Cloning of Self-Major Histocompatibility Complex Antigen-Specific Suppressor Cells from Adult Bone Marrow

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Summary

We examined if suppressor cell clones may be established from adult bone marrow that contains a population of cells capable of specifically downregulating the immune response directed toward self-major histocompatibility complex (MHC) antigens. Freshly prepared adult C3H ($H\text{-}2^k$) marrow cells were cultured in medium containing interleukin 2 (IL-2), IL-3, or a mixture of IL-2 and IL-3. After 7–10 d, cells grown in IL-3-containing medium were screened for their capacity to suppress cytotoxic T lymphocyte (CTL) generation against self-MHC antigens in allogeneic mixed lymphocyte cultures. Cells capable of suppressing anti-C3H CTL generation were cloned by limiting dilution. Several suppressor clones were established that exhibited strong suppression of anti-$H\text{-}2^k$, anti-$H\text{-}2K^k/I^k$, and anti-$H\text{-}2D^k$ CTL generation, but failed to suppress anti-$H\text{-}2^d$ and anti-$H\text{-}2^b$ responses. When tested in a skin allograft model, intravenous injections of these bone marrow–derived anti-self suppressor cells (2.5×10^7 cells) together with IL-3 induced prolongation of C3H skin allografts in anti–mouse lymphocyte serum–treated $B6AF_1$ mice. Injection of IL-3 alone had no effect on allograft survival. Moreover, these cells failed to prolong B10.AKM skin allografts on $B6AF_1$ recipients. Northern blot analysis showed that these cells express full-length transcripts of the T cell receptor (TCR) γ gene, but not those of TCR α, β, or δ genes. However, no rearrangement of γ gene was observed by Southern blot analysis. Flow cytometric analysis revealed that bone marrow–derived suppressor cells are strongly positive for Thy-1 antigen but negative for CD3, CD4 (L3T4), and CD8 (Lyt-2) surface markers, and express only class I MHC antigens. Suppressor cells derived from adult bone marrow may play an important role in extrathymic induction of self-tolerance.

Adult bone marrow contains a population of cells capable of specifically downregulating the immune response directed toward self-MHC antigens. In a mouse skin allograft model, significant prolongation of C3H/He (C3H) skin allograft survival is induced in immunosuppressed $(C57BL/6 \times A)F_1$ ($B6AF_1$) mice by injection of C3H bone marrow cells postoperatively (day +8 relative to grafting on day 0) (1). Immunosuppression is achieved by intraperitoneal injection of rabbit anti–mouse lymphocyte serum (ALS)[1] given on days −1 and +2. Graft survival in ALS-treated, donor bone marrow–injected mice is usually 20–40 d over that in controls given ALS alone. That injection of unrelated C57BL/6 bone marrow cells has no effect in prolonging C3H skin allograft survival on ALS-treated $B6AF_1$ recipient mice (1) illustrates the antigen-specific nature of this bone marrow cell–induced unresponsiveness. The role of suppressor cells specific for their own MHC antigens is indicated in this unresponsive state; adoptive transfer of spleen cells from ALS- and donor bone marrow–treated $B6AF_1$ mice unresponsive to C3H skin allografts to the secondary ALS-treated $B6AF_1$ recipient mice achieves prolongation of C3H skin allograft survival but not that of DBA/2 skin graft survival (2). Further analysis by in vitro coculture MLC experiments demonstrated that these putative suppressor cells bear Thy-1 determinants and MHC antigens of the marrow and skin donor, suggesting that these cells are derived from injected donor bone marrow cells (3).

In an in vitro MLC system, addition of fresh bone marrow cells from normal adult mice or T cell colonies grown from bone marrow cultures causes suppression of generation of CTL directed against MHC antigens shared by the stimulator cells and the added cells (4–6). Suppression is thought to be mediated through prevention of differentiation of CTL precursors to CTL. Subsequently, cloned CTL were also shown to suppress CTL generation against their surface MHC antigens irrespective of the specificity of their receptor for

[1] *Abbreviations used in this paper:* ALS, anti–mouse lymphocyte serum; CTLL, cytotoxic T lymphocyte line; NC, natural cytotoxic.

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antigen (7–9). Because these suppressor cells prevent responses against "self" MHC antigens, these cells were called "veto cells" (10).

In the present paper, we report establishment of suppressor cell clones by culturing adult bone marrow cells in IL-3. These bone marrow–derived clones are strictly dependent on IL-3 for their continuous growth, and are capable of inhibiting in vitro and in vivo cellular immune responses against self-MHC antigens. These cells express Thy-1 determinants but lack any other T cell surface markers, such as CD3, CD4, and CD8 determinants, and TCR proteins. The cells transcribe TCR γ gene without γ gene rearrangement. These bone marrow–derived cells may play an important role in the induction of self-tolerance.

Materials and Methods

Mice. C3H/He (C3H), C57BL/6, DBA/2, (C57BL/6 × A)F_1 (B6AF$_1$), A, and C3H.OH mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were used at 2–7 mo of age. B10.MBR mice were kindly provided by Dr. Martin E. Dorf, Department of Pathology, Harvard Medical School, Boston, MA.

Cytokines. 48-h culture supernatant of WEHI-3 cells (WEHI.CS) was used as a source of IL-3. Purified human IL-2 was purchased from Electro-Nucleonics, Silver Spring, MD. rIL-2 and rIL-3 were purchased from Genzyme, Boston, MA.

Bone Marrow Cell Culture. Fresh bone marrow cells were obtained from the femurs of adult C3H mice and plated in 24-well plates (Flow Laboratories, Inc., McLean, VA) at 10^5 cells/ml/well in RPMI 1640 containing 10% FCS, 5×10^{-5} M 2-ME, and either mouse rIL-2 (50 U/ml) or purified human IL-2 (5 U/ml), IL-3 (WEHI.CS at 25% [vol/vol] or rIL-3 at 10 U/ml), or mixtures of IL-2 and IL-3 (IL-2/3). One half of the culture medium was replaced twice weekly with fresh medium supplemented with appropriate cytokines. After significant cell growth was obtained, the surviving cells were split every 4–7 d. When sufficient numbers of cells grew, cells were assayed for their capacity to suppress generation of CTL in allogeneic MLC. Cells capable of suppressing anti-C3H CTL generation were cloned by limiting dilution. Cloned

