

# Profound Atrophy of the Bone Marrow Reflecting Major Histocompatibility Complex Class II-restricted Destruction of Stem Cells by CD4<sup>+</sup> Cells

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## Summary

The effector functions of CD4<sup>+</sup> cells *in vivo* are presumed to reflect a combination of lymphokine-mediated bystander reactions and direct cytotoxic T lymphocyte activity. To assess the relative importance of these two mechanisms, we studied the effects of transferring small doses of purified unprimed CD4<sup>+</sup> cells to lightly irradiated (600 cGy) recipients expressing major histocompatibility complex class II (Ia) differences. Within the first week after transfer, the host marrow was rapidly repopulated with hemopoietic cells. Thereafter, however, the donor CD4<sup>+</sup> cells caused massive destruction of hemopoietic cells, both in marrow and spleen. Marrow aplasia did not affect stromal cells and was prevented by coinjecting donor but not host bone marrow. The use of allotypic markers and fluorescence-activated cell sorter analysis indicated that the destructive effects of CD4<sup>+</sup> cells were directed selectively to host Ia<sup>+</sup> hemopoietic cells, including stem cells; donor hemopoietic cells and Ia<sup>-</sup> host T cells were spared. No evidence could be found that the ongoing destruction of host cells impaired the capacity of donor stem cells to repopulate marrow, spleen, or thymus. Moreover, CD4<sup>+</sup> cells failed to destroy host-type hemopoietic cells from Ia-deficient mice. Tissue destruction by CD4<sup>+</sup> cells thus did not seem to reflect a bystander reaction. We conclude that, under defined conditions, CD4<sup>+</sup> cells can manifest extremely potent Ia-restricted CTL activity *in vivo*, probably through recognition of covert Ia expression on stem cells and/or their immediate progeny.

CTLs play a vital role in many forms of cellular immunity and are largely responsible for elimination of viruses and rejection of allografts (1). Most of the effector cells for CTL responses are CD8<sup>+</sup> cells. CD4<sup>+</sup> cells make an important contribution to CTL responses, but CD4<sup>+</sup> cells are thought to function largely by providing help (lymphokines) for CD8<sup>+</sup> precursor cells rather than by acting as CTL effector cells. Nevertheless, there are a number of reports that, under defined conditions, CD4<sup>+</sup> cells do exhibit CTL activity (2-9). The cytotoxic properties of CD4<sup>+</sup> cells are most prominent with long-term T cell clones, but CTL activity is also seen for primary responses (2, 6). Most cytotoxic CD4<sup>+</sup> cells display a Th1 phenotype (3, 8). In mice, CTL activity by CD4<sup>+</sup> cells generally involves cell contact leading to apoptosis (7), but TNF-mediated lysis has also been reported (9).

Whether CD4<sup>+</sup> cells act as CTL under normal physiological conditions *in vivo* is unclear. In terms of allograft rejection (10) and induction of lethal GVHD (11), CD4<sup>+</sup> cells

are known to exhibit potent effector function. The prevailing view is that the *in vivo* effector functions of CD4<sup>+</sup> cells probably reflect a combination of lymphokine-mediated "bystander" reactions and direct CTL activity (10). However, the relative importance of these two mechanisms is unknown.

In this paper we investigated whether CD4<sup>+</sup> cells mediate bystander tissue destruction *in vivo* by transferring purified CD4<sup>+</sup> cells to lightly irradiated MHC class II (Ia)-different hosts. The results show that, even in small doses, allogeneic CD4<sup>+</sup> cells are capable of causing massive destruction of host hemopoietic cells, both in spleen and marrow. The surprising finding, however, is that destruction of hemopoietic cells by CD4<sup>+</sup> cells seems to be directed solely to host cells and not to donor-derived cells. Moreover, marrow-derived cells from Ia-deficient (knockout) mice are completely resistant to destruction by allogeneic CD4<sup>+</sup> cells. Tissue destruction by CD4<sup>+</sup> cells thus does not appear to reflect a bystander reaction but rather a highly potent form of direct Ia-restricted CTL activity.

## Materials and Methods

**Mice.** Young adult B6, B6.C-H-2<sup>bm12</sup> (bm12), B6.C-H-2<sup>bm1</sup> (bm1), D1.LP, B10.A(4R), B10.A(2R), B6 Ly 5.1, B6.PL (Thy 1.1) and F<sub>1</sub> hybrids between these strains were obtained from the breeding colony of The Scripps Research Institute. The B6 Ly 5.1 congenic line was derived from breeding pairs obtained from the National Cancer Institute (Frederick, MD). Ia-deficient mice on the B6 background were kindly provided by Dr. L. Glimcher (Harvard Medical School, Boston, MA) (12).

