Stimulation of Mature Unprimed CD8+ T Cells by Semiprofessional Antigen-presenting Cells In Vivo

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Summary

To test whether unprimed CD8+ cells can recognize class I alloantigens presented selectively on non-bone marrow (BM)-derived cells, unprimed parental strain CD8+ cells were transferred to long-term parent → F_1 BM chimeras prepared with supralethal irradiation. Host class I expression in the chimeras was undetectable on BM-derived cells and, in spleen, was limited to low-level staining of vascular endothelium and moderate staining of follicular dendritic cells (a population of nonhemopoietic cells in germinal centers). Despite this restricted expression of antigen, acute blood-to-lymph recirculation of parental strain T cells through the chimeras led to selective trapping of 95% of CD8+ cells reactive to normal F_1 spleen antigen presenting cells (APC) in vitro. Subsequently, a small proportion of the trapped cells entered cell division and gave rise to effector cells expressing strong host-specific CTL activity. The activation of host-specific CD8+ cells was also prominent in double-irradiated chimeras, and cell separation studies showed that the effector cells were generated from resting precursor cells rather than from memory-phenotype cells. It is suggested that the non-BM-derived cells in the chimeras acted as semiprofessional APC. These cells were nonimmunogenic for most host-reactive CD8+ cells but were capable of stimulating a small subset of high-affinity T cells. The possible relevance of the data to the prolonged immunogenicity of vascularized allografts in humans is discussed.

T cell responses leading to allograft rejection are initiated predominantly by BM-derived cells, especially dendritic cells (DC)[1] (1–3). These cells migrate from the graft to the T-dependent areas of the lymphoid tissues and cause "central" sensitization of recirculating host T cells (4). In accordance with the notion that graft rejection is controlled by BM-derived (BMD) cells, it is well established that removing these "passenger leukocytes" from the grafts generally leads to long-term survival (1). This finding also applies when grafts are selectively depleted of DC (5).

Although depleting grafts of BMD cells is often highly efficient in causing graft acceptance in rodent models, this approach is less successful in clinical transplantation (6, 7) and also in a pig model (8). This finding might reflect that the procedures used to deplete BM-derived cells from large allografts, e.g., kidneys, are relatively inefficient. Nevertheless, it is notable that patients receiving vascularized allografts have a high risk of rejection for many years and are usually maintained permanently on immunosuppression. This long-term propensity for graft rejection is surprising because typical DC appear to be relatively short-lived cells (9). So, which cells provide the stimulus for late graft rejection? In the case of large vascular grafts, there is considerable interest in the idea that certain non-BM-derived (NBMD) cells can play a role in T cell sensitization (10). In contrast to the rat, fresh human endothelial cells express MHC molecules and these cells are reported to be immunogenic for unprimed T cells in vitro (11, 12).

Most of the evidence on the immunogenicity of NBMD cells has come from studies with IFN-γ–induced cell lines passaged in vitro. A key issue is whether unprimed T cells can be stimulated by NBMD cells under physiological conditions in vivo. We have approached this question by studying the effects of transferring parent a T cells to a → (a × b)F_1 BM chimeras (BMC) prepared with supralethal irradiation (1,300 cGy) (13). Such parent → F_1 BMC show rapid clearance of host BMD cells and, by 2 mo after transfer, host MHC (Ia) expression is limited to low-level staining of vascular endothelium and follicular dendritic cells (FDC) (a population of NBMD cells in germinal centers). In previous studies, we reported that the environment of parent → F_1 BMC is relatively nonimmunogenic for parental strain CD4+ cells,

[1] *Abbreviations used in this paper:* BMC, bone marrow chimeras; BMD, bone marrow-derived; CTLpf, cytotoxic T lymphocyte precursor frequency; DC, dendritic cells; FDC, follicular dendritic cells; HSA, heat-stable antigen; LDA, limiting dilution analysis; Mϕ, macrophage; NBMD, non-bone marrow-derived; PALS, periarteriolar lymphocyte sheaths; PNA, peanut agglutinin; TDL, thoracic duct lymph.

although prolonged exposure eventually leads to T cell priming (13).

In this paper we show that transferring unprimed parental strain $CD8^+$ cells to long-term parent → F_1 BMC induces specific trapping (sequestration) of 95% of $CD8^+$ cells reactive to normal F_1 spleen cells in vitro. In addition, a small proportion of the trapped $CD8^+$ cells are induced to divide and differentiate into effector cells with highly potent CTL function within 4 d. The data thus imply that, under in vivo conditions, NBMD cells can act as "semiprofessional" APC. These cells are highly efficient in causing T cell trapping but are also able to induce a small proportion of T cells to differentiate into effector cells. We speculate that these latter cells represent a subset of high-affinity cells.

Materials and Methods

Mice. C57BL/6 (B6), B6.PL-Thy 1^a/Cy (Thy 1.1), CBA/CaJ (CBA), (B6 × CBA)F_1, B10.BR, B10.D2, (B6 × B10.D2)F_1, (B6 × B10.BR)F_1, and (B10.BR × B10.D2)F_1 mice were bred at the Scripps Research Institute.

Irradiation. Mice were exposed to various doses of γ-irradiation from a ^{137}Cs source (80 cGy/min) delivered by an irradiator (Gammacell 40; Atomic Energy of Canada, Ottawa, Canada). Cells were irradiated with an irradiator (Gammacell 1000; Atomic Energy of Canada) (450 cGy/min).

Media. HBSS supplemented with 2.5% gamma globulin-free horse serum (Gibco Laboratories, Grand Island, NY) was used for preparation of single cell suspensions. DME supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 5% NCTC-109, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics was used for in vitro culture. Phenol red free HBSS supplemented with 1% gamma globulin-free horse serum and 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) was used for immunofluorescence staining.

mAbs. mAbs specific for Thy 1 (T24, rat IgG) (14); Thy 1.2 (Jlj, rat IgM) (15); Thy 1.1 (19E12, mouse IgG) (16); CD4 (GK1.5, rat IgG and RL172, rat IgM) (17, 18); CD8 (YTS169, rat IgG and 3.168.8, rat IgM) (19); heat-stable antigen (HSA) (J11d, rat IgM) (15); K^k (12.2.2s, mouse IgM (20); D^b (28-14-8s, mouse IgG) (21); I-A^k (10-2.16, mouse IgG) (22); I-A^b (28-16-8s, mouse IgM) (21); a macrophage (Mϕ) marker (F4/80, rat IgG) (23); IL2R p55 (7D4, rat IgM) (24); and MEL-14 (rat IgG) (25) were prepared as ascites fluid or culture supernatant. mAb for FDC (FCD-M1, rat IgG) was a gift from Dr. M. Kosco (Basel Institute, Basel, Switzerland), and mAbs for CD44 (KM201, rat IgG) (26) and very late antigen (VLA)-4α (PS/2, rat IgG) were kindly provided by Drs. K. Miyake and Dr. P. W. Kincade (Oklahoma Medical Research Foundation, Oklahoma City). mAb for CD45RB (23G2, rat IgG) (Pharmingen, San Diego, CA) and PE-conjugated anti-CD8 mAb (Gibco Laboratories) were purchased.