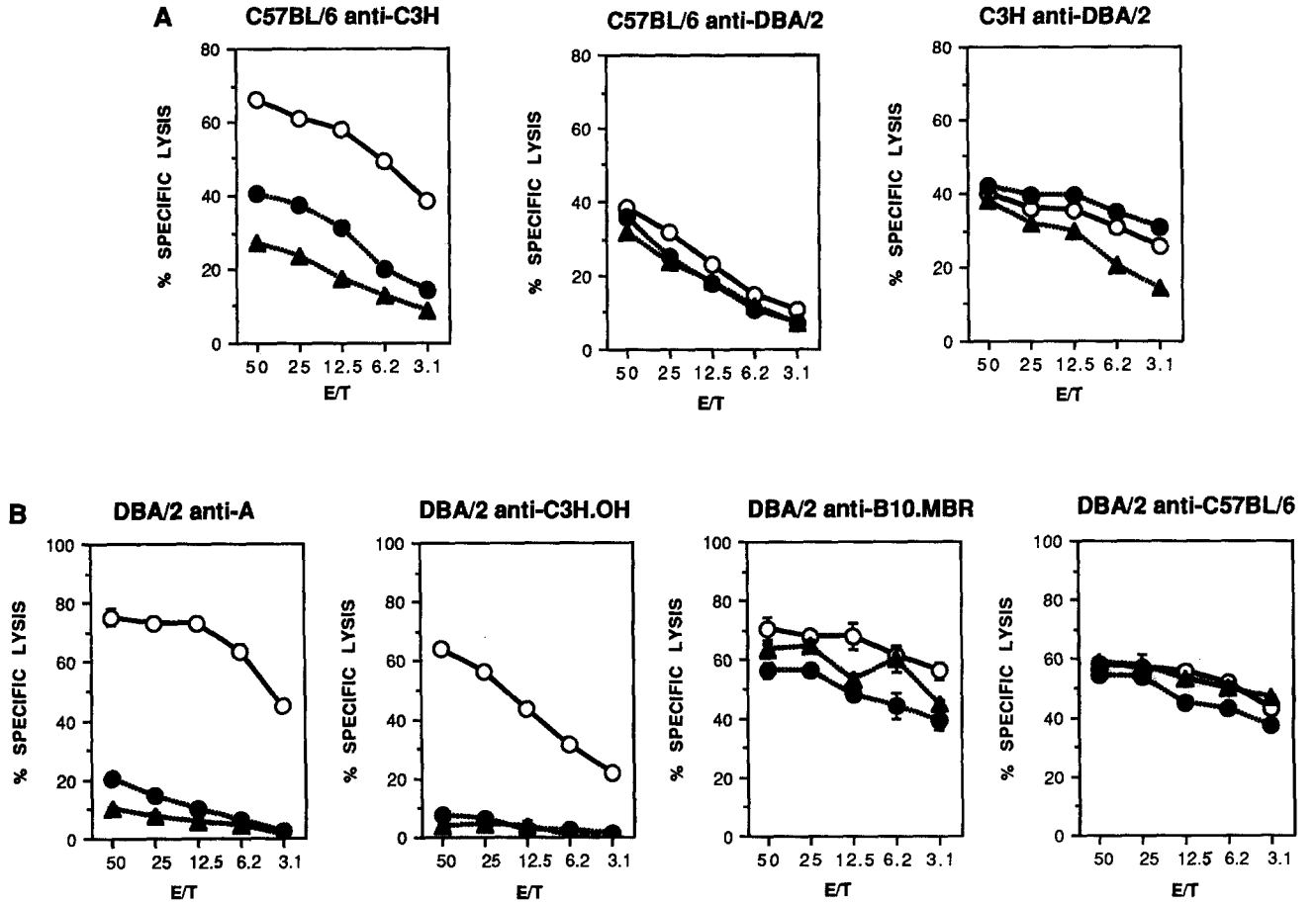

Figure 1. MHC antigen specificity suppression by IL-3-dependent clones 11.7 and 3.2. 5×10^4 irradiated (2,000 rad) clone 11.7 cells (●---●), clone 3.2 cells (▲---▲), or C3H splenocytes (O—O) were added to each 16-mm well containing 5×10^6 responder nylon wool–nonadherent splenocytes and 10^5 irradiated stimulator splenic adherent cells. Responder cell–stimulator cell combinations are as indicated above. After a 5-d culture, surviving cells were assayed at various E/T ratios against ^{51}Cr-labeled 3-d Con A–stimulated spleen cells. C3H blast targets were used for C57BL/6 anti-C3H, DBA/2 anti-A, and DBA/2 anti-C3H.OH, DBA/2 blasts for C57BL/6 anti-DBA/2 and C3H anti-DBA/2, C57BL/6 blasts for DBA/2 anti-C57BL/6, and B10.MBR blasts for DBA/2 anti-B10.MBR effector cells. Results are expressed as percent specific lysis. (*A*) One experiment and (*B*) two experiments in which suppression of DBA/2 anti-C3H and no suppression of DBA/2 anti-C57BL/6 were always confirmed (parts of data not shown). Spontaneous release was ~20–30% of maximal release in all experiments.

cells were further propagated in 24-well plates and then assayed for their capacity to inhibit allogeneic MLC-induced CTL generation. Suppressor clones were maintained in 25-cm^2 flasks. These cells were also frozen and stored in liquid nitrogen.

MLC and Cytotoxicity Assay. MLC was prepared in RPMI 1640 supplemented with 5% FCS, 5×10^{-5} M 2-ME, and penicillin (100 U/ml), and streptomycin (100 μg/ml). For screening of suppressor activity, irradiated cloned cells (5×10^3) were added to both C57BL/6 anti-C3H and C57BL/6 anti-DBA/2 MLC prepared in 96-well U-bottomed plates with 7.5×10^5 responder splenocytes and 5×10^5 irradiated stimulator splenocytes in a total volume of 200 μl. After a 5-d incubation, 100 μl of the cell mixture was transferred to a V-bottomed well containing 10^4 ^{51}Cr-labeled target cells in 100 μl of medium. For the assay of specificity of suppression, 5×10^4 irradiated (2,000 rad) cloned bone marrow cells were added to a 16-mm well containing 5×10^6 responder nylon wool–nonadherent splenocytes and 10^5 irradiated (4,000 rad) stimulator splenic adherent cells. Irradiated normal splenocytes were added to control cultures. After a 5-d incubation, surviving cells were assayed at various E/T ratios against ^{51}Cr-labeled targets. Targets were 3-d Con A blasts of spleen cells. ^{51}Cr-release assay and calculation of percent specific lysis were done as previously described (11).

mAbs. FITC conjugates of mAbs 30-H12 (anti-Thy-1.2) and 53-6.7 (anti-CD8), and PE conjugate of mAb GK1.5 (anti-CD4) were obtained from Becton Dickinson & Co., Mountain View, CA. The hamster mAb 145-2C11 (anti-CD3-ϵ) was used as culture supernatant and an FITC conjugate. The hamster mAb 3A10, which is specific to a murine TCR-δ (12), was used as culture supernatant and biotin conjugates. This antibody was kindly provided by Dr. S. Itohara of the Massachusetts Institute of Technology, Boston, MA. Mouse mAb HB 24 (anti-H-2D^k) was used as culture supernatant. Mouse mAbs 11-4.1 (anti-H-2K^k) and 11-5.2 (anti-I-A^k) were purchased from Becton Dickinson & Co. These antibodies were stained with FITC-conjugated goat anti–mouse IgG (Cappel Laboratories, Malvern, PA).