**Irradiation.** Mice were exposed to a single dose of  $\gamma$ -irradiation (80 cGy/min) by an irradiator (Gammacell 40; Atomic Energy of Canada, Ottawa, Canada).

**Cell Purification.** As described elsewhere (13), using RPMI 1640 or HBSS supplemented with either 5% FCS or  $\gamma$ -globulin-depleted horse serum, highly purified populations of CD4<sup>+</sup> cells were prepared by treating pooled LN with a cocktail of anti-CD8, anti-Ia, and anti-heat-stable antigen (HSA) mAbs plus C at 37°C followed by positive panning at 4°C on plates coated with anti-CD4 mAb. A reciprocal method was used to prepare purified CD8<sup>+</sup> cells. Bone marrow (BM)<sup>1</sup> cells, flushed from the leg bones, were depleted of mature T cells by treatment with anti-Thy-1 mAb plus C (14).

**Assay for BM aplasia.** Adult (6–16-wk-old) recipient mice exposed to 600 cGy 4–6 h before were injected intravenously with T cells (CD4<sup>+</sup> or CD8<sup>+</sup> cells), T-depleted (T<sup>-</sup>) BM cells, or with a mixture of T cells and BM cells (14). At various intervals, the host mice were killed to measure total numbers of nucleated cells in BM (usually both tibiae) and spleen. Nucleated cells were enumerated in 2% acetic acid or by phase-contrast microscopy.

**CFUs.** Using standard techniques (15), the content of CFUs in BM was measured by transferring graded doses of T-depleted BM cells intravenously to heavily irradiated syngeneic hosts (three mice per cell dose). 8–10 d later the recipients were killed and their spleens were fixed in Bouin's solution for colony counting.

**FACS<sup>®</sup> Analysis.** Using FITC-conjugated or unconjugated mAbs followed by FITC-streptavidin, cells were stained for expression of Thy 1.2 (J1j) (15), Thy 1.1 (19E12) (16), Ly 5.1 (A20-1.7), and Ly 5.2 (104-2.1) using standard techniques (16). Conjugated anti-Ly 5 mAbs were kindly provided by Dr. B. J. Fowlkes (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Dead cells were stained with propidium iodide (Sigma Chemical Co., St. Louis, MO) and gated out for analysis. Stained cells were analyzed on a FACScan<sup>®</sup> (Becton Dickinson, Mountain View, CA).

## Results

In previous studies from this laboratory (The Scripps Research Institute), we examined the capacity of CD4<sup>+</sup> cells to cause lethal GVHD in irradiated hosts using the strain combination of B6 and bm12 (14). These two strains are identical except for several point mutations in the  $\beta$  chain of the I-A molecule. When small doses of B6 CD4<sup>+</sup> cells were transferred to lightly irradiated (600 cGy) (B6  $\times$  bm12)F<sub>1</sub> hosts, the recipients died suddenly from GVHD at 3 wk after transfer, apparently from acute hemopoietic failure. Other workers (17) had reported similar findings for Ia-different mice injected with unseparated T cells. The surprising finding was that acute GVHD in lightly irradiated (B6  $\times$  bm12)F<sub>1</sub> hosts failed to occur when B6 CD4<sup>+</sup> cells were supplemented

with donor BM cells or a mixture of donor and host BM cells (14).

The initial aim of the experiments outlined below was to determine why the presence of donor BM cells prevents CD4<sup>+</sup> cells from mediating lethal GVHD in lightly irradiated Ia-different hosts. Unless stated otherwise, doses of  $2 \times 10^6$  highly purified LN CD4<sup>+</sup> cells were transferred intravenously to adult (B6  $\times$  bm12)F<sub>1</sub> hosts exposed to a low dose of 600 cGy 4–6 h before; BM cells ( $2 \times 10^6$ ) were T-depleted and were injected alone or as a mixture with T cells.

