The Development of Autoimmunity in C57BL/6 *lpr* Mice Correlates with the Disappearance of Natural Killer Type 1–positive Cells: Evidence for Their Suppressive Action on Bone Marrow Stem Cell Proliferation, B Cell Immunoglobulin Secretion, and Autoimmune Symptoms

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Summary

F_1 hybrid mice are able to acutely reject parental marrow grafts, a phenomenon that is due to natural killer type 1–positive ($NK1^+$) cells. Circumstantial evidence had suggested that the antigenic determinants recognized by these cells are self-antigens, leading to the hypothesis that the physiological role of $NK1^+$ cells is a downregulatory or suppressive function on bone marrow stem cell proliferation and lymphocyte function. In analyzing this hypothesis it is shown here that in young mice there is a temporal correlation between appearance of $NK1^+$ cells in the spleen and the ability to reject allogeneic marrow or to suppress endogenous stem cell proliferation. The reverse situation exists in mice expressing the homozygous *lpr* gene. Whereas in young mice cells with $NK1^+$ phenotype are demonstrable, these cells disappear with age, i.e., at the time autoimmunity develops. Concomitant with the disappearance of $NK1^+$ cells, the ability to reject marrow grafts and to control endogenous stem cell proliferation also vanishes. The suggestion that the development of autoimmunity is causally related to the disappearance of $NK1^+$ cells is supported by experiments in which $NK1^+$ cells were either eliminated by antibody injection or increased by adoptively transferring cell populations enriched for $NK1^+$ cells into *lpr* mice. It is shown that removal of cells enhances autoimmunity, whereas injection of $NK1^+$ cells delays the onset of autoimmunity. In vitro assays are presented that demonstrate that suppression of autoantibody-secreting B cells is due to two $NK1^+$ cell populations, one that expresses CD3 and causes specific suppression and one that lacks CD3 and causes nonspecific suppression.

Lethally irradiated mice have the ability to acutely reject certain allogeneic and MHC-matched bone marrow grafts. In normal and thymus-deficient nude mice, the principal cell type responsible for the rejection is a cell that expresses cell surface markers characteristic for NK cells as well as the CD3 TCR complex (1–3), whereas in SCID mice cells with a similar phenotype but lacking TCRs perform this function (4). The observation that effector cells with NK phenotype cause the rejection of MHC-matched marrow transplants raises the possibility that the responsible cells recognize self-antigens. In support of this, it had been shown that cells with NK phenotype may cause suppression of endogenous marrow stem cell proliferation (5). This led to the hypothesis that the physiological role of these cells is to control marrow stem cell proliferation. To examine this hypothesis, we sought to correlate the presence of cells with NK phenotype with the ability of mice to suppress stem cell proliferation. Here we report that in young mice the appearance of $NK1^+$ cells correlates with the ability to downregulate stem cell proliferation, whereas in aging mice with the homozygous *lpr* gene, $NK1^+$ cells disappear concomitant with the loss of ability to limit stem cell proliferation. Previous observations reported by Scribner and Steinberg (6) had shown that the ability of mice expressing the *lpr* or *gld* mutations to limit the number of endogenous CFU-spleen (S)[1] declines with age. This suggested that the development of autoimmunity and ability to limit endogenous stem cell proliferation are causally related. We therefore set out to explore whether there is a correla-

[1] *Abbreviations used in this paper:* CFU-S, colony-forming unit–spleen; dDNA, denatured DNA; Sn DNA, small pieces of native DNA.

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tion between the disappearance of cells responsible for acute marrow graft rejection and development of autoimmunity. Here we show that this correlation exists and that in vivo ablation of NK1$^+$ cells leads to an earlier development of autoimmunity. In contrast, adoptive transfer of cell populations enriched for NK1$^+$ cells into *lpr* mice delays the onset of autoimmunity. It is therefore suggested that the physiological role of NK1$^+$ cells is to control both hemopoietic stem cell proliferation and B cell function.

Materials and Methods

Animals and In Vivo Procedures. Female C57BL/6, C3H/OuJ, (C57BL/6 × C3H)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of C57BL/6 *lpr* and C3H *gld* mice purchased from The Jackson Laboratory were bred in our institute. C57BL/6 *nu* mice were purchased from either The Jackson Laboratory or Taconic Farms, Inc. (Germantown, NY).

For assay of bone marrow graft rejection, bone marrow cells were collected by flushing femur and tibia. Marrow transplantation was done by intravenous injection of bone marrow cells into recipient mice that had received total body γ irradiation of 850 rad. Success of grafting was assayed on day 8 posttransplantation by counting the number of stem cell colonies in the recipient spleen (CFU-S) after fixation in Bouin's solution (1). Data are shown as scatter graph of individual mice (Fig. 3) or as mean of CFU-S per spleen of groups of three to five mice. To determine the number of endogenous CFU-S, mice received a dose of 500 rad γ irradiation 8 d before examination for CFU-S (7, 8). Data shown represent mean ± SD of CFU-S per spleen of groups of three to five mice (5). To deplete NK1$^+$ cells, 1-wk-old mice were injected intraperitoneally weekly with an increasing dose of mouse mAb PK136 (anti-NK1.1) ascites according to a previously described protocol (9).

Cell Separations, Antibody Treatment, and Cell Cultures. B cells were removed from spleen cell suspensions by adherence on anti-Ig-coated plates (10). Tissue culture–grade polystyrene plates (Falcon Labware, Oxnard, CA) were coated with 15 μg/ml affinity-purified goat anti–mouse IgG + IgM (Caltag Laboratories, San Francisco, CA) in PBS. The plate was incubated overnight at 4°C and then washed five times with PBS before use. A single cell suspension of spleen cells was suspended in balanced salt solution (BSS) containing 10% FCS, and 5 × 10^7 cells were panned on a 150-mm dish in 15 ml of media. Cells were allowed to settle for 1 h at room temperature. Halfway through the incubation the nonadherent cells were resuspended by gently agitating the plate for 30 s. After incubation nonadherent cells were collected by gently agitating the plate and washing it three times with BSS.

For antibody treatment, cells (2 × 10^7/ml) were incubated for 45 min at 4°C with the respective antibody at optimal concentration, followed by incubation with low toxicity rabbit serum (Low-Tox M; Cedarlane Laboratories, Hornby, Ontario, Canada) as a source of C for 45 min at 37°C. Optimal concentrations for each antibody were determined by treating nylon wool–nonadherent cells with various dilutions of antibody and C, followed by flow cytometric analysis to assay the success of the treatment. The mAbs T24/31.7 (anti-Thy-1 [11]), PK136 (anti-NK1 [12]), 500-A-2 (anti-CD3 [13]), GK1.5 (anti-CD4 [14]), and AD4 (15) (anti-CD8 [15]) were prepared from hybridomas grown in our laboratory.