Flow Cytometric Analysis. Single cell suspensions were stained by the standard procedures. Propidium iodide (0.5 μg/ml) was used to eliminate the dead cells from the data. Two-color immunofluorescence analysis for Thy-1, CD3, CD4, CD8, and TCR-γ/δ determinants was done at the Department of Biology, Massachusetts Institute of Technology, by Dr. Itohara. Cells were stained with FITC- and biotin-conjugated antibodies and streptavidin-PE (Becton Dickinson & Co.). Negative controls were incubated with streptavidin-PE and affinity-purified goat anti–hamster IgG-FITC. Analysis of these samples, as well as single-color analyses for surface MHC antigens, was done with a single-beam flow cytometer, FACScan (Becton Dickinson & Co.) and the FACScan Research Software (Becton Dickinson & Co.).

Analysis of TCR Genes by Northern Blot and Southern Blot Analyses. Total cellular RNAs were isolated by guanidine isothiocyanate and cesium chloride (CsCl) gradient centrifugation (13, 14). Concentration of RNA in Tris/EDTA buffer was measured at 260-nm wave length by DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). 15 μg of total RNAs were subjected to electrophoresis on 1% agarose/formaldehyde gels and transferred to GeneScreenPlus membranes (DuPont, Boston, MA) with 10× SSC. Membranes were baked at 80°C for 2 h, and prehybridized with 50% deionized formamide, 1 M NaCl, 1% SDS, and 10% dextran sulfate for at least 3 h at 42°C. TCR α, β, and γ chain cDNA clones, pHDS 12, pHDS 22, and pHDS 4 (15, 16), respectively, were kindly provided by Dr. H. Saito, Dana Farber Cancer Institute, Boston, MA. TCR δ chain cDNA clone, KN12D1 (17), was kindly provided by Dr. Y. Takagaki, Massachusetts Institute of Technology. The cDNA insert was labeled with ^{32}P by the random primer method (18) and used as a probe. RNA blots were hybridized in 50% deionized formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and labeled cDNA probe (sp act $2–5 \times 10^8$ cpm/μg) at 42°C for overnight. After hybridization, the membranes were washed with a final stringency wash for 30 min at 60°C in 0.1× SSC, and exposed to x-ray films.

For Southern blot analysis, genomic DNA was isolated from the adult C3H mouse liver, cytotoxic T lymphocyte line (CTLL) cells, or cultured bone marrow cells according to the standard procedure (19). 10 μg of DNA was digested with EcoRI or HindIII restriction enzymes (New England Biolabs, Beverly, MA), subjected to electrophoresis on 0.8% agarose gel, and transferred to GeneScreenPlus membranes. Membranes were dipurinated with 0.25 N HCl and then denatured with 0.4 N NaOH and 0.6 M NaCl. Prehybridization and hybridization conditions were the same as those for Northern blot analysis. All of the cDNA probes were labeled by the random primer method. After hybridization, the membranes were washed and exposed to x-ray films as described for Northern blotting.

Skin Grafting. Preparation of ALS and skin grafting techniques have been previously described (1).

Results

In an attempt to study in vitro the differentiation of self-MHC antigen-specific suppressor cells from bone marrow, we cultured freshly prepared adult C3H marrow cells in IL-2, WEHI.CS (IL-3), or a mixture of IL-2 and IL-3 (IL-2/3). After a 7–10 d incubation, significant cell growth was observed in cultures containing IL-3 alone or IL-2/3, but not in cultures containing IL-2 alone. Growing cells were screened for their capacity to suppress CTL generation against self-MHC antigens. Cells that suppressed anti-self CTL generation were cloned by limiting dilution in IL-3-containing medium. Subcloning and screening for specific suppression were repeated. Several suppressor cell clones, such as C3H.BM 11.7, BM 11.12, and BM 3.2, were obtained. Fig. 1 illustrates alloantigen specificity of suppression mediated by clone C3H.BM 11.7 (clone 11.7) and BM 3.2 (clone 3.2). Clone 11.7 and 3.2 cells mediated significant suppression of CTL generation against self-MHC antigens (C57BL/6 anti-C3H). The suppression was on the order of 12–50-fold. There was no suppression of C57BL/6 anti-DBA/2 response by both clone 11.7 and 3.2 cells. Suppression was either absent or minimal (up to twofold) when responder cells were from C3H mice (C3H anti-DBA/2). Specificity of inhibition with regard to the class I and class II MHC antigens was examined. As shown in Fig. 1 *B*, both clones 11.7 and 3.2 mediated suppression when they were added to DBA/2 anti-A (anti-H-2K^k and I^k) and DBA/2 anti-C3H.OH (anti-H-2D^k) sensitization cultures. However, when they were added to DBA/2 anti-B10.MBR (anti-H-2K^b, I-A^k, I-E^k and D^q), no significant inhibition was observed. Since C3H and B10.MBR strains share class II MHC antigens but differ at class I MHC antigens, it appears that suppression was directed towards the responses against class I self-MHC antigens (anti-self-specific suppression). Suppression was dependent on the numbers of

cloned cells added. Significant suppression was observed with a number of cloned cells as few as 1/1,000 of responder cells. Moreover, significant suppression was observed from day 3 of culture and was not due to the shifting of peak response (data not shown).

Natural cytotoxic (NC) cells have been shown to exhibit suppression of in vitro CTL generation (20–22). Some of the suppressor NC cells are IL-3 dependent (23). Therefore, we examined whether clone 11.7 had NC-like lytic activity. L929 target cells, which are susceptible to NC cells (22), were used. Table 1 shows that, in an 18-h ^{51}Cr-release assay, clone 11.7 cells failed to lyse L929 targets that were susceptible to lysis by DBA/2 anti-C3H splenocytes. Clone 11.7 cells also failed to lyse YAC-1 targets in a 4-h ^{51}Cr-release assay, while the same targets were lysed by C57BL/6 splenocytes that are rich in NK cells, as well as by DBA/2 anti-C3H splenocytes. Moreover, electron microscopic analysis showed that the clone 11.7 cells are morphologically lymphoid cells and lack cytoplasmic granules that are characteristic of NC cells.