**Acute Lethal GVHD Induced by CD4<sup>+</sup> Cells in Lightly Irradiated Ia-different Hosts Reflects BM Aplasia.** When a dose of  $2 \times 10^6$  B6 CD4<sup>+</sup> cells was transferred to 600 cGy (B6  $\times$  bm12)F<sub>1</sub> hosts, the mice became acutely ill 16–18 d after transfer and died several days later. This was an invariable finding (seen in >10 experiments) and death rates were virtually 100%. When the mice were killed at days 16–18, the mice were obviously anemic and numbers of white blood cells and erythrocytes in peripheral blood were markedly reduced (data not shown). This pancytopenia was associated with striking atrophy of the host marrow, the numbers of nucleated cells in the long bones (tibiae) being reduced by 20–200-fold relative to mice treated with irradiation alone (Table 1). Splenic atrophy was moderate on day 16 (Table 1) but was marked by days 18–21 (see below).

Supplementing the injected B6 CD4<sup>+</sup> cells with host F<sub>1</sub> (Table 1) or bm12 BM cells (see below) had no effect on BM/spleen atrophy and failed to prevent death. However, adding donor BM cells, or a mixture of donor and host BM cells, caused minimal BM atrophy and resulted in prominent splenomegaly and virtually complete protection against death (Table 1).

In hosts injected with B6 CD4<sup>+</sup> cells in the absence of B6 stem cells, histological examination of the host long bones at days 16–18 revealed an almost complete absence of nucleated hemopoietic cells in the marrow cavities (Fig. 1, *b* and *d*). It is significant, however, that the stromal cells in the marrow were well preserved. When B6 CD4<sup>+</sup> cells were supplemented with B6 BM cells or a mixture of B6 and either F<sub>1</sub> or bm12 BM cells, there was no evidence of marrow atrophy and the marrow was filled with dense accumulations of hemopoietic cells (Fig. 1, *a* and *c*). In this situation, the histology of the marrow was indistinguishable from the marrow of control mice given 600 cGy alone (data not shown).

The dose of B6 CD4<sup>+</sup> cells required to induce BM atrophy in irradiated (B6  $\times$  bm12)F<sub>1</sub> hosts was surprisingly low, since as few as  $7 \times 10^4$  cells were sufficient to cause complete atrophy (Fig. 2 *a*). As a control for these studies with B6 CD4<sup>+</sup> cells, we examined the dose of B6 CD8<sup>+</sup> cells required to cause host marrow atrophy in 600 cGy (B6  $\times$  bm1)F<sub>1</sub> hosts (Fig. 2 *b*). In this strong MHC class I-different combination, induction of marked marrow atrophy necessitated injecting in the order of  $3 \times 10^5$  B6 CD8<sup>+</sup> cells, i.e., fourfold more cells than were needed for CD4<sup>+</sup> cells in the B6  $\rightarrow$  bm12 combination.