Cell cultures were set up in RPMI 1640 supplemented with 10% FCS, 5 × 10^{-5} M β_2-mercaptoethanol, 0.2 mM glutamine, 1 mM Na-pyruvate, and 0.1 mM nonessential amino acids. rIL-2 was obtained from Cetus Corp. (Emeryville, CA). To stimulate expression of NK1 and CD3 antigens, spleen cells depleted of B cells by panning and of CD4, as well as CD8 cells by antibody plus C treatment, were cultured in the presence of IL-2 for the times indicated. Spleen cells from autoimmune mice were cultured for 2 d in the presence of 4 μg/ml LPS to stimulate antibody secretion before enzyme-linked immunospot assay (ELISPOT).

Immunofluorescence Staining and ELISPOT. Indirect double-color immunofluorescence staining was analyzed on a single-laser FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). Rat antibody RA3-3A1/6.1 (anti-B220 [16]) was obtained from American Type Culture Collection (Rockville, MD). Biotin-labeled goat anti–hamster IgG antibody (Caltag Laboratories), FITC-conjugated affinity-purified F(ab')$_2$ fragment of rabbit anti–mouse IgG Fc, and biotin-conjugated affinity-purified F(ab')$_2$ fragment of goat anti–rat IgM (Jackson Immunoresearch, Avondale, PA) were used as second antibodies. PE-conjugated streptavidin was purchased from Tago Corp. (Burlingame, CA). Antibodies were used at optimal concentrations for staining (8). Gates in double staining experiments were set with second antibody controls.

DNA-coated plates were prepared by precoating 96-well flat-bottomed tissue culture polystyrene plates (Falcon 3070; Becton Dickinson & Co.) with 0.02% methylated BSA in carbonate buffer. Plates were incubated overnight at 4°C, and 100 μl of small pieces of native DNA (Sn DNA) and denatured DNA (dDNA) (100 μg/ml) in 0.14 M Tris-buffer saline (TBS), pH 7.4, were added then incubated overnight at 4°C. Both types of DNA were prepared following described methods (17). For Ig ELISPOTs, plates were coated with 100 μl goat anti–mouse IgG + IgM (Caltag Laboratories) (10 μg/ml) in PBS then incubated overnight at 4°C. Plates were washed and blocked by incubation with 3% BSA/PBS (100 μl/well) for 3 h at 37°C. Plates were washed three times with 100 μl BSS and incubated with 0.2–1 × 10^5 cells per well in 100 μl 3% FCS DME at 37°C for 4 h. Plates were washed extensively to remove all cells before adding anti–mouse IgG + IgM biotin-conjugated antibodies (Southern Biotechnology Associates) at 100 μl/well in 3% BSA/PBS/0.05% Tween 20. After incubation overnight at 4°C plates were washed, and streptavidin alkaline phosphatase (Southern Biotechnology Associates), 1:1000, 100 μl/well in 3% BSA/PBS/Tween 20, was added followed by incubation for 2 h at 37°C. Plates were washed, and 100 μl/well bromochloroindolyl phosphate (BCIP; Sigma Chemical Co., St. Louis, MO) was added in substrate buffer, 0.5% agarose (kept at 55°C to prevent solidification). Plates were incubated for 3 h at 37°C (17–19).

Results

In Young Mice the Abilities to Reject Marrow Grafts and to Downregulate Endogenous CFU-S Develop Concomitantly with Appearance of NK1$^+$ Cells. It is well documented that young mice up to the age of 3 wk lack the ability to acutely reject marrow grafts (20). In agreement with this, it is seen in Fig. 1 that BCF1 mice transplanted with BALB/c marrow fail to reject grafts up to the age of 23 d. Thereafter resistance develops gradually so that by day 32 complete ability to reject marrow is demonstrable. To examine whether manifestation of resistance correlates with appearance of NK1$^+$ cells, B as well as T cell–depleted spleen cells from BCF1 mice were cultured in IL-2-containing medium to stimu-

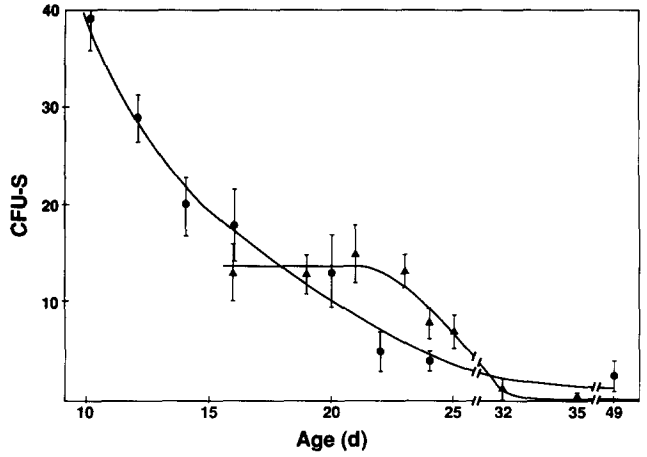

Figure 1. Comparison of appearance of alloresistance and endogenous CFU-S in newborn BCF1 mice. Mice of various ages were irradiated with 850 rad then transplanted with 2 × 10^5 BALB/c bone marrow for determination of alloresistance (▲). Littermates were irradiated with 560 rad to determine endogenous CFU-S (●). Animals were killed 8 d after irradiation or bone marrow transplantation. SD values of groups of three to five mice are plotted.

late antigen expression (1, 3). 5 d later cells were harvested and stained for expression of NK1 and CD3. Fig. 2, *A* and *B*, shows that cell populations from 10- or 17-d-old mice do not possess $NK1^+$ $CD3^-$ or $NK1^+$ $CD3^+$ cells. Cells from 21-d-old mice (Fig. 2 *C*) contain a few dimly staining $NK1^+$ $CD3^-$ and $NK1^+$ $CD3^+$ cells. These two populations are much more clearly defined in cultures from 30- or 36-d-old mice (Fig. 2, *D* and *E*). It therefore appears that cells expressing NK1 or NK1 and CD3 are demonstrable in the spleen of young mice at the time alloresistance to marrow grafts develops, whereas these cells are not present in younger mice not expressing resistance.