In vivo immunosuppressive capacity of these bone marrow–derived anti-self suppressor cells was tested in a skin allograft model. All B6AF$_1$ recipient mice (H-2$^{b/k,d}$) were treated with ALS on days −1 and +2 relative to skin grafting on day 0. In one experiment, a C3H bone marrow–derived clone (clone 3.2 cells, 2.5 × 10^7 cells) was injected intravenously into B6AF$_1$ mice bearing H-2D region–incompatible C3H (H-2D^k) skin grafts starting on day +8 followed by injection intraperitoneally of 1 ml of WEHI.CS (equivalent to ~100 U of rIL 3) (Fig. 2 *A*). Four injections of cloned cells and WEHI.CS on postgrafting days 8, 15, 22, and 29 induced significant prolongation of C3H allograft survival. 7 of 10 allografts (70%) survived for ≥ 40 d in mice given

Table 1. *Cytotoxic Activity of Clone 11.7 Cells*

Effector cells	E/T ratio	Percent specific release	
		YAC-1	L929
		mean ± SEM	
C57BL/6	100:1	13.5 ± 0.7	ND
splenocytes	50:1	8.4 ± 0.5	ND
	25:1	5.1 ± 0.3	ND
DBA/2 anti-C3H*	100:1	36.9 ± 1.0	83.2 ± 3.9
splenocytes	50:1	38.3 ± 2.2	87.8 ± 4.9
	25:1	28.0 ± 0.8	72.0 ± 0.8
Clone 11.7 cells	100:1	−2.7 ± 0.3	−4.5 ± 0.6‡
	50:1	−3.4 ± 0.7	−2.6 ± 2.5
	25:1	−3.3 ± 0.7	1.7 ± 1.1

NK- and NC-like activities of clone 11.7 were examined on NK-sensitive (YAC-1) and NC-sensitive (L929) targets. 8-wk-old C57BL/6 splenocytes and DBA/2 anti-C3H splenocytes were used as positive controls. Effector cells were incubated for 4 h with YAC-1 and 18 h with L929 target cells. Results are expressed as percent specific release.

* DBA/2 anti-C3H splenocytes were generated by 5-d mixed lymphocyte culture.

‡ NC activity of clone 11.7 cells was tested with mouse r-IL-3 (10 U/ml) in the media.

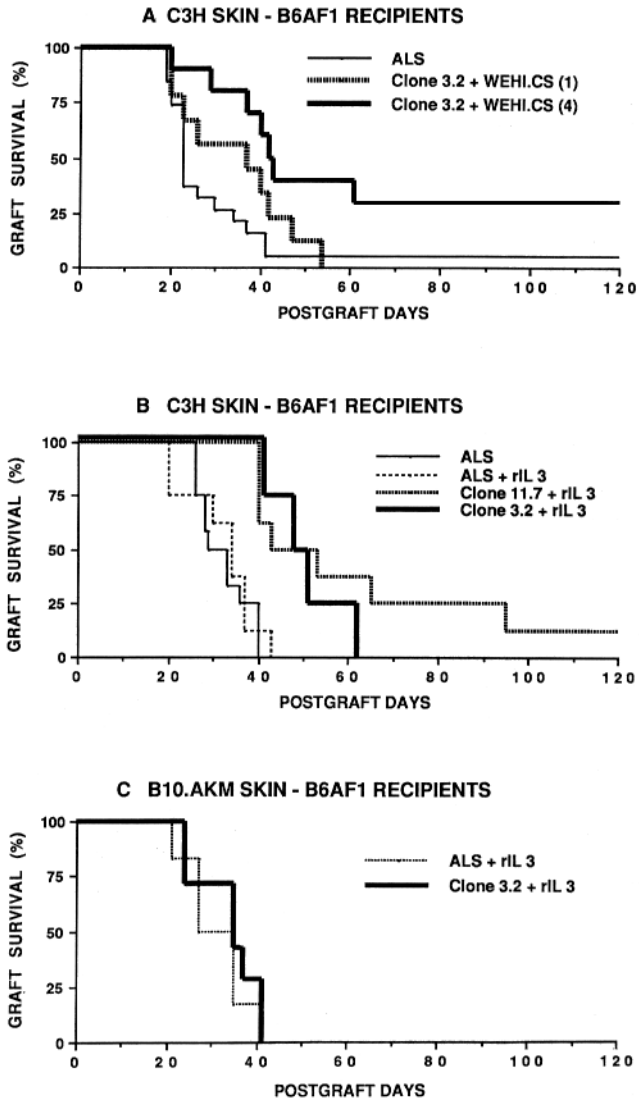

Figure 2. In vivo suppressive activity of IL-3-dependent bone marrow–derived clones. (C57BL/6 × A)F$_1$ (B6AF$_1$) mice were treated with ALS on days −1 and +2 relative to skin grafting on day 0. (*A*) Two groups of B6AF$_1$ mice bearing C3H skin allografts were given either one (n = 9) or four (n = 10) weekly injections of C3H clone 3.2 cells (2.5 × 10^7 cells intravenously) and IL-3 (1 ml of WEHI.CS intraperitoneally) starting on day +8. Control mice received ALS alone (n = 19). (*B*) Two groups of B6AF$_1$ mice bearing C3H skin allografts were given four weekly injections of 2.5 × 10^7 clone 11.7 cells (n = 8) or C3H clone 3.2 cells (n = 4) together with mouse rIL-3 (200 U) starting on day +3. Control mice received ALS alone (n = 12) or ALS and four weekly IL-3 injections (n = 8). (*C*) B6AF$_1$ mice bearing B10.AKM skin allografts were given four weekly injections of rIL-3 (200 U) alone (n = 6) or 2.5 × 10^7 clone 3.2 cells together with IL-3 (n = 7) starting on day +3.