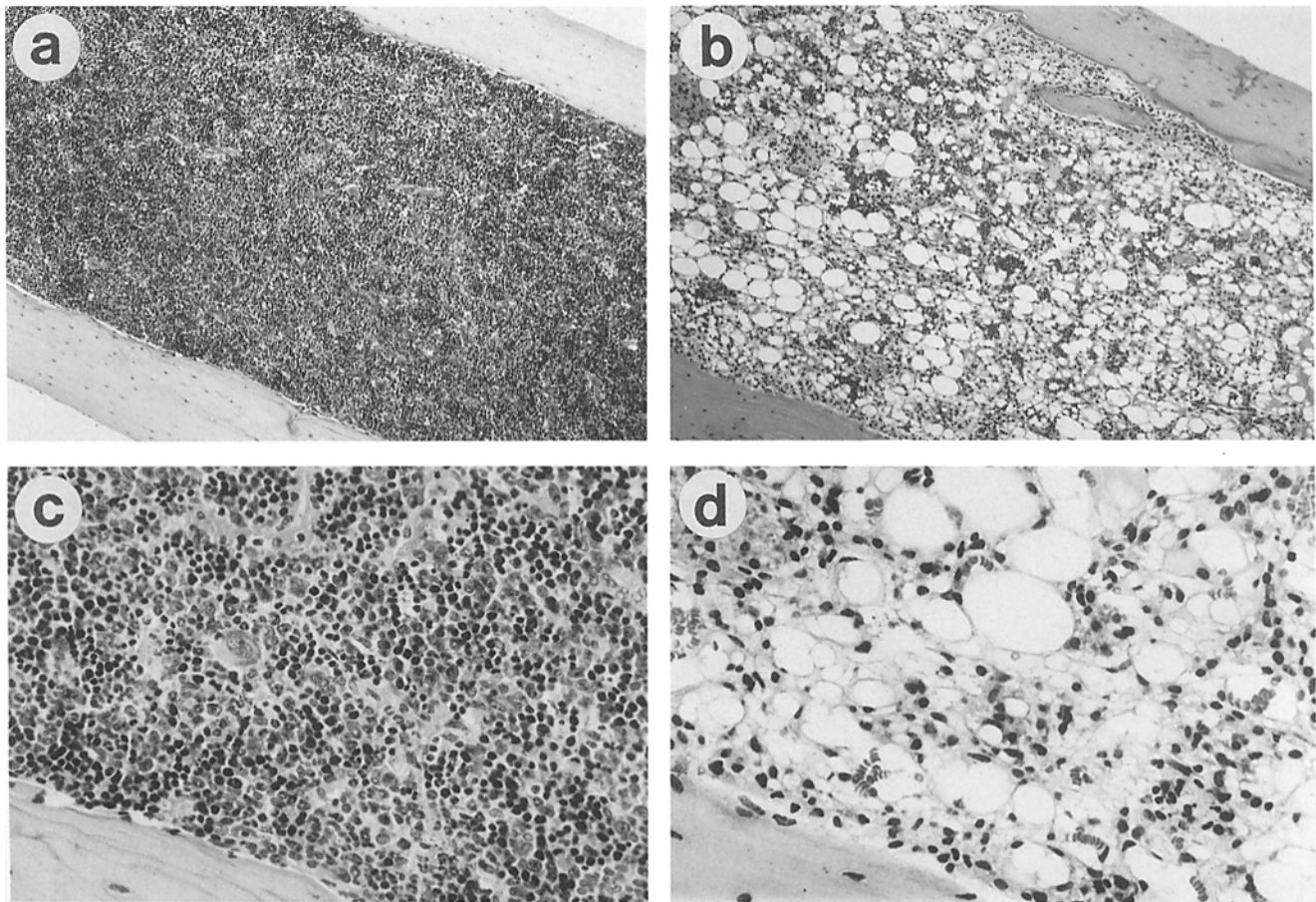
As shown in Table 2, Exp. 1, the capacity of CD4<sup>+</sup> cells

<sup>1</sup> Abbreviation used in this paper: BM, bone marrow.

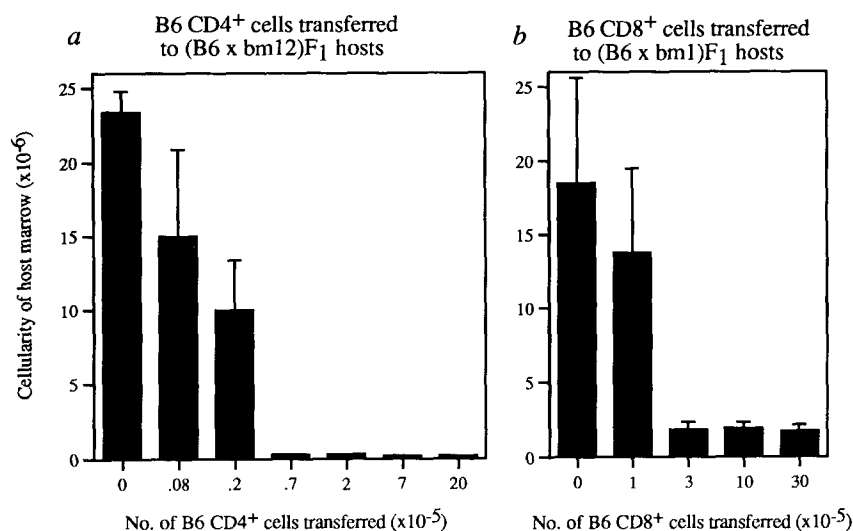
**Table 1.** BM Counts and Spleen Weights of Irradiated (B6 × bm12)<sub>F</sub><sub>1</sub> Hosts Injected 16 d before with B6 CD4<sup>+</sup> Cells Plus Donor vs. Host BM Cells

Cells transferred to 600 cGy (B6 × bm12) <sub>F</sub> <sub>1</sub> hosts	No. of nucleated cells (× 10 <sup>-6</sup> ) (SD) in host marrow (both tibiae) at day 16	Spleen weight (mg) (SD) at day 16	Incidence of lethal GVHD
			%
B6 CD4 <sup>+</sup>	0.1 (< 0.1)	58 (11)	100
B6 CD4 <sup>+</sup> + F <sub>1</sub> BM	0.1 (< 0.1)	63 (9)	100
B6 CD4 <sup>+</sup> + B6 BM	10.5 (6.3)	578 (524)	0
B6 CD4 <sup>+</sup> + B6 BM + F <sub>1</sub> BM	10.9 (0.8)	467 (201)	0
B6 BM	16.2 (1.1)	84 (5)	0
No cells	15.9 (2.4)	86 (6)	0