Preparation of BMC. 8–12-wk-old (B6 × CBA)F_1 mice were exposed to 1,300 cGy γ-irradiation and then injected intravenously with $4–8 \times 10^6$ anti-Thy 1 mAb plus C-treated B6.PL BM cells (13). 2–6 d later the hosts received a cocktail of anti-T cell mAbs (YTS169, GK1.5, and T24 ascites fluid) to eliminate radio-resistant T cells. At 6 mo after reconstitution, some BMC received a second dose of irradiation (900 cGy) followed by reconstitution with T-depleted B6 BM and further in vivo treatment with anti-T cell mAbs. All BMC were maintained on antibiotics added to the drinking water.

Purification of T Cell Subsets. For cell transfer, T cells were purified from LN using pooled cervical, axillary, inguinal, subscapular, and mesenteric nodes. To deplete non-T cells, LN suspensions were treated with anti-HSA and anti-I-A mAb plus C in vitro at 37°C, and then washed before injection. To prepare purified $CD8^+$ cells for MLR and CTL assays, lymph-borne cells were treated with a mixture of anti-HSA, anti-I-A^b and anti-CD4 (RL172) mAb plus C before use. In some experiments, T cells were separated on stepwise Percoll (Pharmacia, Uppsala, Sweden) density gradients as described elsewhere (27).

T Cell Selection in Irradiated Hosts. As described elsewhere (13), long-term BMC and control mice were exposed to 950 cGy irradiation and injected intravenously 4–6 h later with $0.8 - 10^8$ purified T cells. Cannulas were placed in the thoracic duct 10–12 h later and thoracic duct lymph (TDL) was collected on ice for up to 5 d. Lymph-borne cells collected from two to four mice per group were stained for surface markers and treated with anti-CD4 mAb plus C for in vitro assays. For staining analysis, any residual donor BM-derived T cells were gated out with the aid of biotinylated anti-Thy 1.1 mAb (see FACS® analysis). For functional assays, these T cells were removed with anti-Thy 1.1 mAb plus C.

MLR. As described previously (28), doses of $0.4–1.6 \times 10^5$ responder cells were cultured in microtiter plates with 5×10^5 2,000 cGy-irradiated spleen cells as stimulators in a volume of 200 μl for 84 h. Triplicate cultures were pulsed with 1 μCi [^{3}H]TdR during the last 12 h of culture.

In Vitro Generation of CTL. To generate CTL in bulk cultures, doses of 10^6 purified $CD8^+$ cells from TDL were cultured with 4×10^6 2,000 cGy-irradiated (B10.BR × B10.D2)F_1 spleen cells in 2-ml wells together with 2% supernatant from stimulated EL-4 cells as a source of lymphokines. EL-4 cells were kindly provided by Dr. O. Kanagawa (Washington University Medical School, St. Louis, MO). After 5 d, the cultures were harvested, counted, and tested for lysis of 10^4 ^{51}Cr-labeled target cells. Target cells were prepared by stimulating B6, B10.BR, or B10.D2 spleen cells for 64 h with 2.5 μg/ml Con A and 10% (vol/vol) stimulated EL-4 supernatant. ^{51}Cr-release was measured after 4 h, using 0.5% Triton X-100 to measure maximal ^{51}Cr-release. The percent specific lysis was calculated by standard techniques. Spontaneous release was always <20% of maximal release.

Limiting dilution analysis (LDA) was used to measure CTL-precursor frequency. LDA was performed as described elsewhere (29). For each dose of responder cells, T cells were plated in 48 replicate cultures containing 5×10^5 (B6 × B10.BR)F_1 or (B6 × B10.D2)F_1 stimulator cells with 2.0% stimulated EL-4 supernatant in 0.2 ml volumes. CTL activity was tested using both syngeneic and specific target cells. Precursor frequencies were determined according to Poisson distribution.

To measure direct CTL activity of in vivo-stimulated T cells, TDL cells were washed and then tested immediately for lysis of ^{51}Cr-labeled Con A blasts or 2C11 B hybridoma cells (30) as described above.

Assay for Lethal GVHD. Unless stated otherwise, host mice were exposed to 500 cGy irradiation several hours before transfer of donor T cells. Antibiotics were given in the drinking water for the first 6 d, and the host mice were inspected daily until death. The day of death was defined as the day on which mice were unable to take food or water. Such mice were killed to prevent suffering.

FACS® Analysis. TDL cells were incubated with various unconjugated mAbs specific for memory/activation markers, followed by FITC-labeled mouse F(ab)$_2$ anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA), goat F(ab)$_2$ anti-mouse Fcγ (Cappel Laboratories, Durham, NC), or rat F(ab)$_2$

anti-mouse Ig (Jackson ImmunoResearch Laboratories), according to the class of the unconjugated mAbs. After blocking of free binding sites by rat and/or mouse normal sera, cells were stained with PE-conjugated anti-CD8 mAb. For early lymph collections, any residual BM-derived cells were gated out with the aid of biotinylated anti-Thy 1.1 mAb and streptavidin RED613 (Gibco Laboratories). Dead cells were stained with propidium iodide (Sigma Chemical Co.) and gated out for analysis. Stained cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). All instrument settings and the markers used for each staining were fixed during the course of each experiment.

Immunohistochemistry. Freshly cut 5-μm cryostat sections were stained as described (31). Briefly, cryostat sections were acetone fixed and incubated with pretitrated concentrations of unconjugated or biotinylated antibodies or biotinylated peanut agglutinin (PNA) (Biomeda, Foster City, CA), washed and incubated with either peroxidase-conjugated streptavidin or anti-rat IgG (Jackson ImmunoResearch Laboratories). For staining with the unconjugated mAb F4/80, an intermediate incubation step with biotinylated anti-rat IgG (Jackson ImmunoResearch) was used. After staining, sections were washed and and developed with the substrate 3-amino-9-ethylcarbazole (31). Sections were mounted and photographed using a green filter.

Results

Experimental Design. Parent → F_1 BMC were prepared by exposing (B6 × CBA)F_1 (H-2^b × H-2^k) mice to 1,300 cGy and then reconstituting the mice intravenously with T-depleted Thy 1-marked B6.PL (H-2^b, Thy 1.1) BM cells. Within 1 wk, the mice were injected with a mixture of anti-T cell mAb to remove residual radioresistant host T cells. The chimeras were left for at least 6 mo before use as T cell hosts. Some chimeras received a second course of irradiation and BM reconstitution 6 mo after transfer and were used 2–3 mo later. Unless stated otherwise, all chimeras and control hosts were conditioned with 900 cGy just before T cell transfer.

Histology. In previous studies, spleen and thymus suspensions from long-term parent → F_1 chimeras were found to be completely devoid of host-type APC in terms of stimulating MLR in vitro (32). In sections, mononuclear cells expressing a high density of host H-2 class II molecules were undetectable in spleen, LN, skin, and gut (13, and our unpublished results). Host class II expression was limited to low-level staining of vascular endothelium and a subset of germinal center cells; the latter were presumed to be FDC (a class of NBMD cells). Class I expression was not examined.