clone 3.2 cells compared with 3/19 (16%) allografts in ALS controls. A single injection of cells together with WEHI.CS on day +8 was less effective. Injection of WEHI.CS alone or other nonsuppressive IL-3-dependent cells together with WEHI.CS, either once or four times according to the same protocol, failed to prolong graft survival (data not shown). In another experiment, clone 11.7 cells or clone 3.2 cells (2.5×10^7 cells) were injected intravenously four times (postgrafting days 3, 10, 17, and 24) together with mouse rIL-3 (200 U) intraperitoneally (Fig. 2 *B*). All allografts survived in recipient mice given cloned cells and IL-3 for ≥40 d, compared with 3 of 12 allografts (25%) in ALS controls or one of seven allografts (14%) in mice given rIL-3 alone four times. In a separate experiment, clone 3.2 cells (2.5×10^7) were given four times (postgrafting days 3, 10, 17, and 24) together with mouse rIL-3 (200 U) to $B6AF_1$ mice grafted with B10.AKM ($H\text{-}2K^k$, I^k, D^q) skin allografts that differ from the recipient mice only at the H-2D locus. As shown in Fig. 2 *C*, the bone marrow–derived suppressor cells failed to prolong skin allografts over rIL-3 controls. Thus, present data show that these bone marrow–derived, IL-3-dependent cells are capable of specifically suppressing immune responses directed against self-MHC antigens expressed on skin allografts.

Cells of the early T cell lineage have been shown to express TCR α, β, γ, and δ genes (24, 25). Therefore, TCR gene transcription of the marrow-derived suppressor clones was analyzed. Total cytoplasmic RNA was analyzed by Northern blotting with TCR α (pHDS 12), β (pHDS 22), γ (pHDS 4), and δ (KN12D1) chain cDNA probes. cDNA clones pHDS 12, pHDS 22, and pHDS 4 represent $J_\alpha + C_\alpha$, $V_{\beta 7} + C_{\beta 2}$, and $C_{\gamma 2}$ gene segments, respectively. KN12D1 cDNA clone is derived from the $J_\delta 1 + C_\delta$ gene segment. Fig. 3 *A* shows that both clone 11.7 and 11.12 cells express 1.5-kb γ-specific RNA, equivalent to full-length V-J-C transcripts of the γ genes. The same blot failed to hybridize with α, β, and δ chain cDNA clones. In contrast, equivalent amounts of RNA from the IL-2-dependent CTLL cells hybridized to α (1.7-kb), β (1.3- and 1.1-kb), and γ (1.5-kb) chain cDNA probes, while RNA from AS-9 suppressor T cell hybridoma cells (11) hybridized to γ (not shown) and δ (1.6-kb) chain cDNA probes. These results indicate that only the TCR γ gene, but not TCR α, β, or δ genes, is transcribed in IL-3-dependent anti-self-specific suppressor cells. The cells were further examined for rearrangement of the TCR β and γ genes by Southern blot analysis. Rearrangement of the $\beta 1$ gene complex was determined with the pHDS 22 probe ($V_{\beta 7} + C_{\beta 2}$) and HindIII-digested DNA from C3H liver cells, clones 11.7 and 11.12 cells, and CTLL cells (Fig. 3 *B*, *a*). Rearrangement of the γ gene was determined with the TCR γ C region ($C_{\gamma 2}$) probe (pHDS 4) and EcoRI-digested DNA (Fig. 3 *B*, *b*). Clones 11.7 and 11.12 showed no rearrangements of TCR β and γ gene clusters. In contrast, CTLL cells showed both TCR β and γ gene rearrangements as previously reported (15).

The phenotype of clone 11.7 was determined by immunofluorescence staining and flow cytometric analysis using a panel of mAbs. As shown in Fig. 4, clone 11.7 cells were strongly positive for Thy-1 determinants, but had no detectable CD4 or CD8 T cell surface markers. Despite the presence of TCR γ gene transcript, clone 11.7 cells express neither cell surface CD3 nor TCR-γ/δ determinants. They do not express IL-2R glycoproteins nor antigens associated with B cells (Lyt-5) and macrophages (Mac 1) (data not shown). With regard to the expression of MHC antigens, both clones 11.7 and 3.2 are positive for class I $H\text{-}2D^k$ antigens, but negative for class II antigens ($I\text{-}A^k$) (Fig. 5). Expression of $H\text{-}2K^k$ was always much less than that of $H\text{-}2D^k$, and ranged from 0 to 10%.

Discussion

The results described here demonstrate that continuous culture of adult bone marrow in IL-3 leads to development of lymphoid cell populations capable of suppressing in vitro as well as in vivo responses to self-MHC antigens. IL-3, which is also known as multilineage CSF (multi-CSF), supports the growth and development of hematopoietic cells from every

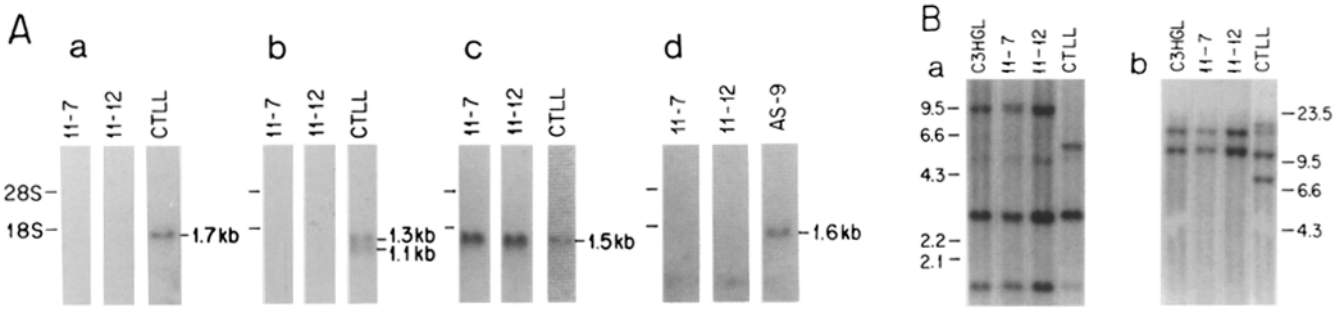

Figure 3. (*A*) Analysis of TCR gene expression by Northern blotting. Total cellular RNA was isolated from bone marrow–derived, IL-3-dependent clones 11.7 and 11.12, IL-2-dependent CTLL cells, and a suppressor T cell hybridoma line, AS-9. After electrophoresis through 1% agarose gels, RNA blots were hybridized to ^{32}P-labeled ($2\text{–}5 \times 10^8$ cpm/μg) TCR α (*a*), β (*b*), γ (*c*), and δ (*d*) chain cDNA clones. Position of rRNA markers are as indicated. (*B*) Analysis of TCR gene rearrangement by Southern blotting. Genomic DNA was isolated from the adult C3H mouse liver, clones 11.7 and 11.12, and CTLL cells. (*a*) HindIII-digested DNA was subjected to electrophoresis on a 0.8% agarose gel and hybridized to ^{32}P-labeled TCR β probe. (*b*) EcoRI-digested DNA was hybridized with a TCR-γ probe. Position of HindIII-cut λ-size markers are as indicated.