Purified LN CD4<sup>+</sup> cells and T-depleted BM cells (2 × 10<sup>6</sup> of each population) were transferred intravenously 4–6 h after irradiation of the hosts. The data (mean of three to five mice tested individually) are from a single experiment and are representative of several other experiments.



**Figure 1.** Histology of BM aplasia. (B6 × bm12)<sub>F</sub><sub>1</sub> mice were exposed to 600 cGy and injected with B6 CD4<sup>+</sup> cells plus a mixture of B6 and bm12 BM cells (2 × 10<sup>6</sup> of each) (a and c) or with B6 CD4<sup>+</sup> cells plus bm12 BM cells (b and d). After 16 d, femurs from the two groups of mice were decalcified; sections were prepared and stained with hematoxylin and eosin. (a and c) Low and high power views of femur from a mouse given B6 CD4<sup>+</sup> cells plus B6 BM plus bm12 BM cells. The marrow is filled with nucleated hemopoietic cells. (b and d) Low and high power views of femur from a mouse given B6 CD4<sup>+</sup> cells plus bm12 BM cells. The marrow is largely devoid of nucleated hemopoietic cells, but the stromal cells are well preserved. (a and b) ×100; (c and d) ×400.



**Figure 2.** Dose of B6 CD4<sup>+</sup> cells required to induce marrow atrophy in (B6 × bm12)F<sub>1</sub> hosts. (a) Total numbers of nucleated cells recovered from both tibiae of 600 cGy (B6 × bm12)F<sub>1</sub> mice injected 16 d before with graded doses of B6 CD4<sup>+</sup> cells. (b) Total numbers of nucleated cells recovered from both tibiae of 600 cGy (B6 × bm1)F<sub>1</sub> mice injected 14 d before with graded doses of B6 CD8<sup>+</sup> cells. Mean of data from three mice per group tested individually.

**Table 2.** Induction of Marrow Atrophy by CD4<sup>+</sup> Cells in 600 cGy (B6 × bm12)F<sub>1</sub> Hosts Requires Recognition of Host Ia Antigens.

Exp. No.	Cells transferred	Hosts (600 cGy)	Target antigen	No. of nucleated cells (× 10 <sup>-6</sup> ) (SD) in both tibiae at days 15-18
1	D1.LP CD4 <sup>+</sup>	(B6 × bm12)F <sub>1</sub>	I-A <sup>bm12</sup>	0.5 (0.1)
	D1.LP CD4 <sup>+</sup> + D1.LP BM		I-A <sup>bm12</sup>	23.2 (2.9)
	bm12 CD4 <sup>+</sup>		I-A <sup>b</sup>	0.9 (0.2)
	bm12 CD4 <sup>+</sup> + bm12 BM		I-A <sup>b</sup>	19.0 (3.2)
	No cells		-	17.3 (3.3)
2	B6 CD4 <sup>+</sup>	(B6 × bm12)F <sub>1</sub>	I-A <sup>bm12</sup>	0.3 (0.1)
	(B6 × bm12)F <sub>1</sub> CD4 <sup>+</sup>		-	16.6 (2.4)
	Chimera B6 CD4 <sup>+</sup>		I-A <sup>bm12</sup>	16.7 (3.7)
	No cells		-	15.0 (2.4)
3	B6 CD4 <sup>+</sup>	(B6 × bm12)F <sub>1</sub>	I-A <sup>bm12</sup>	0.1 (<0.1)
	B6 CD8 <sup>+</sup>		I-A <sup>bm12</sup>	9.4 (1.8)
	No cells		-	9.4 (1.2)
4	B6 CD4 <sup>+</sup>	(B6 × 4R)F <sub>1</sub>	I-A <sup>k</sup>	1.2 (0.6)
	B6 CD4 <sup>+</sup> + B6 BM		I-A <sup>k</sup>	20.3 (3.9)
	4R CD4 <sup>+</sup>		I-A <sup>b</sup>	1.3 (0.5)
	4R CD4 <sup>+</sup> + 4R BM		I-A <sup>b</sup>	24.0 (1.7)
	No cells		-	21.6 (2.5)
5	4R CD4 <sup>+</sup>	2R	I-E <sup>k</sup>	1.7 (0.4)
	4R CD4 <sup>+</sup> + 4R BM		I-E <sup>k</sup>	25.3 (4.7)
	No cells		-	22.0 (0.8)
6	B6 CD8 <sup>+</sup> cells	(B6 × bml)F <sub>1</sub>	K <sup>bm1</sup>	0.2 (0.1)
	B6 CD4 <sup>+</sup> cells		K <sup>bm1</sup>	20.3 (1.9)
	No cells		-	18.3 (6.0)

