced IgM synthesis by C57BL/6 spleen cells (95% inhibition) and by splenocytes (85% inhibition) derived from young (4 wk) NZB/NZW mice. In contrast, Con A at this concentration caused significantly less (P < 0.001) suppression (27%) of IgM synthesis by adult (18 wk) NZB/NZW mice. This result suggested that cultures from adult BALB/c, young and adult C57BL/6, and young NZB/NZW mice contain cells which can be activated by Con A to become suppressor cells and that such precursors of suppressor cells are markedly diminished in adult NZB/NZW mice.

Suppressor Effect of Con A-Pulsed Spleen Cells on PWM-Stimulated IgM Biosynthesis. To further investigate the suppressor T-cell activity of adult NZB/NZW mice and of normal mice, the cells from the spleens of various strains of mice were pulsed with Con A for 24 h and then washed to remove the Con A. The resultant Con A-pulsed cells were then co-cultured with equal numbers of previously untreated spleen cells from mice in the presence of PWM, and the IgM synthesis of the resultant co-cultures was measured. As shown in Fig. 2 and Table I, Con A-pulsed splenocytes derived from BALB/c or C57BL/6 mice led to an 83–88% suppression of the PWM-induced IgM synthesis of co-cultured BALB/
c or C57BL/6 splenocytes. In connection with these studies, a series of control experiments were performed to exclude the possibility that sufficient Con A was carried over with pulsed cells to inhibit the PWM-stimulated target cells. It was shown that splenocytes mixed with Con A and washed immediately did not significantly suppress the PWM-induced IgM synthesis of co-cultured splenocytes ($P > 0.3$). Similarly, when Con A was added directly to PWM-stimulated splenocytes at a concentration calculated to be present in the washed Con A-pulsed cells, there was no significant suppression of IgM biosynthesis ($P > 0.4$).

The concentration of residual Con A after washing was determined in all cases using tritiated Con A as indicated in the Materials and Methods section. The Con A-induced suppressor cells were shown to be T cells in accord with the previous observations of Dutton (16, 17) and Rich and Pierce (18, 19). It was
found that anti-θ treatment of the spleen cells before exposure to Con A completely abrogated the suppressor activity of the BALB/c cells (Fig. 3). In another approach, spleen cell populations were fractionated by anti-Fab Sephadex G-200 columns, and the resultant T- and B-cell fractions were then pulsed with Con A and tested for suppressor activity in co-culture studies. It was found that the T-cell fraction and not the B-cell fraction was suppressive.

Loss of Suppressor Capacity by Con A-Pulsed Spleen Cells with Age in NZB/NZW Mice. The ability of spleen cells from NZB/NZW animals to be activated to become suppressor cells after pulsing with Con A for 24 h was then examined. The Con A-pulsed spleen cells from young (4 wk) NZB/NZW mice caused a level of suppression of IgM synthesis by spleen cells from a variety of sources that was comparable to that seen when Con A-pulsed spleen cells were obtained from BALB/c or C57BL/6 strains (Fig. 2, Table I). In contrast, Con A-pulsed spleen cells from adult (18 wk) NZB/NZW mice lacked significant suppressor activity ($P = 0.25$). We conclude that NZB/NZW mice lose suppressor cell activity as they age. This age-dependent loss of Con A suppressor T-cell activity is not a normal age-dependent phenomenon as demonstrated by the fact that spleno-
cytes derived from both young and adult C57BL/6 mice could be induced by incubation with Con A to form suppressor cell populations (Fig. 2, Table I). It should be noted that even though adult NZB/NZW mice splenocytes cannot be induced to form suppressor cells by incubation with Con A, such NZB/NZW splenocytes have proliferative responses induced by this dose of mitogen (2 μg/ml) that were not significantly different from the normal (P = 0.2).