We had previously provided evidence for a physiological function of $NK1^+$ cells in the suppression of endogenous CFU-S proliferation (5). If $NK1^+$ cells indeed have this function, one would predict that the ability of young mice to reject allogeneic marrow should correlate with the ability to control the proliferation of endogenous CFU-S. To examine this, mice of various ages were irradiated with 560 rad then assayed for endogenous CFU-S. Fig. 1 shows that 10-d-old mice have very high numbers of CFU-S but that with increasing age the number of CFU-S decreases so that at the age of 22 d values common to adult mice are reached. This shows that in young mice the appearance of $NK1^+$ cells correlates not only with the ability to reject allogeneic marrow grafts but also with the ability to suppress endogenous marrow stem cell proliferation.

In C57BL/6 lpr Mice the Development of Autoimmunity Correlates with Loss of Ability to Suppress Endogenous Stem Cell Proliferation and Ability to Reject Allogeneic Bone Marrow. It had been reported that C57BL/6 mice with the homozygous *lpr* gene

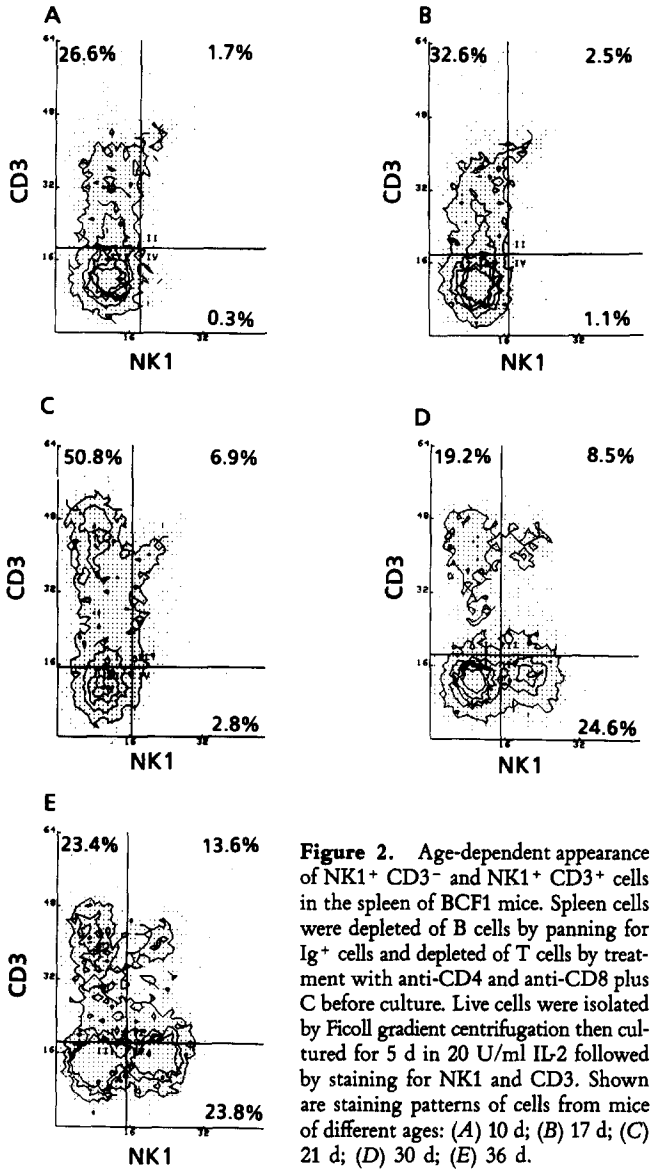

Figure 2. Age-dependent appearance of $NK1^+$ $CD3^-$ and $NK1^+$ $CD3^+$ cells in the spleen of BCF1 mice. Spleen cells were depleted of B cells by panning for Ig^+ cells and depleted of T cells by treatment with anti-CD4 and anti-CD8 plus C before culture. Live cells were isolated by Ficoll gradient centrifugation then cultured for 5 d in 20 U/ml IL-2 followed by staining for NK1 and CD3. Shown are staining patterns of cells from mice of different ages: (*A*) 10 d; (*B*) 17 d; (*C*) 21 d; (*D*) 30 d; (*E*) 36 d.

lack the ability to suppress endogenous stem cell proliferation (6). Therefore, if indeed $NK1^+$ cells are responsible for regulation of stem cell proliferation, one would predict that in *lpr* mice the ability to suppress endogenous CFU-S disappears concomitantly with ability to reject allogeneic marrow grafts. To examine this prediction, C57BL/6 *lpr* mice of varying ages were assayed for B cells secreting autoantibody to monitor the development of autoimmunity. Litter mates were irradiated and assayed for endogenous CFU-S. Table 1 shows that 7-wk-old normal mice possess only a low number of anti-DNA antibody-secreting B cells in their spleens. This is in contrast to C57BL/6 *lpr* mice of this age, which contain significantly higher number of cells that secrete anti-DNA antibody. Moreover, this number increases dramatically be-

Table 1. *In Aging C57BL/6 lpr Mice the Number of Anti-DNA Antibody-producing B Cells Increases Concomitantly with an Increase of Endogenous CFU-S*

Animals		Endogenous CFU-S	ELISPOTS per 10^5 cells			
			Fresh		Culture*	
Strain	Age		Sn DNA	dDNA	Sn DNA	dDNA
C57BL/6	7 wk	2.3 ± 0.60	1.3 ± 1.2	3.3 ± 1.5	8.3 ± 1.5	10.0 ± 1.0
C57BL/6	7 mo	2.0 ± 0.01	1.3 ± 1.2	5.3 ± 1.5	10.6 ± 1.5	18.0 ± 2.0
C57BL/6 *lpr*	7 wk	1.0 ± 1.00	6.7 ± 3.5	6.3 ± 0.6	25.5 ± 0.7	26.0 ± 2.0
C57BL/6 *lpr*	7 mo	23.1 ± 11.2	46.3 ± 3.8	44.0 ± 5.3	107.0 ± 7.8	107.0 ± 3.6

* Cells were cultured for 2 d in the presence of LPS. Data given were derived from three individual mice.