Host mice were exposed to 600 cGy and injected with purified CD4<sup>+</sup> cells (2 × 10<sup>6</sup>/mouse) ± syngeneic BM cells (2 × 10<sup>6</sup>/mouse); chimera B6 CD4<sup>+</sup> cells were prepared from LN of heavily irradiated (1,100 cGy) (B6 × bm12)F<sub>1</sub> mice injected 6 mo previously with T-depleted B6 BM cells. BM counts refer to the mean data from three mice/group.

**Table 3.** Marrow Aplasia Induced by CD4<sup>+</sup> Cells Develops Late and Is Not Apparent during the First 7 d after Transfer

Cells injected into 600 cGy (B6 × bm12)F <sub>1</sub> hosts	No. of nucleated cells (× 10 <sup>-6</sup> ) (SD) in both tibiae		
	day 2	day 7	day 16
-	1.0 (0.8)	18.0 (2.0)	20.2 (4.2)
F <sub>1</sub> BM (2 × 10 <sup>6</sup> )	2.0 (1.1)	20.6 (1.6)	23.4 (7.4)
F <sub>1</sub> BM (2 × 10 <sup>6</sup> ) + B6 CD4 <sup>+</sup>	1.1 (0.1)	20.4 (2.9)	0.2 (0.1)
F <sub>1</sub> BM (2 × 10 <sup>7</sup> ) + B6 CD4 <sup>+</sup>	-	17.3 (2.2)	0.4 (0.2)
B6 CD4 <sup>+</sup>	1.3 (0.2)	15.8 (3.3)	0.3 (0.2)

As for Table 1, three mice per group. CD4<sup>+</sup> cells were injected in a dose of 2 × 10<sup>6</sup> cells/mouse.

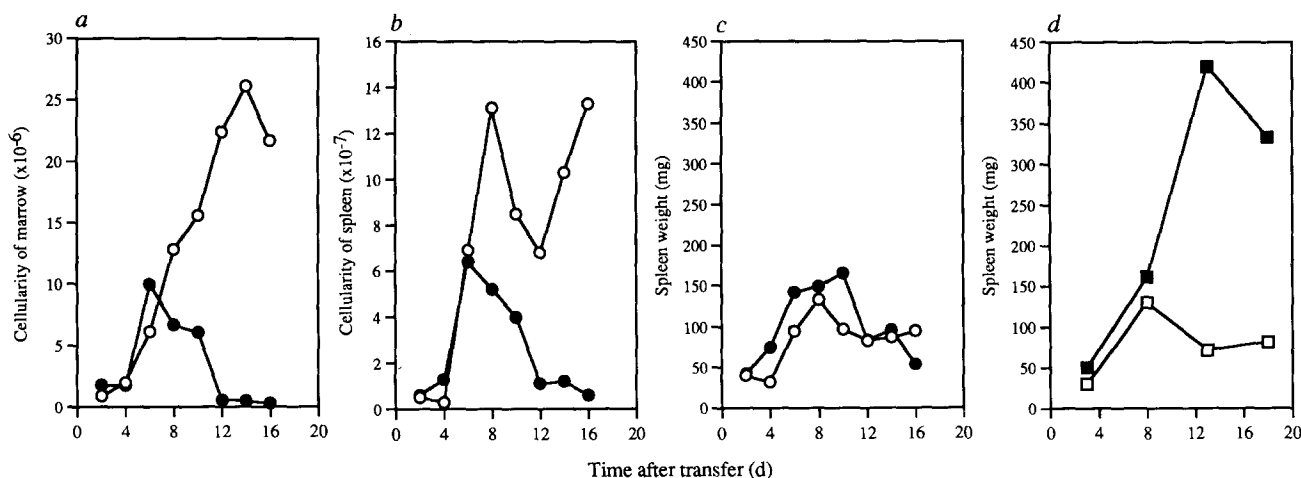
to induce host marrow atrophy in irradiated (B6 × bm12)F<sub>1</sub> hosts applied to both parental strains and also extended to D1.LP (I-A<sup>b</sup>) CD4<sup>+</sup> cells (B6 and D1.LP are MHC identical but have different genetic backgrounds). Recognition of host antigens appeared to be essential because no marrow atrophy was observed when (B6 × bm12)F<sub>1</sub> hosts were injected with host F<sub>1</sub> CD4<sup>+</sup> cells or with B6 CD4<sup>+</sup> cells tolerized to host antigens in B6 BM → 1100 cGy (B6 × bm12)F<sub>1</sub> chimeras (Table 2, Exp. 2). In terms of T cell subsets, marrow atrophy in (B6 × bm12)F<sub>1</sub> hosts was restricted to CD4<sup>+</sup> cells: no atrophy occurred with injection of B6 CD8<sup>+</sup> cells (Table 2, Exp. 3).