The Production of SIRS by Con A-Activated Spleen Cells from Normal Mice Strains and from Young and Old NZB/NZW Mice. Supernatant fluids from Con A-activated spleen cells have previously been shown to be potent suppressors of antibody synthesis (20). We therefore investigated the ability of spleen cells from normal mice and from NZB/NZW mice to produce SIRS for IgM biosynthesis following Con A stimulation. Con A was removed from the supernatant fluid by absorption with Sephadex G-75. When 5 μl of the supernatant fluids from Con A-activated spleen cells of BALB/c or C57BL/6 mice was added to 1-ml cultures of spleen cells of a variety of sources, IgM synthesis was inhibited by 79–81% in all cultures tested (Fig. 4, Table II). This amount of supernatant fluid (5 μl) from Con A-activated spleen cells of young NZB/NZW mice added to PWM-stimulated cultures was similarly inhibitory with a suppression of 72±16%. In contrast, the supernatant fluids derived from Con A-pulsed adult NZB/NZW spleen cells failed to significantly inhibit IgM synthesis by cultures of autologous spleen cells (inhibition 14±20%, P > 0.3). We conclude that the adult NZB/NZW mice spleen produced less active SIRS than normal spleen cells or the spleen cells of young NZB/NZW mice. Thus, NZB/NZW mice appear to lose precursors of Con A-activatable suppressor T cells and the ability to produce the T-cell suppressor humoral product (SIRS) as they age.

Ability of Spleen Cells from NZB/NZW Mice to Respond to Suppressor Signals. The ability of PWM-simulated spleen cells from young and adult
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**Figure 4** Percentage suppression (reading down) of PWM-stimulated IgM synthesis of BALB/c responders with SIRS from Con A-stimulated splenocytes of the strains listed above (final SIRS concentration 1:200).

**Table II**

Suppression of IgM Synthesis of Various Responding Cells Induced by SIRS from Various Murine Sources (Final SIRS Concentration = 1:200)

| Sources of sirs  | BALB/c | Young NZB/NZW | Adult NZB/NZW |
|------------------|--------|----------------|----------------|
| BALB/c           | 81 ± 19| 84 ± 18        | 80 ± 14        |
| C57BL/6          | 79 ± 16| -              | -              |
| Young NZB/NZW    | 72 ± 16| 74 ± 22        | 72 ± 20        |
| Adult NZB/NZW    | 14 ± 20| 14 ± 20        | 8 ± 24         |

NZB/NZW mice to be inhibited by suppressor signals was then investigated. It was demonstrated (Fig. 5, Tables I and II) that both young and adult NZB/NZW spleen cells stimulated with PWM could be suppressed by Con A-activated spleen cells from BALB/c, C57BL/6, or young NZB/NZW animals. The IgM production of PWM-stimulated spleen cells from the young and adult NZB/NZW mice could also be suppressed by supernates of Con A-activated spleen cells (SIRS) from normal strains or from young NZB/NZW mice (Fig. 4). Thus, although adult NZB/NZW mice lose suppressor T-cell activity as they age, these cells are still responsive to the normal regulatory suppressor signals provided by Con A-activated T cells or their products.

**Discussion**

The concept that autoimmunity in NZB/NZW mice is related to loss of suppressor T-cell activity has been supported in the past by a variety of data that bear indirectly on the question of suppressor T-cell function. In the most compelling studies, both humoral and cellular immune responses were shown to increase in NZB/NZW animals when responses at 4 wk of age were compared
with the responses at 10–26 wk of age (5, 12, 14, 24). Thus, NZB/NZW antibody responses to the thymic-independent antigens, pneumococcal polysaccharide and poly I-C, increased (4, 12, 14) and, in addition, the ability of NZB/NZW spleen cells to mediate graft vs. host reactions in appropriate recipients was enhanced as NZB/NZW animals aged (7, 11, 13). In both cases this increased response with age was abrogated by the injection of thymocytes obtained from the young NZB animals into the adult NZB animals under study. These data are most easily interpreted by assuming that suppressor cell activity in NZB/NZW animals declines with age and that the addition of thymocytes from young animals replenishes suppressor cell activity. However, an alternative explanation is that the enhanced responses are due to activation of helper T-cell activity with age, and the helper T cells can be competitively inhibited by the addition of young thymocytes not yet activated by the disease process.