tween the age of 7 wk and 7 mo. Assay for endogenous CFU-S reveals a similar effect. 7-wk-old *lpr* mice express a normal number of endogenous CFU-S that is increased 10 times in 7-mo-old mice, a result that is not seen in normal mice. To assay the ability of C57BL/6 *lpr* mice to reject allogeneic marrow grafts, mice of varying ages were irradiated and then transplanted with BALB/c bone marrow. Fig. 3 shows that normal C57BL/6 mice regardless of age reject a dose of 10^6 BALB/c bone marrow. Quite a different result is seen in C57BL/6 *lpr* mice. Recipients up to the age of 2 mo reject the graft, whereas animals aged 4, 7, or 9 mo grow increasing numbers of CFU-S, i.e., they lose the ability to reject the transplant. These results therefore show that in mice expressing the homozygous *lpr* gene the development of autoimmunity

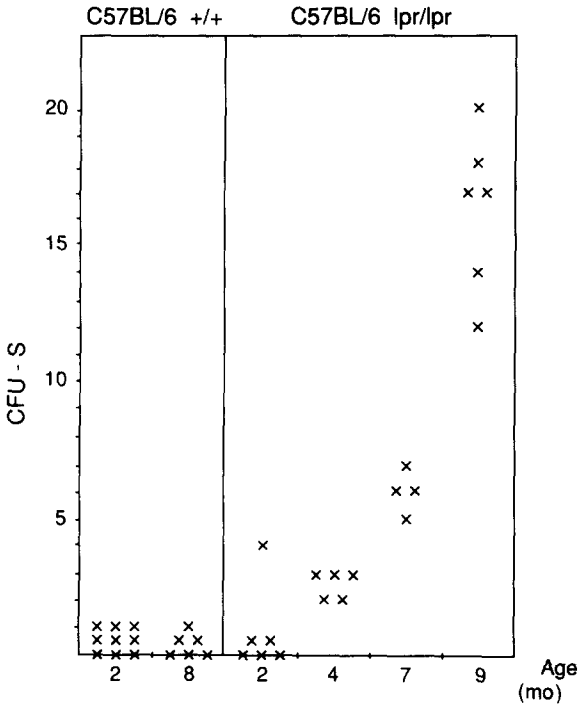

Figure 3. C57BL/6 *lpr* mice lose the ability to reject allogeneic marrow grafts with age. C57BL/6 or C57BL/6 *lpr* mice were irradiated with 850 rad and transplanted with 10^6 BALB/c bone marrow cells. On day 8 animals were killed and spleen colonies counted. CFU-S of individual mice of different ages are plotted. Loss of ability to reject marrow is seen in old *lpr* but not in normal mice.

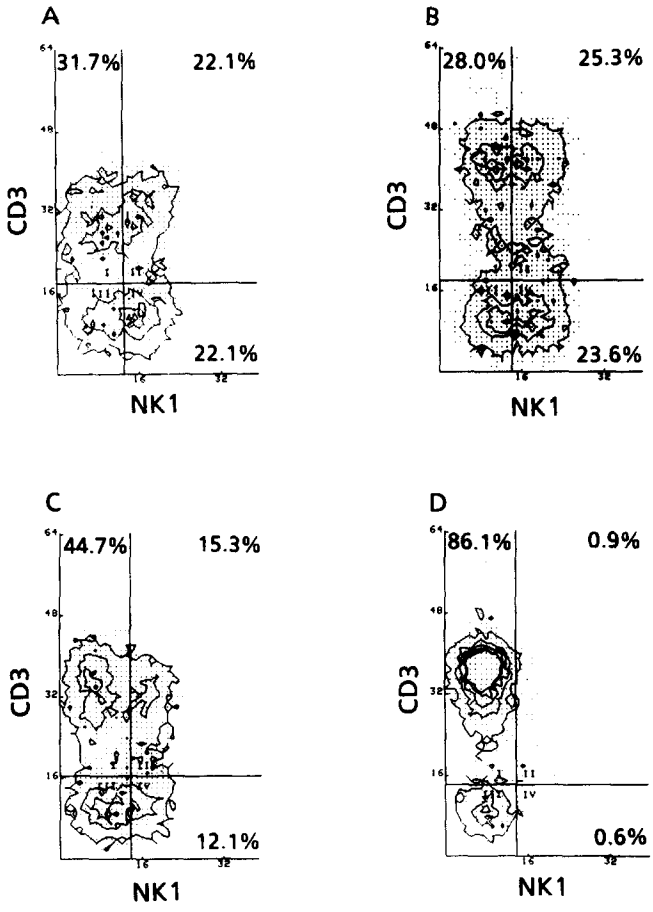

Figure 4. $NK1^+$ $CD3^-$ and $NK1^+$ $CD3^+$ cells disappear with age in C57BL/6 *lpr* (*C* and *D*) but not in normal mice (*A* and *B*). Spleen cells were depleted of B cells and T cells then cultured for 5 d in 20 U/ml IL-2 followed by staining with NK1 and CD3. (*A*) C57BL/6, 7 wk old; (*B*) C57BL/6, 9 mo old; (*C*) C57BL/6 *lpr*, 7 wk old; (*D*) C57BL/6 *lpr*, 9 mo old.

Table 2. *Injection of CD3 B220 Cells Does Not Suppress the Ability to Reject Allogeneic Marrow Grafts in Young C57BL/6 lpr Mice*

Bone marrow			
Recipients	Donor	Adoptive cell transfer*	CFU-S
C57BL/6 *lpr*	10^5 C57BL/6	-	24 ± 4.5
C57BL/6 *lpr*	10^5 C57BL/6	2.5 × 10^7 B220 CD3	25 ± 5.5
C57BL/6 *lpr*	10^6 BALB/c	-	<1
C57BL/6 *lpr*	10^6 BALB/c	2.5 × 10^7 B220 CD3	<1

* B220 CD3 cells were enriched from 8-mo-old C57BL/6 *lpr* spleen by B cell panning followed by treatment with anti-CD4 and anti-CD8 + C. Cells were injected 1 d before marrow transplanation into 8-wk-old recipients.

correlates with loss of ability to control endogenous stem cell proliferation as well as ability to reject allogeneic marrow.

In Aging C57BL/6 lpr Mice NK1⁺ Cells Disappear. The observation that a correlation exists between development of autoimmunity and loss of functions attributed to NK1⁺ cells raises the question whether this loss of function is reflected in the disappearance of cells with this phenotype from the spleen of mice developing autoimmunity. To find out, B and T cell–depleted spleen cells from *lpr* mice were cultured in IL-2 for 5 d, as in Fig. 2, followed by staining with NK1- and CD3-specific antibody. Results show that the staining pattern of cells from normal (Fig. 4 *A*) and *lpr* (Fig. 4 *C*) mice aged 7 wk is essentially the same and that a significant proportion of cells express either NK1 or NK1 and CD3. Quite in contrast, the cell population from 9-mo-old *lpr* (Fig. 4 *D*) but not normal mice (Fig. 4 *B*) lacks both NK1⁺ CD3⁻ as well as NK1⁺ CD3⁺ cells. It therefore appears that in *lpr* mice developing autoimmunity, NK1⁺ CD3⁻ as well as NK1⁺ CD3⁺ cells disappear.