Host class I (K^k) expression in the spleen of parent → F_1 chimeras is shown in Fig. 1. In striking contrast to normal (B6 × CBA)F_1 mice which display strong class I (K^k) expression throughout the spleen (Fig. 1 *A*), host K^k expression in B6.PL → (B6 × CBA)F_1 BMC is limited to low-level staining of vascular endothelium and quite strong staining of germinal centers (Fig. 1, *C* and *D*). The staining of germinal centers is probably restricted to FDC because in serial sections an essentially similar pattern of staining is seen with an FDC-specific antibody (Fig. 1 *F*). The germinal center areas are PNA^+ (Fig. 1 *E*) and also express host class II (I-A^k) molecules (13, and Fig. 1 *G*). The possibility that radioresistant host Mϕ accounted for the staining in germinal centers seems most unlikely because, as detected by F4/80 expression, Mϕ are restricted to the red pulp (Fig. 1 *H*).

Negative Selection. As discussed elsewhere (13), the technique of acute blood-to-lymph recirculation of T cells through allogeneic hosts is a highly sensitive method of studying the earliest stages of allorecognition. When parental strain T cells are transferred to irradiated F_1 hybrid mice, T cells with host reactivity migrate to the lymphoid tissues and contact host BMD cells, presumably DC. Recognition of MHC antigens on DC traps the host-reactive T cells, with the result that the donor T cells entering TDL are selectively depleted of host reactivity. This period of negative selection lasts for 1–2 d after transfer and affects both $CD4^+$ and $CD8^+$ T cells.

To study trapping of parental strain $CD8^+$ cells in BMC hosts, long-term B6.PL → (B6 × CBA)F_1 BMC (henceforth termed B6.PL → F_1 BMC) were exposed to 900 cGy and injected intravenously 2–4 h later with a large dose of unseparated B6 LN T cells. Lymph-borne cells were collected 16–32 h after T cell injection, and donor-derived $CD8^+$ cells were purified by mAb plus C treatment (see Materials and Methods). The data in Table 1 show primary MLR of B6 $CD8^+$ cells after blood-to-lymph recirculation through normal B6.PL mice vs. B6.PL → F_1 BMC. MLR were measured in vitro using normal spleen cells as stimulators. As expected, B6 $CD8^+$ cells filtered through H-2 (H-2^b)-identical B6.PL hosts gave high MLR to F_1 stimulator cells expressing H-2^k or H-2^d antigens. The surprising finding was that filtering B6 $CD8^+$ cells through B6.PL → F_1 BMC caused complete removal of the host-reactive cells: the lymph-borne $CD8^+$ cells responded strongly to third-party H-2^d spleen cells, but gave no detectable response to H-2^k spleen cells. The same finding occurred when twice-irradiated BMC were used as hosts (Table 1).

Similar data were obtained when trapping was measured in terms of CTL precursors. As shown in Fig. 2 *A*, B6 $CD8^+$ cells filtered through syngeneic B6.PL hosts generated strong CTL activity against both H-2^k (B10.BR) and H-2^d (B10.D2) target cells after stimulation with (B10.BR × B10.D2)F_1 spleen stimulators in vitro. By contrast, B6 $CD8^+$ cells filtered through the BMC generated only H-2^d-reactive CTL and not H-2^k-reactive CTL.

The above data refer to CTL generated in bulk cultures. To seek quantitative information on the extent of trapping in BMC hosts, we used LDA. This approach gives an accurate estimate of CTL precursor frequency (CTLpf). As shown in Fig. 2 *B* and Table 2, in vitro stimulation of B6 $CD8^+$ cells filtered through B6.PL mice generated a high CTLpf for both H-2^k and H-2^d (about 1% for each). Filtering B6 $CD8^+$ cells through normal F_1 hosts reduced the CTLpf for H-2^k by 2 logs but caused only a small reduction in the CTLpf for H-2^d. Based on the ratio of CTLpf for H-2^k vs. H-2^d (Table 2), there was a >99% specific reduction in CTLp for H-2^k. Very similar findings applied when B6 $CD8^+$ cells were filtered through B6.PL → F_1 BMC. Even when the BMC were conditioned with two doses of BM plus irradiation, there was a 94% specific reduction in CTLp for H-2^k (Table 2).

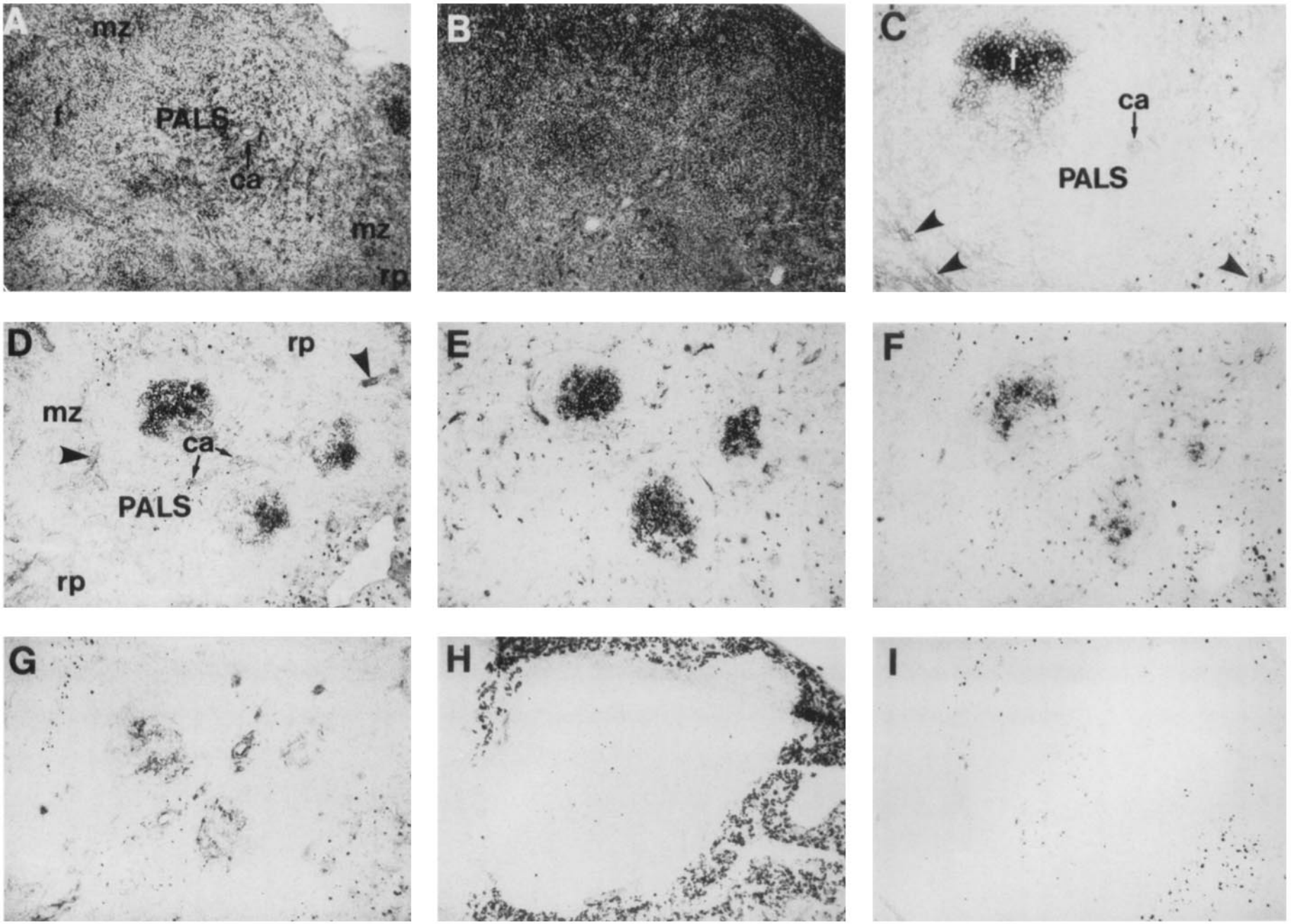
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Table 1. *MLR by B6 CD8⁺ Cells Filtered from Blood to Lymph through B6.PL → F_1 BMC Hosts for 16–32 h: Complete Unresponsiveness to Host H-2^k Antigens*