The results obtained in the present studies provide direct evidence that suppressor cell function in NZB/NZW mice is in fact lost as such animals age. In these studies we utilize a direct test of suppressor-cell function, the ability of Con A-activated cells to mediate immunosuppression, to study suppressor cell activity in NZB/NZW mice. We found that young NZB/NZW animals have normal suppressor activity, as judged by the ability of Con A-activated spleen cells from young animals to inhibit PWM-driven IgM biosynthesis in vitro. In contrast, adult NZB/NZW mice have reduced suppressor activity, as judged by the inability of Con A-activated cells from adult animals to inhibit PWM-driven IgM biosynthesis in vitro. Parallel with the work of Dutton (16, 17) and Rich and Pierce (18, 19), the suppressor cells under study were shown in these experiments to be T cells. In addition, the suppression could be mediated by a soluble material (SIRS) present in the supernatant fluid of Con A-activated normal spleen cells. As one would expect from the data on Con A-activated cells themselves, the inhibitory potential of SIRS derived from Con A-activated spleen cells of young NZB/NZW mice was similar to that of supernates of Con A-
pulsed cells obtained from other mouse strains, whereas the inhibitory potential of SIRS obtained from adult NZB/NZW mice had markedly reduced inhibitory activity. These studies with supernatant suppressor material, therefore, confirm the fact that adult NZB/NZW mice have reduced suppressor T-cell activity. Although the adult NZB/NZW mice lose the precursors of cells that can be activated to become suppressors by Con A, they are still normally responsive to negative signals in that their PWM-driven IgM biosynthesis could be inhibited by co-culturing cells obtained from adult animals with Con A-activated T cells or with SIRS from normal animals.

The occurrence of an abnormality in suppressor T-cell function in NZB/NZW mice suggests that a similar phenomenon may be characteristic of human systemic lupus erythematosus and other human conditions associated with autoimmunity. Such a finding would be complementary to our previous observation that a subset of patients with common variable hypogammaglobulinemia demonstrate excessive T-cell activity and that at least in some instances this may be the basis of the hypogammaglobulinemia (25). The findings in hypogammaglobulinemia are based on studies parallel to those reported here in that in both instances PWM-driven immunoglobulin synthesis is studied in the presence or absence of cells whose suppressor capability is being tested. In the case of some of the patients with hypogammaglobulinemia, unstimulated peripheral T cells were found to suppress immunoglobulin production by normal cells, whereas normal peripheral T cells did not suppress immunoglobulin production. In the case of adult NZB/NZW mice, Con A-activated spleen cells were found to lack suppressor activity that is normally present. Thus, NZB/NZW mice and hypogammaglobulinemic patients represent two ends of a spectrum of T-cell regulatory defects.

A major question yet to be answered is whether the loss of suppressor T-cell activity is the primary event in the autoimmune phenomenon of NZB/NZW animals or whether it is a secondary event related to infection of these animals with viruses or to the development of natural antithymocyte antibodies. In favor of the idea that loss of suppressor T cells is the primary event is the fact that NZB/NZW mice have a hyperresponsiveness to sheep erythrocytes within the first 10 days of life (5). Since the natural antithymocyte antibody found in NZB/NZW mice is an IgM antibody (8) that is not transferred to the fetus, one would not expect to have an effect of such an antibody so early in life. In addition, the loss of suppressor cells in NZB/NZW mice occurs between 3 and 10 wk of age before significant titer of natural antithymocyte antibody appears (7). Whatever the cause of suppressor T-cell activity loss in NZB/NZW mice, it is easy to suppose that suppressor T cells usually play an important role in negative feedback of antibody responses and that loss of suppressor T cells results in autoantibody formation with the consequent development of Coombs' positive hemolytic anemia, immune complex nephritis, and other manifestations of autoimmune disease. In this sense, if loss of suppressor T cells is not the primary event in NZB/NZW autoimmunity, it is at least a proximal event in a chain of causation.