In addition to the B6/bm12 combination, CD4<sup>+</sup> cells induced marked marrow atrophy in a number of other Ia-different strain combinations, including strains with isolated allelic I-A differences (4R → B6, B6 → 4R) and I-E differences (4R → 2R) (Table 2, Exp. 4 and 5). Ia disparity ap-

peared to be essential because B6 CD4<sup>+</sup> cells failed to cause marrow atrophy in class I-different (B6 × bm1)F<sub>1</sub> hosts (Table 2, Exp. 6).

In all of the above situations, marrow atrophy in Ia-different hosts only applied when CD4<sup>+</sup> cells were injected in the absence of donor BM cells. When CD4<sup>+</sup> cells were supplemented with syngeneic donor BM cells, marrow atrophy was mild or undetectable (Table 2).

**Kinetics of Marrow Aplasia.** The above data refer to marrow counts measured 14–18 d after transfer of CD4<sup>+</sup> cells. The kinetics of the onset of marrow aplasia are shown in Table 3 and Fig. 3. When (B6 × bm12)F<sub>1</sub> mice were treated with 600 cGy alone, the cellularity of the marrow on day 2 was reduced by 10–20-fold relative to unirradiated mice (Table 3). Thereafter, cell counts in the marrow increased progressively to reach near-normal levels by days 7–12 (indicating repopulation by radioresistant host stem cells). When 600



**Figure 3.** Kinetics of marrow and spleen atrophy in irradiated (B6 × bm12)F<sub>1</sub> mice given B6 CD4<sup>+</sup> cells. Groups of (B6 × bm12)F<sub>1</sub> mice were exposed to 600 cGy and injected with 2 × 10<sup>6</sup> F<sub>1</sub> BM cells (○) or a mixture of 2 × 10<sup>6</sup> B6 CD4<sup>+</sup> cells and 2 × 10<sup>6</sup> F<sub>1</sub> BM cells (●). Total numbers of nucleated cells in BM (both tibiae) (a), spleen (b), and spleen weights (c) were then measured at 2-d intervals. In a separate experiment (d), spleen weights in 600 cGy (B6 × bm12)F<sub>1</sub> mice were measured at various stages after transferring 2 × 10<sup>6</sup> B6 BM cells (□) or 2 × 10<sup>6</sup> B6 CD4<sup>+</sup> cells and 2 × 10<sup>6</sup> B6 BM cells (■). In this experiment, spleen weights in mice given B6 CD4<sup>+</sup> cells and B6 BM cells returned to near normal levels by day 30. The data represent the mean of three mice per group.

cGy F<sub>1</sub> mice were injected with B6 CD4<sup>+</sup> cells, either alone or with F<sub>1</sub> BM cells, the increase in marrow counts between days 2 and 7 was the same as in uninjected irradiated mice, indicating that CD4<sup>+</sup> cells did not prevent initial repopulation of the host marrow. Between days 7 and 16, however, the cellularity of the marrow dropped precipitously. Adding large doses of F<sub>1</sub> BM cells ( $2 \times 10^7$ ) failed to prevent this late onset of marrow atrophy (Table 3).