Filtration hosts for B6 CD8⁺ cells	No. of responder cells for MLR ($\times 10^5$)	[^{3}H]TdR incorporation (cpm $\times 10^3$) with irradiated spleen stimulators		
		B6 H-2^b	(B6 × CBA)F_1 H-2^{bxk}	(B6 × B10.D2)F_1 H-2^{bxd}
Normal B6.PL	1.6	0.2*	65.4	67.0
	0.8	0.1	37.5	46.5
	0.4	0.1	9.7	12.7
B6.PL → F_1 BMC (1,300 cGy)	1.6	0.3	0.5	57.6
B6.PL → F_1 BMC (1,300 + 900 cGy)	1.6	0.1	0.2	64.8

Normal B6.PL mice and long-term BMC prepared with one or two doses of irradiation plus BM were injected intravenously with 10^8 B-depleted B6 LN T cells 4 h after exposure to 900 cGy. Thoracic duct cannulation was performed 10–12 h later and the donor cells were collected from TDL from 16–32 h after injection. The lymph-borne T cells were treated with a mixture of anti-CD4, anti-HSA, anti-I-A and anti-Thy 1.1 mAb plus C to prepare purified donor-derived CD8⁺ cells. These cells were used as responder cells for MLR using irradiated (2,000 cGy) spleen cells as stimulators. MLR were measured on day 3.
* Mean response of triplicate cultures.

The above data indicate that the limited expression of host class I antigens on the stromal (NBMD) cells of BMC is highly efficient at causing trapping of CD8⁺ cells. In considering this finding, it is important to emphasize that T cell trapping in the lymphoid tissues is not necessarily followed by overt stimulation (cell division). In normal F_1 hosts where antigen is presented by DC, ~50% of the trapped host-reactive cells fail to divide (33). The remaining cells proliferate extensively and reenter the circulation as blast cells. This stage of positive selection begins at about 2 d after transfer and reaches a peak at 4–5 d (13). Positive selection of CD8⁺ cells in BMC hosts is discussed below.

Positive Selection. In the case of CD8⁺ cells, positive selection of parental strain T cells in normal F_1 hosts generates large numbers of circulating CD8⁺ blast cells with direct CTL activity (34, 35). These blasts cause rapid lysis of ^{51}Cr-labeled host-type target cells in vitro. This is illustrated in Fig. 3, *C* and *D* where it can be seen that at day 3 (69–82 h) and day 4 (90–106 h) after transfer of B6 T cells to normal F_1 hosts, the donor cells in TDL were highly efficient at causing direct lysis of target cells expressing host-type class I antigens (B10.BR, H-2^k) in vitro. Lysis of B10.BR targets was somewhat higher on day 4 than on day 3. Lysis of syngeneic (B6) targets was negligible (Fig. 3, *A* and *B*).

With transfer of B6 T cells to B6.PL → F_1 BMC, the donor CD8⁺ cells recovered from TDL on day 3 caused only low-level lysis of B10.BR targets (Fig. 3 *C*). At later stages, however, the CTL activity of the lymph-borne cells increased dramatically. Thus, by day 4 after transfer, the anti-B10.BR CTL activity of T cells stimulated in BMC hosts was appreciably higher than for T cells stimulated in normal F_1 hosts (Fig. 3 *D*). The generation of strong CTL activity

Figure 1. Host class I expression in spleens of normal (B6 × CBA)F_1 and long-term B6.PL → (B6 × CBA)F_1 BMC. Cryostat sections of spleens were stained as described in Materials and Methods. Except for A and C, serial sections are shown. All sections were photographed at × 100 except C. (*A*) Normal F_1 stained for K^k; staining is evident throughout the spleen with strong expression on the DC in periarteriolar lymphocyte sheaths (*PALS*), the B cells in the follicles (*f*), and the Mϕ in the marginal zone (*mz*) and the red pulp (*rp*). (*B*) BMC stained for D^b (donor class I). As in A, staining is evident throughout the spleen. (*C*) High power (×160) view of BMC stained for K^k (host class I). There is strong staining of a follicle/germinal center and weak staining of the red pulp sinusoids (*arrowheads*), whereas the PALS is conspicuously negative. (*D*) BMC stained for K^k (host class I). Three areas of strong germinal center staining are seen in the outer white pulp. There is moderate staining of vascular endothelial cells in the marginal zone (*arrowheads*) and the central arteriole (*ca*), whereas the PALS and the red pulp are largely negative. (*E*) BMC stained with PNA. Strong staining of germinal centers in the outer white pulp is evident (compare with *D*). (*F*) BMC stained with an FDC-specific mAb FDC-M1. The pattern of staining in the outer white pulp closely resembles staining for K^k and PNA. (*G*) BMC stained for I-A^k (host class II). Staining is limited to germinal center areas and vascular endothelial cells. (*H*) BMC stained for F4/80 (Mϕ). Staining is restricted to the marginal zone and the red pulp with no staining in the white pulp. (*I*) BMC stained with an irrelevant mAb. Staining is limited to cells with endogenous peroxidase activity. These cells are most prominent in the red pulp, but are also found in the PALS.