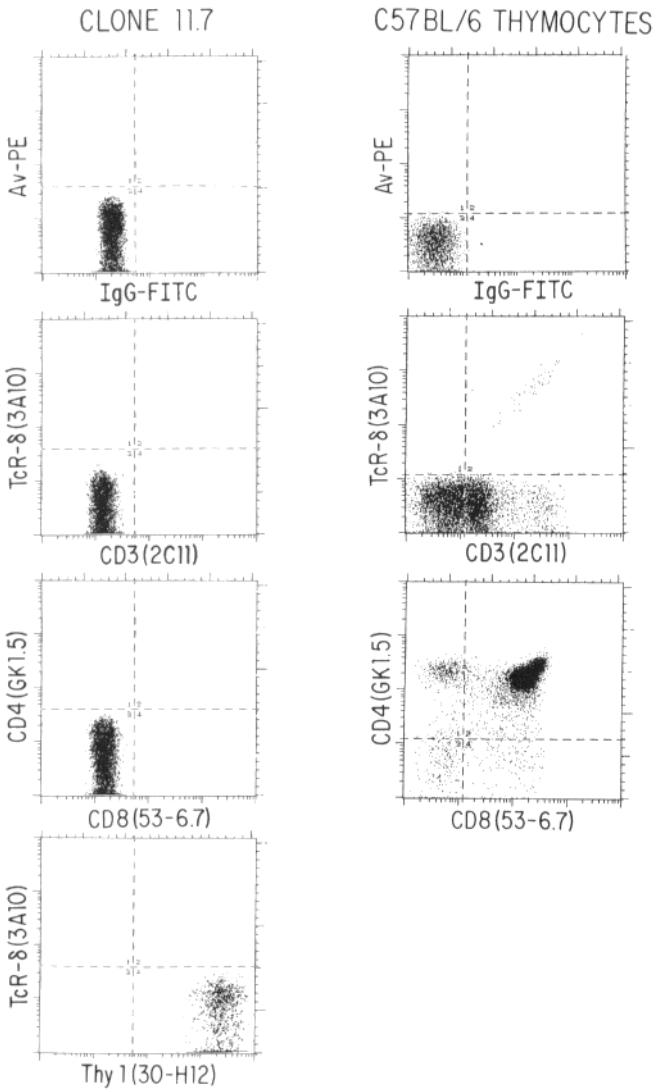

Figure 4. Two-color immunofluorescence analysis of Thy-1, CD3, CD4, CD8, and TCR-γ/δ determinants on IL-3-dependent bone marrow–derived clone 11.7 cells. The clone cells were stained with FITC-conjugated anti-Thy-1 (30-H12) and anti-CD3 (2C11) antibodies, biotin-conjugated anti-δ (3A10) antibodies, and streptavidin-PE. The cells were also stained with anti-CD4 (GK1.5)-PE and anti-CD8 (53-6.7)-FITC. Control cells consisted of thymocytes of a 1-wk-old C57BL/6 mouse. Markers indicated by broken lines were settled according to the negative controls incubated with streptavidin-PE and affinity-purified goat anti-hamster IgG-FITC. Fluorescence intensities are shown in $\log_{10}$ scales.

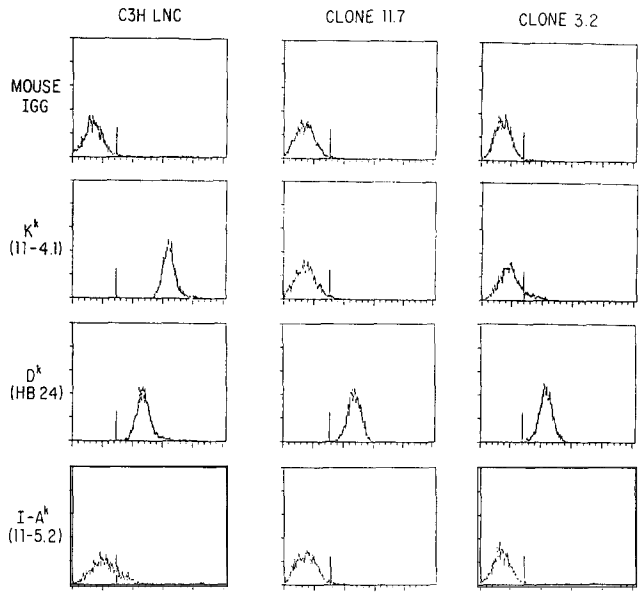

Figure 5. Single-color immunofluorescence analysis of surface MHC antigen expression. Clone 11.7 and 3.2 cells and C3H lymph node cells were treated with mouse IgG (control), anti-H-2K^k (11-4.1), anti-H-2D^k (HB 24), and anti-I-A^k (11-5.2) antibodies. These antibodies were stained with FITC-conjugated goat anti-mouse IgG.

major hemopoietic lineage in vitro and generates multi-lineage colonies from bone marrow cells (26, 27). There had been no well-substantiated evidence that indicated that this cytokine interacts with cells of a lymphoid lineage. Recently, however, Palacios et al. (28) demonstrated that IL-3 is also capable of differentiating early T progenitor cells from adult bone marrow. They have established several cell clones (pro T cells) by in vitro culture of bone marrow from CBA/N-*nu/nu* mice in IL-3. Pro T cells have low levels of Thy-1 and no detectable Lyt-2 or L3T4 surface markers, express short RNA transcripts (~1.0–1.2 kb) of the γ gene, and no RNA transcripts of either the α or the β genes. Their δ gene transcription was not examined. Upon repopulation into the thymus of sublethally irradiated host mice, these cells give rise to phenotypically and functionally mature T cells.