It is well documented that in *lpr* mice with autoimmune symptoms there is a concomitant development of lymphadenopathy reflected in a dramatic increase of cells expressing B220 and CD3 (see below). It was therefore possible that the disappearance of NK1⁺ cells from aging *lpr* mice is due to the emergence of B220⁺ CD3⁺ cells. To examine whether B220⁺ CD3⁺ cells cause functional loss of effectors responsible for acute marrow graft rejection, cell populations from old *lpr* mice enriched for B220⁺ CD3⁺ cells were injected into young *lpr* mice. Recipient mice were then tested for ability to reject allogeneic marrow grafts. Results show (Table 2) that mice that had received one spleen equivalent of CD3⁺ B220⁺ cells and then were transplanted with either syngeneic or allogeneic bone marrow reject the allogeneic but not the syngeneic graft. This demonstrates that B220⁺

Table 3. *Cell Populations from Normal and Nude Mice but Not from Old lpr Mice Suppress Autoantibody Secretion in lpr B Cells*

Cell culture*		ELISPOTS per 10^5 cells	
NK1⁺ cells	B cells	Sn DNA (inhibition)	dDNA (inhibition)
-	C57BL/6 *lpr*	76.2 ± 6.4	85.8 ± 4.6
C57BL/6 (7 wk)	-	2.4 ± 2.6	2.6 ± 1.8
C57BL/6 (7 wk)	C57BL/6 *lpr*	52.8 ± 3.9 (31%, p <0.001)	54.0 ± 3.0 (37%, p <0.001)
C57BL/6 *lpr* (7 wk)	-	4.2 ± 0.4	4.2 ± 1.1
C57BL/6 *lpr* (7 wk)	C57BL/6 *lpr*	55.2 ± 8.7 (28%, p <0.01)	54.7 ± 2.6 (36%, p <0.001)
C57BL/6 *lpr* (9 mo)	-	3.5 ± 2.3	2.4 ± 1.8
C57BL/6 *lpr* (9 mo)	C57BL/6 *lpr*	71.7 ± 5.4 (6%, NS)	81.4 ± 6.4 (5%, NS)
C57BL/6 nude (11 wk)	-	2.0 ± 1.6	1.6 ± 1.1
C57BL/6 nude (11 wk)	C57BL/6 *lpr*	30.1 ± 4.8 (61%, p <0.001)	30.1 ± 6.2 (65%, p <0.001)

* NK1⁺ cells were enriched from spleen cells by B cell panning and treatment with anti-CD4 and anti-CD8 + C, then mixed with B cells at a ratio of 1:1 followed by culture in medium containing 4 μg/ml LPS for 2 d. To isolate B cells, spleen cells were treated with anti-Thy-1 + C. NK1⁺-containing populations were from spleens of normal, nude, or *lpr* mice of different ages and B cells were from 9-wk-old *lpr* mice.

Table 4. *(C57BL/6 × C3H)F$_1$ Cells Suppress B Cells from C57BL/6 More Efficiently than B Cells from C3H*

Cell culture*		ELISPOTS per 2 × 10^4 cells	ELISPOTS per 10^5 cells	
NK1$^+$ cells	B cells	Ig (inhibition)	Sn DNA (inhibition)	dDNA (inhibition)
BCF1	–	2.0 ± 1.0		
–	C57BL/6	34.7 ± 3.1		
BCF1	C57BL/6	15.3 ± 6.4 (56%, p <0.01)		
BCF1	–	2.0 ± 2.3		
–	C3H	27.0 ± 2.6		
BCF1	C3H	21.5 ± 2.0 (20%, p <0.05)		
BCF1	–		4.6 ± 2.7	5.0 ± 2.3
–	C57BL/6 *lpr*		83.0 ± 6.8	88.6 ± 6.4
BCF1	C57BL/6 *lpr*		39.2 ± 4.6 (53%, p <0.001)	43.6 ± 2.7 (51%, p <0.001)
BCF1	–		5.8 ± 3.8	8.0 ± 1.9
–	C3H *gld*		79.2 ± 7.8	89.2 ± 1.3
BCF1	C3H *gld*		57.3 ± 7.7 (28%, p <0.01)	66.5 ± 1.6 (26%, p <0.001)

* Culture conditions and enrichment of NK1$^+$ cells were identical to those in Table 3.

CD3$^+$ cells have no effect on the ability to reject marrow grafts, consistent with the interpretation that B220$^+$ CD3$^+$ cells do not interfere with the function of cells responsible for acute marrow graft rejection.

NK1$^+$ Cells Are Able to Cause Specific as Well as Nonspecific Suppression of B Cell Ig Secretion In Vitro. Since in *lpr* mice the disappearance of NK1$^+$ cells correlates with development of autoimmunity, we sought to explore whether cells with this phenotype express suppressive effects on B cell Ig secretion. To examine this, NK1$^+$ cells were enriched from normal or C57BL/6 *lpr* spleen by depletion of B and T cells, then mixed with B cells from C57BL/6 *lpr*. Cell mixtures were cultured for 2 d in the presence of LPS to stimulate Ig secretion. Table 3 shows that cells from normal C57BL/6 mice suppress Ig secretion of C57BL/6 *lpr* B cells 31–37%, and a very similar suppression is seen when cells from 7-wk-old *lpr* mice were tested (28–36%). In contrast, cells from 9-mo-old *lpr* mice have very little suppressive effect

Table 5. *Cells That Coexpress NK1 and CD3 Are Responsible for Specific Suppression B Cell Ig Secretion*