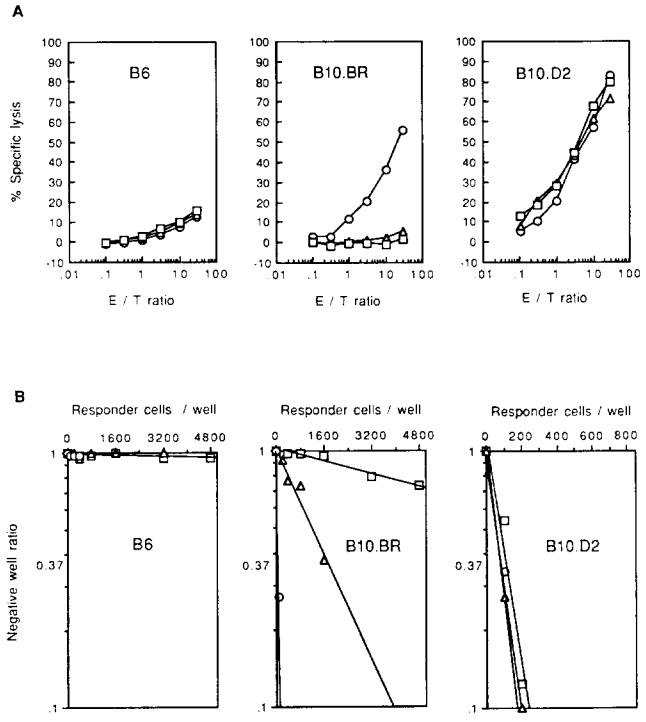

Figure 2. Acute blood-to-lymph recirculation of B6 T cells through irradiated long-term B6.PL → F_1 BMC selectively removes CTL precursors specific for host class I antigens. Normal B6 T cells were transferred intravenously to normal B6.PL mice (○), normal (B6 × CBA)F_1 mice (□), and long-term B6.PL → F_1 BMC (△). The hosts were conditioned with 950 cGy 4–6 h before T cell transfer. Donor cells were collected from TDL of the host mice 16–32 h later and treated with anti-HSA mAb and anti-CD4 mAb plus C to prepare $CD8^+$ cells. (*A*) Lymph-borne B6 $CD8^+$ cells were stimulated with (B10.BR × B10.D2)F_1 (H-2^k × H-2^d) spleen stimulators in vitro for 5 d in bulk cultures and then tested for lysis of ^{51}Cr-labeled B6 (syngeneic), B10.BR (host), and B10.D2 (third-party) Con A blasts as target cells. Specific ^{51}Cr-release for triplicate cultures is known. (*B*) CTLpf were measured by LDA. Graded doses of lymph-borne $CD8^+$ cells were cultured with (B6 × CBA)F_1 spleen stimulators. After 7 d, the cells in each well were split into two portions and tested for lysis of B10.BR and B6 targets. In parallel, B6 $CD8^+$ cells were cultured for 7 d with (B6 × B10.D2)F_1 stimulators and then tested for lysis of B10.D2 and B6 targets. Note that, to prevent overcrowding of the data in the figure, the scale for lysis of B10.D2 targets (*bottom right*) is different than for B10.BR and B6 targets.

in BMC hosts also applied to BMC prepared with double irradiation (Fig. 3, *C* and *D*). CTL activity was specific for B10.BR targets, lysis of B6 targets being very low. No lysis of B10.BR targets occurred when B6 T cells were transferred to syngeneic B6.PL mice (Fig. 3, *C* and *D*).

Similar results were found in a second experiment in which CTL activity in TDL was monitored sequentially on days 3, 4, and 5 after transfer (Fig. 4). In this experiment, it can be seen that the CTL activity of T cells generated in normal F_1 hosts increased progressively from days 3 to 5 (Fig. 4, *D–F*). For T cells transferred to BMC hosts, CTL activity for B10.BR targets was lower than the control T cells on day 3, higher on day 4, and equivalent on day 5. On days 4 and 5, the CTL from both groups of mice caused equivalent nonantigen-specific (redirected) lysis of the anti-CD3 hybridoma, 2C11.

Hourly outputs of cells collected from TDL during the course of the above experiment are shown in Fig. 4, *J–L*. With T cell transfer to syngeneic hosts, cell outputs remained low throughout the experiment. By contrast, with cell transfer to normal F_1 hosts, cell yields were clearly elevated on day 3, and reached peak values on days 4 and 5. With BMC hosts, cell yields were low on day 3 but then increased significantly on days 4 and 5. At this stage, cell outputs were three to fourfold higher than in the control B6 → B6.PL group. Nevertheless, cell yields from the BMC hosts were about sixfold lower than from the normal F_1 hosts. These data refer to total T cells. For both the BMC and normal F_1 group,

Table 2. *Acute Blood-to-Lymph Recirculation of B6 $CD8^+$ Cells through Long-Term B6.PL → F_1 BMC: CTL Precursor Frequencies in $CD8^+$ Cells Recovered at 16–32 h after Injection*

Expt.	Irradiated hosts used for acute blood-to-lymph recirculation of B6 T cells	CTLpf ($\times 10^{-4}$) for target cells: B6 (H-2^b)	B10.BR (H-2^k)	B10.D2 (H-2^d)	Ratio of response to B10.BR vs. B10.D2	Percent specific reduction of response to B10.BR
1	Normal B6.PL	<0.3	140.0	120	1.17	–
	Normal (B6 × CBA)F_1	<0.3	0.6	90	<0.01	>99
	B6.PL → F_1 BMC (1,300 cGy)	<0.3	5.0	120	0.04	97
2	Normal B6.PL	<0.3	120.0	110	1.09	–
	Normal (B6 × CBA)F_1	<0.3	0.4	60	<0.01	>99
	B6.PL → F_1 BMC (1,300 cGy)	<0.3	4.0	90	0.04	96
	B6.PL → F_1 BMC (1,300 + 900 cGy)	<0.3	5.0	80	0.06	94

See Fig. 1 legend and Materials and Methods for details.

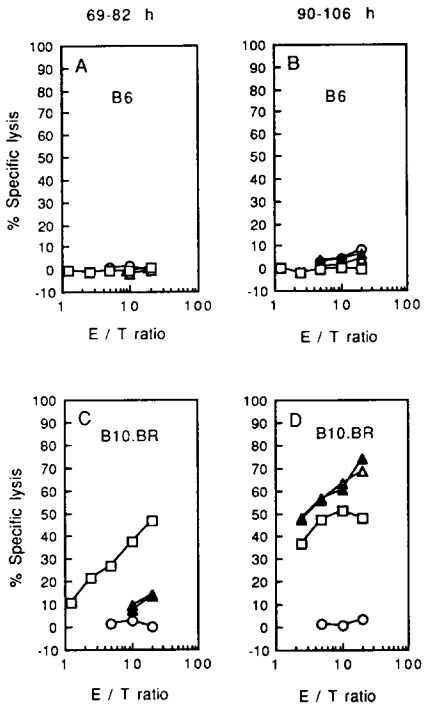

Figure 3. Direct CTL activity by lymph-borne CD8+ cells collected from B6.PL → F_1 BMC at days 3 and 4 after injection of normal B6 T cells. B6 T cells were transferred to normal B6.PL mice (○), normal F_1 mice (□), and long-term B6.PL → F_1 BMC prepared with either a single dose of 1,300 cGy (△) or two consecutive doses of 1,300 cGy and 900 cGy (▲). Mice of each group received 950 cGy 4–6 h before T cell transfer. Thoracic duct cannulas were inserted at 12 h after injection and lymph was collected continuously thereafter. The data show direct CTL activity for ^{51}Cr-labeled B6 and B10.BR Con A blast target cells in vitro for CD8+ cells collected from TDL at 69–82 h (*left*) and 90–106 h (*right*) after initial T cell injection. Specific ^{51}Cr-release for triplicate cultures is shown.

the proportion of CD8+ cells in TDL increased progressively from days 3 to 5, reaching 70–80% on day 5 (see Fig. 4 legend).

Fig. 5 shows the phenotype of CD8+ cells recovered from TDL of the above three groups of host mice at days 1–5 after T cell injection. In the case of CD8+ cells entering the lymph of the control B6 → B6.PL group, the vast majority of the cells in each lymph collection displayed the typical phenotype of recirculating virgin/resting T cells, i.e., CD45RB^{hi}, MEL-14^{hi}, and Pgp-1 (CD44)^{lo}. With T cell transfer to normal F_1 hosts, nearly all of the cells recovered during the stage of negative selection (≤32 h) had a resting phenotype. Thereafter, the lymph contained increasing proportions of blast cells expressing a memory/activated phenotype, i.e., CD45RB^{lo}, MEL-14^{lo}, and Pgp-1^{hi} cells. By day 4–5, nearly all of the cells in the lymph were typical blast cells, most of which displayed activation markers. With T cell transfer to BMC hosts, cells with an activated phenotype began to appear in the lymph at about day 3 after transfer and were the predominant population by day 5.