It appears that bone marrow–derived suppressor clones, represented by clone 11.7, are early T lymphocyte lineages similar to the cells described by Palacios et al. (28). Clone 11.7 cells have a high degree of Thy-1 expression but lack other T cell markers such as L3T4 (CD4) and Lyt-2 (CD8). They express class I antigens but no class II antigens. The reason for more expression of H-2D^k antigen than H-2K^k antigen is not immediately known. It could be nonspecific suppression due to long-term culture, or specific suppression through an event occurring in or near the genes controlling H-2K^k antigen expression. While they do not have a cell surface CD3/TCR complex, they express 1.5-kb RNA transcripts of the TCR γ gene. TCR α, β, or δ genes were not transcribed in these cells. To our surprise, Southern blot analysis revealed no TCR γ gene rearrangement in these cells. Significance of TCR γ RNA expression without TCR γ gene rearrangement is not immediately known. Recent reports by Weinstein et al. (29) described expression of high-level γ gene transcripts in IL-3-dependent leukemia cell lines, suggesting that IL-3 may influence the ability of lymphoid/myeloid progenitors to commit to the T cell lineage. Interestingly, like clones 11.7 and 11.12 in our study, IL-3-dependent leukemia cells in Weinstein's study (29) showed no genomic γ gene rearrangement despite apparent full-size transcription of the γ gene. Transcription of TCR γ gene was shown to be dependent on the presence of IL-3 because TCR γ gene tran-

scripts were lost in the absence of IL-3 and reappeared when IL-3 was added back. Although the TCR gene rearrangements are thought to occur primarily in the thymus, extrathymic γ gene rearrangements have been reported. For example, γ gene rearrangement occurs in fetal liver (30), in bone marrow culture in the presence of Con A–conditioned medium (31), and in the long-term cultured bone marrow cells of athymic nude mice (32).

What are the functions of these early extrathymic cells expressing the TCR γ gene? While no function has been assigned to the cells described in the previous reports (28, 29, 32), present studies demonstrate that IL-3-dependent, bone marrow–derived, TCR γ RNA-positive clones such as clone 11.7 are capable of suppressing the immune response directed against their own MHC antigens; i.e., they function as veto cells. When clone 11.7 cells were added to allogeneic MLC, they inhibited CTL generation against their surface antigens shared by stimulator cells. They had no effect in MLC of third-party responder-stimulator combinations. In addition, administration of bone marrow–derived clones into immunosuppressed recipients prolonged survival of skin allografts that shared the same MHC antigens as those of the added cells. These marrow-derived clones were not effective in prolonging third-party skin allografts of comparable histoincompatibility (i.e., B10.AKM skin allografts in $B6AF_1$ mice). The relatively short prolongation observed in these experiments could be attributed to too low an IL-3 dose (100–200 U/wk) and/or inappropriate cell doses (2.5×10^7 cells/injection) used in those experiments. Because these marrow-derived clones have been in culture for a long period, it is possible that the majority of injected cells may be trapped and destroyed in the reticuloendothelial system before they exert their function. Further experiments are in progress to determine the optimal dose (numbers) and timing of IL-3 and cells to achieve maximal suppressive effect. Nevertheless, the present results suggest that these immature cells may play an important role in the induction of self-tolerance very early in T cell ontogeny.

While these cells might eventually home to the thymus and differentiate into mature T cells, repeated attempts to repopulate clone 11.7 cells ($H\text{-}2^k$, Thy-1.2) in the thymus of irradiated AKR mice ($H\text{-}2^k$, Thy-1.1) have thus far failed. Therefore, it is possible that these cells may represent a unique population of immature lymphoid cells that remain in the extrathymic environment. Suppression of anti-self immunity by these cells could represent an alternative mechanism to intrathymic elimination of self-reactive T cells (33–36) for the induction of self-tolerance. These cells may remain dormant in the bone marrow except when suppression of anti-self-MHC reactivity is required during the early ontogeny of T cell differentiation. In a bone marrow–induced allograft unresponsiveness model (1), activation and proliferation of these cells in donor marrow occur as a result of increased production of IL-3 by the host T lymphocytes after alloantigen stimulation.

What is the mechanism of the observed suppression of CTL generation by clone 11.7 cells? It is unlikely that suppression is mediated by lysis of stimulator cells by these cloned cells. Clone 11.7 lacked lytic activity of NC and NK cells, and failed to lyse syngeneic $H\text{-}2^k$ targets (L929 cells) in an 18-h assay. Alternatively, the suppression by clone 11.7 cells may take place through their recognition of receptors that can recognize self-antigens, i.e., through recognition of anti-self idiotypes. However, this is also unlikely because the bone marrow–derived suppressor cells lack the conventional CD3/TCR complex. Lack of TCR γ gene rearrangement suggests that the TCR γ gene transcript is probably nonfunctional. It cannot be ruled out, however, that the cells possess a unique receptor for anti-self idiotypes that is yet to be identified. A more likely possibility is, as has been suggested previously (37, 38), that the alloreactive T cells bearing receptor to the MHC antigen of stimulator cells recognize the same MHC antigen on suppressor cells that in turn send out an inactivating signal to the recognizing T cells. The final inactivating signal could be an antigen-nonspecific soluble factor, whereas the specificity of suppression is achieved through recognition of class I antigens that are expressed by the suppressor cells. Similarly, in the case of self-tolerance induction, a self-reactive cell, probably a T cytotoxic precursor cell, triggers its own suppression by recognizing the class I MHC antigen shared by the suppressor cell and itself (the self-reactive cell). This hypothesis is supported by the in vitro experiments in which significant suppression of CTL generation occurred when stimulator cells possessed $H\text{-}2K^k$ and/or $H\text{-}2D^k$ antigens but not H-2 $I\text{-}A^k$ and $I\text{-}E^k$ antigens alone. Marked suppression of anti-$H\text{-}2K^k$ CTL generation by these suppressor cells despite their low expression of $H\text{-}2K^k$ antigens needs further investigation. It could be that very low expression of class I MHC antigens is sufficient to induce suppression. Alternatively, suppression may be mediated through possible crossreactivity of anti-$H\text{-}2K^k$ T cytotoxic precursor cells with $H\text{-}2D^k$ determinants.