Cell culture*			ELISPOTS per 10^5 cells	
NK1$^+$ cells				
Strain	Treatment	B cells	Sn DNA (inhibition)	dDNA (inhibition)
–	–	C57BL/6 *lpr*	86.6 ± 3.4	90.4 ± 4.2
BCF1	–	C57BL/6 *lpr*	45.4 ± 8.0 (48%, p <0.001)	48.8 ± 6.8 (46%, p <0.001)
BCF1	αCD3 + C	C57BL/6 *lpr*	70.2 ± 5.0 (19%, p <0.001)	69.0 ± 5.0 (24%, p <0.001)
BCF1	αNK1 + C	C57BL/6 *lpr*	79.0 ± 8.3 (9%, NS)	81.4 ± 7.0 (10%, NS)
BCF1	Mix of NK1 and CD3 populations	C57BL/6 *lpr*	74.2 ± 3.0 (14%, p <0.001)	70.0 ± 7.0 (23%, p <0.001)
–	–	C3H *gld*	78.4 ± 5.5	80.2 ± 2.3
BCF1	–	C3H *gld*	59.0 ± 7.4 (25%, p <0.01)	65.0 ± 6.5 (19%, p <0.01)
BCF1	αCD3 + C	C3H *gld*	65.0 ± 5.6 (17%, p <0.01)	61.8 ± 5.5 (23%, p <0.01)
BCF1	αNK1 + C	C3H *gld*	73.6 ± 6.2 (6%, NS)	74.4 ± 6.7 (7%, NS)
BCF1	Mix of NK1 and CD3 populations	C3H *gld*	63.2 ± 2.9 (19%, p <0.01)	60.2 ± 9.1 (25%, p <0.01)

* Cell culture and enrichment of cells were identical to those in Table 3. Cell populations containing NK1$^+$ cells were treated before culture with anti-CD3 or anti-NK1 + C as indicated. A mixture of cells treated with either anti-CD3 + C or anti-NK1 + C was also assayed for its effects on B cells.

Table 6. *Injection of Anti-NK1 Causes an Increase in Cells Secreting Autoantibody*

C57BL/6 *lpr* mice injected with:	Time of assay	ELISPOTS per 10^5 cells			
		Fresh		Culture	
		dDNA	Sn DNA	dDNA	Sn DNA
	wk				
–	6	1 ± 1.1	1 ± 1.5	15 ± 1.5	16 ± 1.1
Anti-NK1	6	2 ± 0.8 (NS)	3 ± 1.1 (NS)	22 ± 1.9 (p <0.001)	21 ± 2.4 (p <0.01)
–	8	5 ± 1.6	6 ± 1.2	29 ± 3.6	28 ± 2.7
Anti-NK1	8	9 ± 1.8 (p <0.01)	9 ± 1.5 (p <0.01)	43 ± 5.4 (p <0.001)	42 ± 3.9 (p <0.01)
–	12	12 ± 1.7	12 ± 1.5	48 ± 7.8	49 ± 5.5
Anti-NK1	12	18 ± 1.1 (p <0.001)	17 ± 0.8 (p <0.001)	75 ± 5.6 (p <0.001)	67 ± 3.1 (p <0.001)

* Cells were cultured for 2 d in the presence of LPS.

(5–6%), consistent with the observation that old *lpr* mice lack NK1$^+$ cells. We had previously shown that nude mice possess in their spleens a very high number and activity of cells responsible for acute marrow graft rejection (3). It was therefore interesting to assay the effects of cell populations from these mice. Table 3 shows that cells from C57BL/6 nude mice cause by far the strongest inhibition (61–65%).

Next the effects of spleen cell populations from BCF1 enriched for NK1$^+$ cells were tested. BCF1 mice reject parental C57BL/6 but not C3H marrow (1), therefore, NK1$^+$ cells from these mice should suppress B cells from C57BL/6 but not from C3H. To test this, NK1$^+$ cells enriched from BCF1 spleen by depletion of B and T cells were mixed with B cells from either C57BL/6 or C3H/OuJ mice. Results (Table 4) show that Ig secretion of C57BL/6 cells is suppressed 56%, whereas that of C3H cells is suppressed 20%. A very similar result is seen when effector cells are mixed with B cells from C57BL/6 *lpr* or C3H *gld* mice. Again there is a 51–53% suppression of C57BL/6 as opposed to a 26–28% inhibition of C3H B cells. These results show that cell populations from BCF1 spleen express stronger suppressive effects on H-2^b compared with H-2^k B cells.

The effector cell populations tested in these assays are heterogeneous, which makes it difficult to know precisely which cells are responsible for the observed effects. To analyze this question cell populations were treated with antibody and C to eliminate cells expressing NK1 and CD3. Table 5 shows that the ability of BCF1 cells to inhibit Ig secretion of

Table 7. *Injection of Cells from Nude Mice Enriched for NK1$^+$ Cells into lpr Mice Delays the Onset of Autoimmunity*

C57BL/6 *lpr* mice‡ injected with:	Time of assay	ELISPOTS per 10^5 cells			
		Fresh		Culture	
		dDNA	Sn DNA	dDNA	Sn DNA
	wk				
–	12	14 ± 1.1	14 ± 1.2	47 ± 2.6	45 ± 4.3
C57BL/6 nude spleen	12	4 ± 1.0 (p <0.001)	4 ± 0.7 (p <0.001)	26 ± 1.5 (p <0.001)	15 ± 2.2 (p <0.001)
–	16	44 ± 3.0	38 ± 4.1	103 ± 8.3	96 ± 7.4
C57BL/6 nude spleen	16	17 ± 3.1 (p <0.001)	16 ± 2.7 (p <0.001)	65 ± 8.7 (p <0.001)	60 ± 6.8 (p <0.001)

* Cells were cultured for 2 d in the presence of LPS.

‡ C57BL/6 *lpr* mice (also analyzed in Fig. 5, *E* and *F*) were injected with cells from C57BL/6 nude spleen. NK1$^+$ cells were enriched from nude spleen by removing B cells by panning, resulting in 30–40% NK1$^+$ CD3$^+$ and 20–30% NK1$^+$ CD3$^-$ cells. Cells (5×10^6 per mouse) were injected intravenously at the age of 7, 9, and 13 wk.

C57BL/6 *lpr* B cells is decreased by treatment with anti CD3 + C from 46–48% to 19–24%, but that there is no effect of antibody treatment on suppression of C3H *gld* B cells (19–25% vs. 17–23%). This indicates that $CD3^+$ cells are responsible for suppression of C57BL/6 *lpr* but not of C3H *gld* B cells. Treatment of BCF_1 cells with anti-NK1 + C abrogates suppressive activity on both C57BL/6 *lpr* and C3H *gld* B cells. A mixture of BCF_1 effector cells treated independently with either anti-NK1 + C or anti-CD3 + C is not able to suppress C57BL/6 *lpr* B cells more efficiently than cells treated with anti-CD3 + C, which suggests that the cells responsible for suppression of C57BL/6 *lpr* B cells coexpress NK1 and CD3. When the same mixture of cells is assayed on C3H *gld* B cells, suppression is similar to that seen with cells treated with anti-CD3 + C. These results suggest that whereas specific suppression of C57BL/6 B cells is due to $NK1^+$ $CD3^+$ cells, nonspecific suppression by $NK1^+$ $CD3^-$ cells acts on C3H B cells and probably also on C57BL/6 B cells.