Similar findings applied in a second experiment (Fig. 6). In this experiment, BMC hosts prepared with the usual single

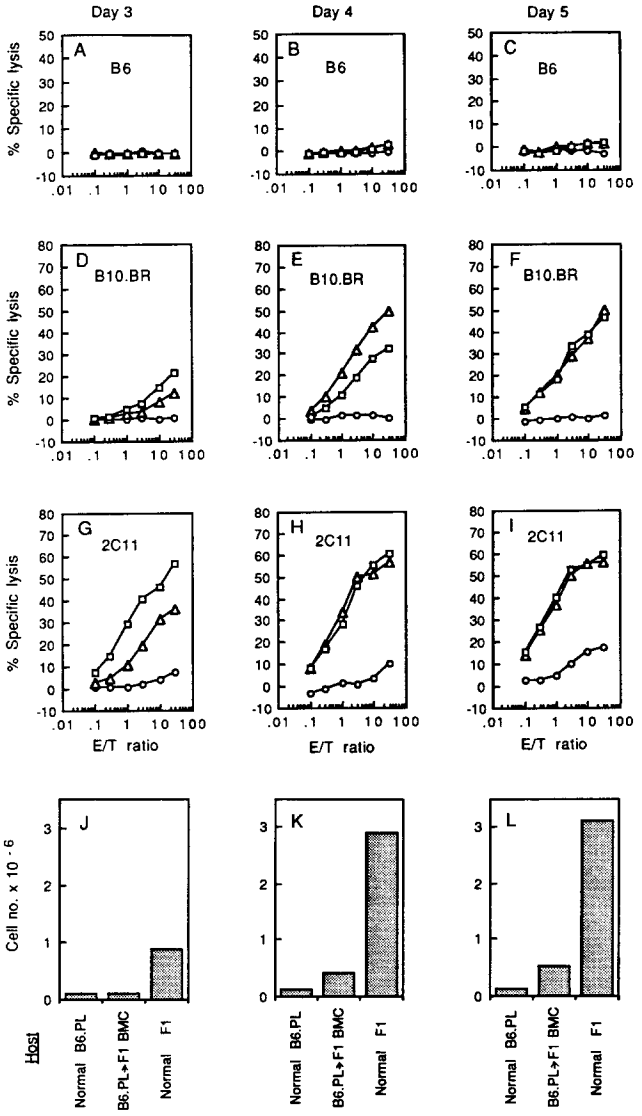

Figure 4. Direct CTL activity and total cell counts in TDL of normal B6.PL mice (○), normal F_1 mice (□), and long-term B6.PL → F_1 BMC (1,300 cGy) (△) at days 3, 4, and 5 after injection of normal B6 T cells (see Fig. 3). At each time point, TDL from the three groups of mice were tested for direct CTL activity on B6 and B10.BR Con A blasts and also on the 2C11 cell line (a B hybridoma producing anti-CD3 mAb). Cell yields in TDL refer to total cells. On days 3, 4, and 5, the proportion of CD8+ cells in the lymph samples was 22, 49, and 72% for the BMC hosts, and 38, 64, and 79% for the normal F_1 group.

dose of irradiation (1,300 cGy) were compared with BMC hosts prepared with double irradiation (1,300 + 900 cGy). For convenience the data are shown in terms of percent positive cells. Two points emerge from the data. First, the rate of appearance of CD8+ cells with a memory/activated phenotype (including IL-2R+ cells) in TDL was virtually identical for the two groups of BMC hosts. Second, the entry of activated CD8+ cells into TDL of the BMC hosts occurred about 1 d later than in the control normal F_1 group. For the BMC group, it should be noted that the rapid increase in the proportion of activated CD8+ cells in the

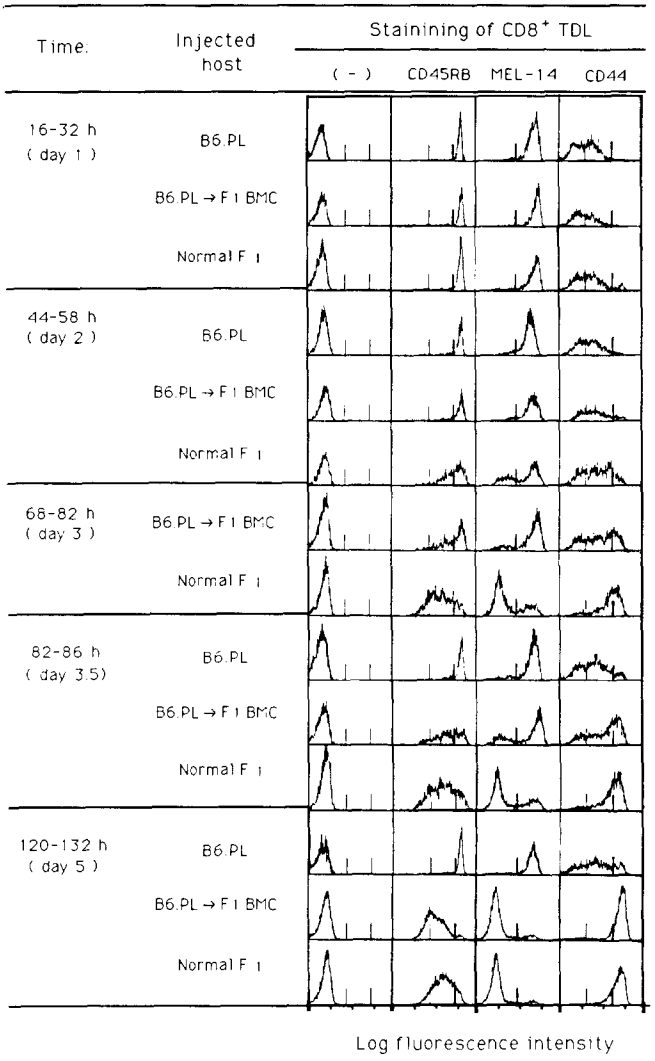

Figure 5. CD8+ cells entering TDL of long-term B6 → F_1 BMC hosts injected with B6 T cells begin to show memory/activation markers on day 3 after transfer. Lymph-borne cells from the three groups of mice in Fig. 4 were collected continuously from days 1–5. At the time of intervals shown, aliquots of cells were stained for expression of CD8 vs. CD45RB, MEL-14, or Pgp-1 (CD44).