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References

1. Monaco, A.P., and M.L. Wood. 1970. Studies on heterologous ALS in mice. VII. Optimal cellular antigen for induction of immunologic tolerance in ALS. *Transplant. Proc.* 2:489.
2. Wood, M.L., and A.P. Monaco. 1980. Suppressor cells in specific unresponsiveness to skin allografts in ALS-treated, marrow injected mice. *Transplantation (Baltimore).* 29:196.
3. Maki, T., R. Gottschalk, M.L. Wood, and A.P. Monaco. 1981. Specific unresponsiveness to skin allografts in antilymphocyte serum-treated, marrow-injected mice: participation of donor marrow-derived suppressor T cells. *J. Immunol.* 127:1433.
4. Miller, R.G., and H. Derry. 1979. A cell population in nu/nu spleen can prevent generation of cytotoxic lymphocytes by normal spleen cells against self antigens of the nu/nu spleen. *J. Immunol.* 122:1502.
5. Miller, R.G. 1980. An immunological suppressor cell inactivating cytotoxic T lymphocyte precursor cells recognizing it. *Nature (Lond.).* 287:544.
6. Muraoka, S., and R.G. Miller. 1980. Cells in bone marrow and in T cell colonies grown from bone marrow can suppress generation of cytotoxic T lymphocytes directed against self antigen. *J. Exp. Med.* 152:54.
7. Fink, P.J., H.-G. Rammensee, and M.J. Bevan. 1984. Cloned cytolytic T cells can suppress primary cytotoxic responses directed against them. *J. Immunol.* 133:1775.
8. Fink, P.J., H.-G. Rammensee, J.D. Benedetto, U.D. Staerz, L. Lefrancois, and M.J. Bevan. 1984. Studies on the mechanism of suppression of primary cytotoxic responses by cloned cytotoxic T lymphocytes. *J. Immunol.* 133:1769.
9. Rammensee, H.-G., P.J. Fink, and M.J. Bevan. 1984. Functional clonal deletion of Class I-specific cytotoxic T lymphocytes by veto cells that express antigen. *J. Immunol.* 133:2390.
10. Miller, R.G. 1980. How a specific anti-self deletion mechanism can affect the generation of the specificity repertoire. *In* Strategies of Immune Regulation. E.E. Sercarz and A.J. Cunningham, editors. Academic Press, New York. 507.
11. Maki, T., S. Satomi, M. Gotoh, and A.P. Monaco. 1986. Contra-IL2; a suppressor lymphokine that inhibits IL2 activity. *J. Immunol.* 136:3298.
12. Itohara, S., N. Nakanishi, O. Kanagawa, R. Kubo, and S. Tonegawa. 1989. Monoclonal antibodies specific to native murine T-cell receptor $\gamma\delta$: analysis of $\gamma\delta$ T cells during thymic ontogeny and in peripheral lymphoid organs. *Proc. Natl. Acad. Sci. USA.* 86:5094.
13. Glisin, V., R. Crkvenjakow, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry.* 13:2633.
14. Chirgwin, J.M., A.E. Prylbyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294.
15. Saito, H., D.M. Kranz, Y. Takagaki, A.C. Hayday, H.N. Eisen, and S. Tonegawa. 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature (Lond.).* 309:757.
16. Saito, H., D.M. Kranz, Y. Takagaki, A.C. Hayday, H.N. Eisen, and S. Tonegawa. 1984. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature (Lond.).* 312:36.
17. Takagaki, Y., N. Nakanishi, I. Ishida, O. Kanagawa, and S. Tonegawa. 1989. T cell receptor-γ and -δ genes preferentially utilized by adult thymocytes for the surface expression. *J. Immunol.* 142:2112.
18. Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
19. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
20. Jadus, M.R., and R. Parkaman. 1986. The selective growth of murine newborn-derived suppressor cells and their probable mode of action. *J. Immunol.* 136:783.
21. Pope, B.L. 1987. Secretion of a suppressor cell-inducing factor by an Interleukin 3-dependent cell line with natural cytotoxic activity. II. Range, potency, and kinetics of suppressive activity. *Immunobiology.* 174:107.
22. Jadus, M.R., G. Schmunk, J.Y. Djeu, and R. Parkman. 1986. Morphology and lytic mechanisms of interleukin 3-dependent natural cytotoxic cells: tumor necrosis factor as a possible mediator. *J. Immunol.* 137:2774.
23. Djeu, J.Y., E. Lanza, S. Pastore, and A.J. Hapel. 1983. Selective growth of natural cytotoxic but not natural killer effector cells in interleukin-3. *Nature (Lond.).* 306:788.
24. Kronenberg, M., G. Siu, L.E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.* 4:529.
25. Chien, Y.-h., M. Iwashima, D.A. Wettstein, K.B. Kaplan, J.F. Elliot, W. Born, and M.M. Davis. 1987. T-cell receptor δ gene rearrangements in early thymocytes. *Nature (Lond.).* 330:722.
26. Ihle, J.N., L. Pepersack, and L. Rebar. 1981. Regulation of T cell differentiation: in vitro induction of 20-a-hydroxysteroid dehydrogenase in splenic lymphocytes is mediated by a unique lymphokine. *J. Immunol.* 126:2184.
27. Ihle, J.N., J. Keller, S. Oroszlan, L.E. Henderson, T.D. Copeland, F. Fitch, M.B. Prystowsky, E. Goldwasser, J.W. Schrader, E. Palaszynski, M. Dy, and B. Lebel. 1983. Biologic properties of homogeneous interleukin 3. *J. Immunol.* 131:282.
28. Palacios, R., M. Kiefer, M. Brockhaus, K. Karjalainen, Z. Dembic, P. Kisielow, and H. von Boehmer. 1987. Molecular, cellular, and functional properties of bone marrow T lymphocyte progenitor clones. *J. Exp. Med.* 166:12.
29. Weinstein, Y., K. Morishita, J.L. Cleveland, and J.N. Ihle. 1989. Interleukin 3 (IL-3) induces transcription from nonrearranged T cell receptor γ loci in IL-3-dependent cell lines. *J. Exp. Med.* 169:2059.
30. Haars, R., M. Kronenberg, W.M. Gallatin, I.L. Weissman, F.L. Owen, and L.E. Hood. 1986. Rearrangement and expression of T cell antigen receptor and gamma genes during thymic development. *J. Exp. Med.* 164:1.
31. Hurwitz, J.L., J. Samaridis, and J. Pelkonen. 1988. Progression of rearrangements at T cell receptor β and γ gene loci during athymic differentiation of bone marrow cells *in vitro*. *Cell.* 52:821.
32. Yoshikai, Y., Y. Takeda, S. Ohga, K. Kishihara, G. Matsuzaki, and K. Nomoto. 1989. Rearrangements of T-cell antigen receptor γ and δ chain genes are detected in the long-term cultured bone marrow cells of athymic nude mice but not in those of euthymic mice. *Immunology.* 66:512.
33. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273.
34. Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature (Lond.).* 333:742.

35. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73.
36. Sprent, J., and S.R. Webb. 1987. Function and specificity of T cell subsets in the mouse. *Adv. Immunol.* 41:39.
37. Fink, P.J., R.P. Shimonkevitz, and M.J. Bevan. 1988. Veto cells. *Annu. Rev. Immunol.* 6:115.
38. Miller, R.G., S. Muraoka, M.H. Claesson, J. Reinmann, and P. Benveniste. 1988. The veto phenomenon in T-cell regulation. *Ann. NY Acad. Sci.* 532:170.