Removal of $NK1^+$ Cells in lpr Mice by Antibody Injection Accelerates the Development of Autoimmunity, Whereas Injection of Cells Enriched for $NK1^+$ Cells Inhibits Autoimmunity. Our experiments in *lpr* mice show that disappearance of $NK1^+$ cells correlates with appearance of autoimmunity symptoms. This raised the possibility that $NK1^+$ cells play a direct role in the control of autoimmunity and that in their absence the development of autoimmunity is accelerated. In support of this notion, in vitro assays with B cells from normal or *lpr* mice show that both $NK1^+$ $CD3^+$ and $NK1^+$ $CD3^-$ cells suppress Ig secretion. Therefore, if indeed these two cell populations were responsible for the in vivo regulation of B cells and suppression of autoimmunity, it would be interesting to know whether elimination of $NK1^+$ cells from *lpr* mice accelerates the development of autoimmunity. To find out, young *lpr* mice were injected with anti-NK1 antibody PK136. Continued injection of this antibody leads to complete elimination of $NK1^+$ cells (9, and data not shown). Assay of splenic B cells from injected or control mice for anti-DNA antibody–secreting cells revealed a small but significant increase at 6 wk. This increase was more pronounced at later time points, when antibody-treated mice had 50% more cells producing autoantibody (Table 6). Assay of antibody-injected or control *lpr* mice by fluorometry for cells expressing B220 and CD3 revealed no significant differences (data not shown). These results show that removal of cells expressing NK1 stimulates the appearance of autoantibody-secreting B cells in *lpr* mice but has no demonstrable effect on the appearance of $B220^+$ $CD3^+$ cells.

Since removal of $NK1^+$ cells in vivo causes accelerated development of autoimmunity, one might expect that adoptive transfer of cell populations enriched for $NK1^+$ cells into *lpr* mice should delay its development. To examine this prediction $NK1^+$ cells were enriched from C57BL/6 *nu* spleen cells by depleting spleen cells of B cells (3). The resulting population typically consists of 30–40% $NK1^+$ $CD3^+$ cells and 20–30% $NK1^+$ $CD3^-$ cells (3). This cell population was adoptively transferred into C57BL/6 *lpr* mice at the age of 7 and 9 wk. At 12 wk animals were killed and tested for autoantibody-secreting B cells. Results in Table 7 show that uninjected *lpr* mice have 14 and 45–47 B cells per 10^5 fresh and cultured spleen cells, respectively, whereas animals that received adoptive cell transfers have 4 and 15–26 anti-DNA antibody-secreting B cells, respectively. This demonstrates that whereas 12-wk-old *lpr* mice have significantly elevated numbers of cells producing autoantibody compared with 7-wk-old *lpr* mice (Table 1), animals that received adoptive cell transfers have numbers of autoantibody-secreting B cells comparable to those of normal mice or young *lpr* mice. By the age of 16 wk, control *lpr* mice have significantly elevated numbers of autoantibody-secreting B cells in fresh and cultured spleen cells (Table 7), whereas again mice that received adoptive cell transfers have much lower numbers of autoantibody-secreting cells (Table 7). However, the number of autoantibody-secreting B cells is significantly higher than that of normal mice or young *lpr* mice, which shows that adoptive cell transfers cause a significant delay in appearance but not a complete suppression of autoimmunity.

As mentioned above, one of the manifestations of autoimmunity in *lpr* mice is the development of lymphadenopathy caused by accumulation of $B220^+$ $CD3^+$ cells. It was therefore interesting to examine whether *lpr* mice that received adoptive cell transfers have high or low numbers of these cells. Fig. 5, *A* and *B*, shows that 6- or 24-wk-old normal C57BL/6 mice have between 4.1 and 8.9% $B220^+$ $CD3^+$ cells in their B cell–depleted spleen cells. Similarly, 6-wk-old *lpr* mice possess 8.6% $B220^+$ $CD3^+$ cells, but the percentage of these cells increases to 38.5% in 12-wk-old mice (Fig. 5, *C* and *D*). 12-wk-old mice that received adoptive cell transfers have only 6.0% $B220^+$ $CD3^+$ cells in their spleen, and this percentage increases to 13.1% by 16 wk (Fig. 5, *E* and *F*). These results show that adoptive cell transfer of cells enriched for $NK1^+$ cells suppresses the appearance of $B220^+$ $CD3^+$ cells in *lpr* mice and therefore has a preventive effect on the development of autoimmune symptoms.

Discussion

The ability of lethally irradiated F_1 hybrid mice to acutely reject parental marrow grafts has been somewhat of an enigma in transplantation immunology. A long-standing explanation for this phenomenon has been that hemopoietic histocompatibility (Hh) antigens are responsible for the rejection and that expression of these antigens requires homozygosity (21). While presently it is not known with certainty whether Hh antigens indeed exist, an alternative possibility is that the rejection mechanism responsible involves effector cells that recognize self-antigens. In support of this, we had previously shown that removal of $NK1^+$ cells from normal mice by various means may lead to failure to control proliferation of endogenous CFU-S in lightly irradiated animals (5). In keeping with this observation, we show here that in young mice up to the age of 3 wk the abilities to reject allogeneic marrow and to control endogenous CFU-S proliferation are not demonstrable and that both functions appear at a time when $NK1^+$ cells are demonstrable.