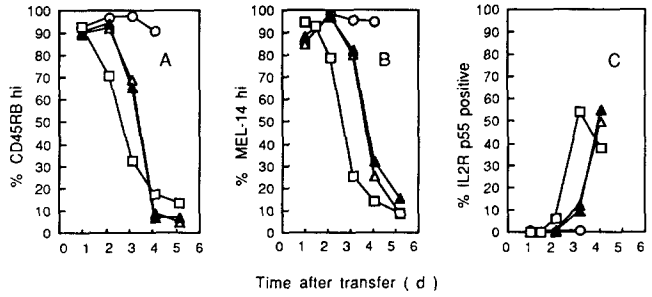

Figure 6. Similar rate of appearance of activated CD8+ cells in TDL of BMC hosts prepared with one vs. two doses of irradiation. The data show CD45RB, MEL-14, and IL-2R expression on lymph-borne CD8+ cells collected from the four groups of mice discussed in Fig. 3. The data are presented in terms of the percentage of cells expressing the marker concerned. Cells were stained as described in Materials and Methods.

lymph between days 3 and 4 corresponded closely with the marked increase in CTL activity discussed above.

The above findings document that transferring parental strain CD8+ cells to long-term parent → F_1 BMC causes a small but significant proportion of the donor T cells to proliferate and differentiate into blast cells with potent anti-host effector function. Although the donor LN T cells used in these experiments consisted largely of typical resting T cells, it remained possible that a minority population of memory-phenotype cells accounted for the proliferative response of the T cells in BMC hosts. To examine this possibility, B6 LN T cells were layered on stepwise Percoll density gradients before injection. High buoyant density T cells were recovered from the band between the two highest density layers (p 1.08 and 1.90); under the conditions used, only 10% of the T cells entered this band. As shown in Fig. 7, *G–J*

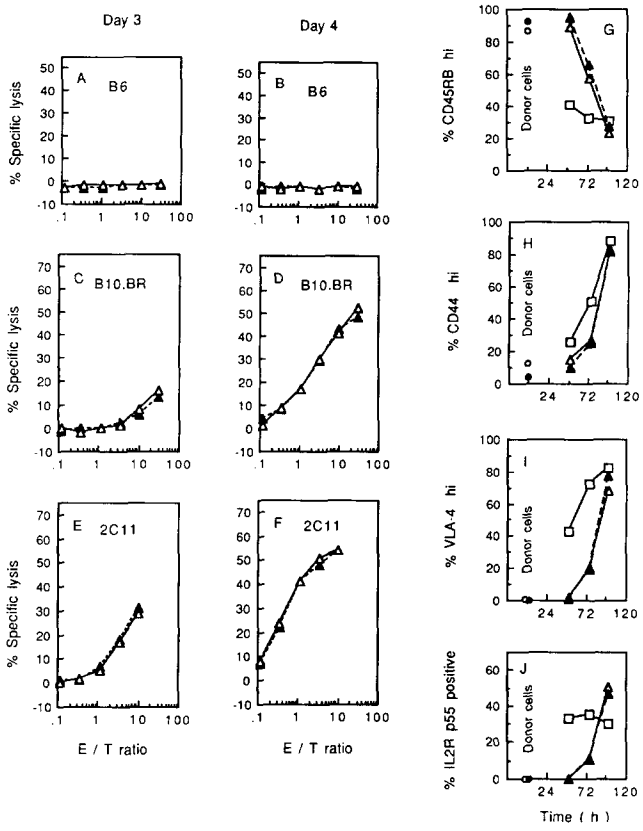

Figure 7. CTL activity and surface markers of lymph-borne CD8+ cells collected from BMC hosts injected with high-density T cells. Groups of B6.PL → F_1 BMC were injected with normal B6 T cells (△) or high-density B6 T cells (▲) prepared on Percoll gradients (Materials and Methods). Control normal F_1 mice received normal B6 T cells (□). To measure direct CTL activity, lymph-borne cells collected on day 3 (*A*, *C*, and *E*) or day 4 (*B*, *D*, and *F*) after transfer were tested for their capacity to lyse ^{51}Cr-labeled B6, B10.BR, and 2C11 target cells in vitro (see Fig. 4). To detect activation markers on CD8+ cells (*G–J*), lymph-borne cells collected on days 2, 3, and 4 after transfer were double stained for expression of CD8 vs. the four markers shown. Initial expression of the four markers on CD8+ cells in the injected populations of normal B6 T cells (○) and high-density T cells (●) is shown on the left side of the figures.

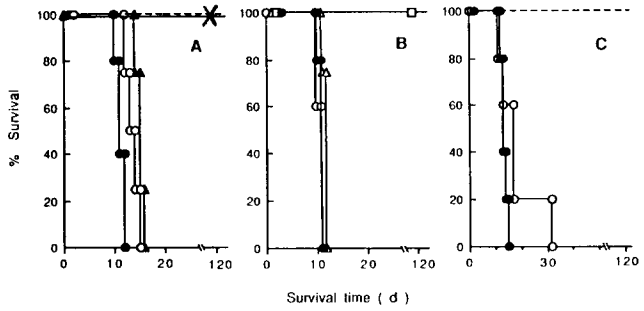

Figure 8. Susceptibility of BMC to lethal GVHD. (*A* and *B*) The host mice were conditioned with 500 cGy a few hours before T cell transfer. (*C*) The host mice were not conditioned with irradiation. The source and number of T cells injected were as follows. (*A*) (▲) Normal F_1 mice given 5×10^6 B6 T cells; (●) normal F_1 mice given 8×10^7 B6 T cells; (○) B6.PL → F_1 BMC given 8×10^7 B6 T cells, (x), B6.PL → F_1 BMC given 8×10^7 B6 $CD4^+$ T cells; and (- - -) uninjected B6.PL → F_1 BMC (500 cGy only). (*B*) (●) Normal F_1 mice given 5×10^7 B6 T cells; (○) B6.PL → F_1 BMC given 8×10^7 B6 T cells, (x), B6.PL → F_1 BMC given 5×10^7 CBA T cells; and (□) B6.PL → F_1 BMC given 5×10^7 normal F_1 T cells. (*C*) (●) Normal F_1 mice given 5×10^7 B6 T cells; (○) B6.PL → F_1 BMC given 5×10^7 B6 T cells; and (- - -) uninjected, untreated B6.PL → F_1 BMC.

(*left*) nearly all of these high-density T cells expressed the typical markers of virgin/resting T cells.

The results of injecting B6.PL → F_1 BMC with normal B6 T cells vs. purified high-density B6 T cells are shown in Fig. 7. It is apparent that these two T cell populations were indistinguishable in terms of generating host-reactive CTL (Fig. 7, *C* and *D*) and forming $CD8^+$ blast cells expressing activation markers such as IL-2R, Pgp-1, and VLA-4 (Fig. 7, *G–J*). This was apparent even during the early exponential phase of the response. The possibility that the activated cells generated in the chimeras were selectively derived from memory-phenotype cells contaminating the initial T cell inoculum thus seems unlikely.

Susceptibility to GVHD. The activation and differentiation of parental strain T cells in parent → F_1 BMC constitutes a GVH reaction. To examine whether this reaction proceeds to overt GVHD, groups of B6.PL → F_1 BMC were exposed to light irradiation (500 cGy) and injected with large doses of B6 T cells. The recipients showed signs of acute GVHD at 1 wk after transfer and all of the mice died within 2 wk (Fig. 8 *A*). No deaths occurred in control BMC hosts given B6 $CD4^+$ cells. Induction of lethal GVHD was thus $CD8^+$ cell dependent.