To date the suggestion that NZB/NZW mice have a reduction of negative regulators of the immune responses has led to various therapeutic attempts to
augment the capacity of these animals to produce suppressor signals. One such attempt involves the use of thymosin and is related to the observation of Dauphinee and Talal (26) that both NZB/NZW thymosin levels and the proliferative response of NZB/NZW thymocytes in allogeneic recipients decrease as the animals age. Dauphinee and Talal (26) found that thymosin does in fact correct the proliferative response of older NZB/NZW thymocytes; however, when Gershwin et al. (27) treated NZB/NZW mice with thymosin in various schedules starting at different ages, they did not observe a dramatic delay in onset of autoimmunity. In another attempt to augment suppressor T-cell activity, Gershwin and Steinberg treated NZB/NZW mice in vivo with Con A. They observed that Con A treatment given to young mice does in fact result in a more prolonged tolerance to foreign protein antigens than in untreated mice, but nevertheless the animals lose tolerance at a time when normal animals maintain tolerance (28). This relative failure of Con A treatment is in accord with the observations of the present studies wherein we show that there is progressive decline in the number of cells in the spleen of NZB/NZW animals that can be activated to become suppressor cells by Con A treatment. Finally, NZB/NZW autoimmunity has been treated by the administration of thymocytes or splenocytes from young NZB/NZW mice. Such treatment inhibited the development of Coombs' positive hemolytic anemia but was only marginally successful in preventing other autoimmune phenomena. In addition, initiation of young thymocyte treatment at 10 wk of age when the autoimmunity was becoming established rather than at 4 wk of age was ineffective (11, 13). This may be due to the relatively early development in NZB/NZW animals of cytotoxic natural antithymocyte antibodies that destroy the transferred T cells.

The demonstration in this study of a soluble suppressor substance derived from T cells by Con A stimulation (i.e., SIRS) and the observation that adult NZB/NZW mice are responsive to this negative signal raise the intriguing possibility of treating these mice and, by extension, of treating humans with systemic lupus erythematosus in a more physiological manner with this soluble suppressor protein. Indeed, in preliminary studies, the administration of SIRS to NZB/NZW animals from an early age led to the development of hypogammaglobulinemia in these animals as well as a marked delay and reduction in extent of the development of anti-DNA antibody, Coombs' positive hemolytic anemia, and immune complex nephritis.

Summary

We have investigated suppressor T-cell activity in female NZB/NZW F1 mice using PWM-driven IgM biosynthesis in vitro as an indicator system. In initial studies we observed that spleen cells from normal mice (BALB/c, C57BL/6), as well as from young (4 wk) and adult (18 wk) NZB/NZW mice, cultured in the presence of PWM synthesize 860±120 ng IgM/10^6 cells/7 days. However, when Con A (at 2 μg/ml) was added directly to the cultures (along with PWM), cells obtained from adult normal mice and young NZB/NZW mice showed a 94% suppression of IgM synthesis, whereas cells obtained from adult NZB/NZW mice were suppressed significantly less.

To analyze these findings we studied the effect of Con A-induced suppressor
cells (cells cultured with Con A for 24 h and washed free of Con A) on PWM-driven IgM biosynthesis. Spleen cells obtained from normal mice cultured in the presence of Con A-pulsed cells obtained from normal mice and young NZB/NZW mice showed an 83-88% suppression of PWM-driven IgM synthesis. Similarly, supernates obtained from Con A-pulsed cells of normal mice or of young NZB/NZW mice suppressed PWM-driven IgM synthesis. This suppression by Con A-pulsed cells and their supernates required T cells since T-cell fractions but not B-cell fractions eluted from anti-Fab Sephadex columns mediated suppression of co-cultured normal cells; in addition, Con A-pulsed cells treated with anti-0 and complement do not mediate suppression. These studies of Con A-induced suppressor cell activity in normal mice and young NZB/NZW mice contrast with studies of Con A-induced suppressor cell activity in adult NZB/NZW mice. We found that adult NZB/NZW Con A-pulsed cells and supernates obtained from such Con A-pulsed cells had vastly decreased suppressor potential; in this case the Con A-pulsed cells and supernatant fluids derived from such cells did not suppress PWM-driven IgM synthesis by normal cells. Finally, whereas spleen cells from young and adult NZB/NZW mice differ in their suppressor cell potential, cells from both sources could respond equally to suppressor signals in that Con A-pulsed normal cells or supernates derived from such cells caused equivalent suppression of PWM-driven IgM synthesis by young and adult NZB/NZW cells. These observations allow us to conclude that NZB/NZW mice lose suppressor T-cell activity as they age.

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