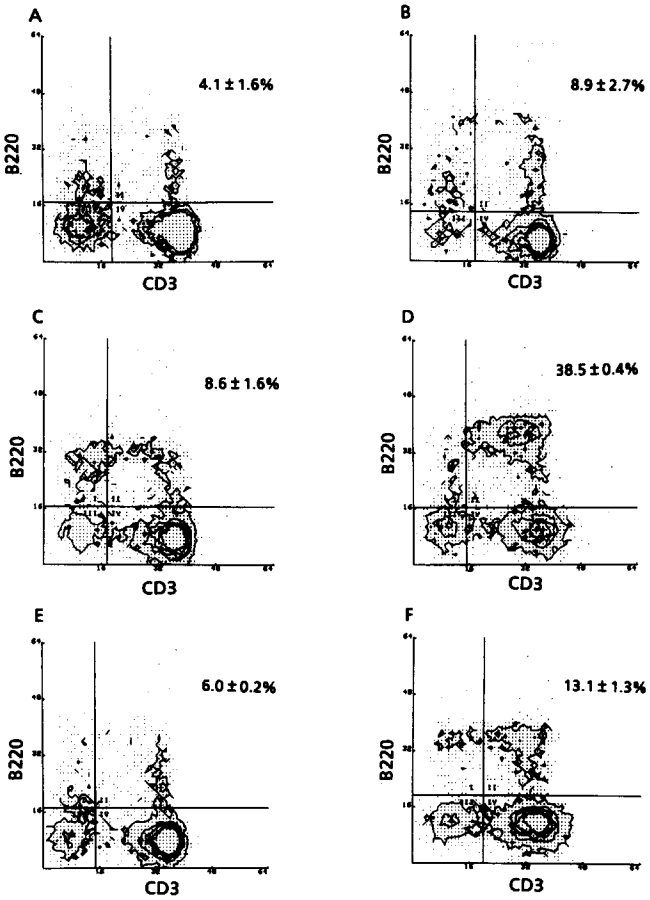

Figure 5. $CD3^+$ $B220^+$ cells in C57BL/6 and C57BL/6 *lpr* mice. All spleen cells were depleted of B cells by panning and then stained for CD3 and B220. (*A*) C57BL/6, 6 wk old; (*B*) C57BL/6, 24 wk old; (*C*) C57BL/6 *lpr*, 6 wk old; (*D*) C57BL/6 *lpr*, 12 wk old; (*E*) C57BL/6 *lpr*, 12 wk old. These mice had been injected (5×10^6 cells per mouse) with cells enriched for $NK1^+$ cells from C57BL/6 nude at the age of 7 and 9 wk. (*F*) C57BL/6 *lpr*, 16 wk old, had been injected with the same cell populations and dose as in *E* at the age of 7, 9, and 13 wk.

In normal mice the ability to reject marrow grafts and to control endogenous marrow stem cells persists, whereas in mice homozygous for the *lpr* gene these functions disappear concomitant with a loss of $NK1^+$ cells. This pointed to the possibility that $NK1^+$ cells play a role not only in controlling marrow stem cell proliferation but also in downmodulating immune functions. In attempts to gather support for this, young *lpr* mice were treated with antibody to remove $NK1^+$ cells. This resulted in a significant increase in autoantibody-secreting B cells. The reverse was accomplished by injection of cell populations enriched for $NK1^+$ cells. There was suppression to normal levels of the number of autoantibody-secreting B cells consistent with the interpretation that these cells have downregulatory effects on B cells. An interesting observation is that injection of cell populations enriched for $NK1^+$ cells into *lpr* mice causes complete suppression of $B220^+$ $CD3^+$ cells responsible for lymphadenopathy at the later stages of the disease. This could indicate that $NK1^+$ cells have immunosurveillance functions and control the proliferation of $B220^+$ $CD3^+$ cells. The observation that treatment of *lpr* mice with anti-NK1 has no demonstrable effect on $B220^+$ $CD3^+$ cells has to be interpreted in view of the fact that $NK1^+$ cells develop from $NK1^-$ precursors (3, 22). Therefore, injection of anti-NK1 will not remove precursors of $NK1^+$ cells but may suffice to cause effects on autoantibody-secreting B cells.

A very interesting question is precisely which subpopulation of $NK1^+$ cells is responsible for the observed effects. It is a well-established observation that $NK1^+$ cells have homeostatic effects on antibody responses (23), but the relative contributions of $NK1^+$ $CD3^-$ and $NK1^+$ $CD3^+$ cells have not been elucidated. Our in vitro experiments provide clues to answer this question. We show that suppression of C3H B cells by BCF1 cells is due to $NK1^+$ $CD3^-$ cells, whereas suppression of C57BL/6 B cells is due to $NK1^+$ $CD3^+$ effector cells. This is consistent with the interpretation that $NK1^+$ $CD3^-$ cells have nonspecific, whereas $NK1^+$ $CD3^+$ cells have specific, suppressive effects. One could speculate from this that the specific suppressive effects demonstrable with $NK1^+$ $CD3^+$ cells are a reflection of the in vivo phenomenon of hybrid resistance. This is strongly supported by the previous demonstration that $NK1^+$ $CD3^+$ cells are responsible for acute parental marrow graft rejection in BCF1 mice (1). The finding that $NK1^+$ $CD3^+$ cells have specific effects is most likely explained by the expression of TCRs. Recent experiments have shown that $NK1^+$ $CD3^+$ cells possess TCR α/β chains and that a change in one of the chains interferes with the function of these cells. Thus, in C57BL/6 mice expressing the TCR $V\beta5$ transgene, $NK1^+$ $CD3^+$ cells are present but specific acute marrow graft rejection is not demonstrable (24). The absence of TCRs on $NK1^+$ $CD3^-$ cells is consistent with their nonspecific suppressive effect although this does not exclude that they express specificities yet to be uncovered. The observation that SCID mice that possess $NK1^+$ $CD3^-$ but not $NK1^+$ $CD3^+$ cells reject marrow grafts may point to this possibility (4).

Both $NK1^+$ $CD3^-$ as well as $NK1^+$ $CD3^+$ cells show suppressive effects in our in vitro assays. It therefore is likely that both cell types act synergistically in vivo. It is difficult to design experiments to assess the relative contributions of either cell type in the development of autoimmunity. The reason for this is that both cells develop from $NK1^-$ $CD3^-$ precursors (1, 3, 22) and in normal mice they are not readily demonstrable by FACS® staining (1, 3) because they express low amounts of antigen. Culture of cells for 2–5 d in IL-2 causes stimulation of antigen expression, which makes possible identification of both phenotypes by double staining (1, 3, 22). Despite this, purification of phenotypically stable cell populations from IL-2-cultured cells turned out to be impossible because of antigen modulation (our unpublished results). Therefore, isolation of homogeneous cell populations for in vivo adoptive cell transfer experiments is not feasible.

Our finding that $NK1^+$ cells have a downregulatory function on the proliferation of bone marrow stem cells is in agreement with a previous result by Hansson et al. (25). These authors reported that depletion of $NK1^+$ cells in normal mice by continued injection of anti-NK1 antibody results in

a failure of injected mice to downregulate myeloid precursors in the spleen. These observations as well as those reported here point to an important regulatory function of $NK1^+$ cells in the immune system and may open the door to the treatment of autoimmune disorders.

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