In a second experiment, groups of lightly-irradiated B6.PL → F_1 BMC were injected with B6 ($H\text{-}2^b$) vs. CBA ($H\text{-}2^k$) T cells. Here, the stimulus for GVHD was provided by both BMD and NBMD cells in the case of CBA T cells but only by NBMD cells for B6 T cells. In both situations, the recipients succumbed to lethal GVHD within 2 wk (Fig. 8 *B*). No deaths occurred when (B6 × CBA)F_1 T cells were injected.

The above data refer to BMC given irradiation before T cell transfer. When this conditioning dose of irradiation was omitted, GVHD induction in B6.PL → F_1 BMC recipients of B6 T cells occurred more slowly. Nevertheless, all of the BMC hosts died within 5 wk (Fig. 8 *C*). In control experiments, transferring B6 T cells to nonirradiated normal F_1 hosts led to a rapid onset of GVHD, and all of the mice died within 2 wk.

Discussion

As mentioned in the introductory section, patients receiving vascularized allografts remain susceptible to rejection for many years. The types of T cells controlling late rejection are not well defined. Rejection might be controlled principally by presensitized T cells primed soon after grafting through contact with graft-derived DC. Long-lived memory T cells could cause rejection episodes by directly attacking the graft or through lymphokine release. Alternatively, late rejection might be largely under the control of newly-formed naive T cells, sensitization of these T cells being elicited through contact with NBMD cells in the graft. The present finding that unprimed $CD8^+$ cells differentiate into effector cells when exposed to NBMD cells in long-term BMC is consistent with this idea.

One has to consider the possibility that the donor T cells responded to "processed" antigens in the chimeras, i.e., to host class I peptides presented by donor-derived APC. This idea is implausible for three reasons. First, peptides resulting from the external pathway of antigen processing generally associate only with class II and not class I molecules (36). Second, there is no evidence that T cells reactive to native class I molecules (plus endogenous peptides) have crossreactive specificity for allo class I peptides presented by self class I molecules. Third, previous studies showed that the donor-derived APC from parent → F_1 BMC are unable to stimulate parental strain T cells in vitro (32). A more serious objection is that the immunogenicity of the chimeras was controlled by residual host BMD cells. This idea seems very unlikely because host BMD cells were undetectable either in cell suspensions or in sections (13, 32, and this paper). All of the chimeras studied were prepared with very heavy irradiation (1,300 cGy) and were left for at least 6 mo before use. If significant numbers of host APC survived this treatment, subjecting the chimeras to further irradiation and BM reconstitution would be expected to cause a reduction in immunogenicity. In practice, however, the immunogenicity of chimeras prepared with one vs. two doses of irradiation was indistinguishable.

Although host class I expression in the chimeras was quite limited, it is interesting that acute blood-to-lymph recirculation of parental strain T cells through the chimeras selectively removed $\geq 95\%$ of $CD8^+$ cells reactive to normal F_1 spleen cells in vitro. This finding implies that the density of antigen required to induce trapping of T cells, which is a manifestation of initial T cell contact with APC, is surprisingly low. The data also imply that, if alloreactivity is directed predominantly to various endogenous self peptides (rather than to allo MHC epitopes per se) (37), the overlap of self peptides between NBMD cells and BMD cells must be extensive.

Although the selective trapping of host-reactive $CD8^+$

cells in BMC recipients approached 100%, only a small proportion of the trapped cells were induced to proliferate. This is apparent from the finding that the generation of $CD8^+$ blast cells in the chimeras was quite low, i.e., about sixfold lower than in normal F_1 hosts. Nevertheless, the fact that the chimeras did stimulate appreciable proliferative responses and led to the production of $CD8^+$ cells with potent effector function is highly significant. Blast cells with typical activation markers appeared in TDL at about 3 d after transfer and then increased exponentially to account for nearly all of the lymph-borne cells by day 5. Since similar findings applied after injection of purified high-density T cells, the possibility that the effector cells arose selectively from contaminating memory-phenotype precursors seems most unlikely. The finding that the chimeras eventually developed lethal GVHD indicates that the effector cells were functionally relevant under in vivo conditions. Although most of the experiments involved BMC hosts conditioned with irradiation just before T cell transfer (to facilitate detection of donor T cells), it should be noted that omitting this dose of irradiation did not prevent the induction of lethal GVHD. This finding makes it unlikely that the immunogenicity of the chimeras depended upon an adjuvant-like effect of acute irradiation.

It is notable that the effector cells generated in BMC hosts were extremely potent in terms of CTL activity. In fact, on day 4 after injection, the CTL activity of blasts from the chimeras was higher than for blasts collected from normal F_1 hosts. How can this be explained? The simplest possibility is that overt stimulation of $CD8^+$ cells in the chimeras applied only to a minor subset of cells with the highest affinity for host class I antigens. Because of their high intrinsic affinity, these T cells avoided the requirement for professional APC and were able to interact with host NBMD cells with sufficient avidity to cause T cell triggering and differentiation into CTL. Cells of average affinity failed to be stimulated and, for this reason, the CTL generated in the chimeras were skewed towards high affinity cells. In normal F_1 hosts, by contrast, T cell contact with antigen on host DC caused activation of cells with a wide range of affinities. The average affinity of CTL generated in these mice was thus lower than in BMC hosts.

Proving the above model hinges on demonstrating that CTL generated in BMC hosts do indeed have above-average affinity. Some support for this idea is provided by the finding that the enhanced CTL activity of cells from the chimeras only applied to antigen-specific lysis. Thus, for "redirected" (nonantigen-specific) lysis of an anti-CD3 hybridoma line, cells from BMC hosts and normal F_1 hosts were indistinguishable. Despite this finding, attempts to prove a difference in affinity on the basis of susceptibility to inhibition with anti-CD8 mAb have been largely unsuccessful (data not shown). For this reason, at present the notion that the CTL generated in the chimeras represent a subset of high-affinity cells has to be viewed more as hypothesis than fact.

It should be emphasized that the precise identity of the NBMD cells controlling APC function in BMC hosts is still unclear. The level of host class I expression was quite low in sections and, in spleen, was largely restricted to vascular endothelium and the FDC of germinal centers. Proving whether these cell populations express APC function in situ is difficult. In future experiments, we hope to address this issue by defining the precise microenvironments in which $CD8^+$ cells undergo initial blast transformation in BMC hosts. Since the highest density of host class I (and class II) was found on FDC, it will be particularly interesting to test whether the activation of $CD8^+$ cells (and/or $CD4^+$ cells) in chimeras is prominent in germinal centers. Although there is currently no direct evidence that FDC act as APC for T cells, it is striking that FDC are a major reservoir for HIV infection (38), which raises the possibility that the T lymphocytopenia in AIDS is a reflection of T–FDC interaction.

As a final comment, it may be mentioned that our initial aim in studying allorecognition in BMC hosts was to test the notion that T cell contact with antigen on nonprofessional APC is nonimmunogenic and leads to tolerance (anergy) induction (39, 40). In practice, the finding that T cell contact with antigen in the chimeras led to overt immune responses made it impossible to assess this idea: the NBMD APC in the chimeras turned out to be semiprofessional rather than nonprofessional. Since T cell activation in the chimeras appeared to be restricted to a minor subset of cells, what happened to the remainder of the host-reactive cells? Were these cells rendered anergic or did the cells remain unstimulated? Further studies will be needed to resolve this important question.

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