When the cellularity of the marrow was measured at 2-d intervals, the onset of marrow atrophy in F<sub>1</sub> mice given B6 CD4<sup>+</sup> cells (data not shown) or B6 CD4<sup>+</sup> cells plus F<sub>1</sub> BM cells (Fig. 3 a) first became evident 8–10 d after transfer. Thereafter marrow counts declined abruptly and reached very low levels by day 12. Similar kinetics applied to cell counts in spleen (Fig. 3 b). Spleen weights were mildly increased relative to uninjected control mice 6–10 d after transfer and then decreased to below the control levels after day 14 (Fig. 3 c). This applied to B6 CD4<sup>+</sup> cells transferred in the absence of B6 BM cells. When B6 CD4<sup>+</sup> cells were supplemented with syngeneic B6 BM cells, massive splenomegaly occurred: spleen weights were maximal ~2 wk after transfer and then gradually declined towards normal levels over the next 2–3 wk (Fig. 3 d and data not shown); marrow counts remained close to control levels throughout the period studied (data not shown).

**Marrow Aplasia Includes Stem Cells.** To examine whether the destruction of host marrow cells by CD4<sup>+</sup> cells included stem cells, the few cells remaining in the host marrow at 14 d after transfer were assayed for CFUs using standard procedures (see Materials and Methods); the cells (pooled from 9 mice) were treated with anti-Thy-1 mAb plus C before

transfer to remove mature T cells. As shown in Table 4, Exp. 1, the total content of CFUs in the hind legs of F<sub>1</sub> mice injected with B6 CD4<sup>+</sup> cells (without B6 BM cells) was reduced by about 300-fold relative to marrow cells recovered from uninjected irradiated control mice. To determine whether the destruction of stem cells applied to pluripotential stem cells, host BM cells harvested from a group of 20 F<sub>1</sub> host mice injected 15 d before with B6 CD4<sup>+</sup> cells were T-depleted and then tested for their capacity to protect lethally irradiated (1,100 cGy) F<sub>1</sub> mice. Of three mice injected with a dose of  $5 \times 10^5$  BM cells, two mice died 3 wk after transfer and one mouse survived. With control BM cells from mice treated with irradiation alone, a dose of  $5 \times 10^5$  cells led to 100% survival and  $10^5$  cells caused 80% survival. These data imply that the residual marrow cells harvested from F<sub>1</sub> mice given B6 CD4<sup>+</sup> cells were largely depleted of pluripotential stem cells.

**Marrow Aplasia Induced by CD4<sup>+</sup> Cells Is Associated with Migration of Donor T Cells into the Host Marrow.** To examine whether the donor CD4<sup>+</sup> cells entered the host long bones, 600 cGy (B6 × bm12)F<sub>1</sub> hosts (Thy 1.2) were injected with B6.PL (B6-Thy-1.1) CD4<sup>+</sup> cells. When the cells recovered from the host marrow were analyzed by FACS® at days 14–16, 40–70% of the few nucleated cells recovered were Thy 1.1<sup>+</sup> (Table 4, Exp. 2); 5–10% of the cells were Thy 1.2<sup>+</sup>. These findings indicate that the donor CD4<sup>+</sup> cells did indeed reach the marrow of the host. Donor (Thy 1.1<sup>+</sup>) CD4<sup>+</sup> cells were also evident in the spleen (10–20% Thy 1.1<sup>+</sup> cells by day 18) (data not shown). With regard to host cells, the few viable cells recovered from the spleen 16–18 d after transfer consisted predominantly of radioresistant host (Thy 1.2<sup>+</sup>) T

**Table 4.** CFUs and Donor T Cells in Aplastic Marrow from Irradiated (B6 × bm12)F<sub>1</sub> Mice Injected with B6 CD4<sup>+</sup> Cells 14 d before

Exp No.	Treatment of (B6 × bm12)F <sub>1</sub> hosts	Cells recovered from host marrow at day 14		
		No. of nucleated cells in both hind legs	CFUs/10 <sup>6</sup> cells	Total CFUs in both hind legs
1	-	42 × 10 <sup>6</sup>	66	2,772
	600 cGy	32 × 10 <sup>6</sup>	32	1,024
	600 cGy + B6 CD4 <sup>+</sup> cells	0.5 × 10 <sup>6</sup>	6	3
2	-	% Thy 1.1 <sup>+</sup> cells in marrow	% Thy 1.2 <sup>+</sup> cells in marrow	
	600 cGy + B6.PL CD4 <sup>+</sup> cells	<2	4	
		65	7	

In Exp. 1, pooled marrow cells were treated with anti-Thy 1.2 mAb plus C to remove mature T cells and then transferred in graded doses to 1,000 cGy B6 mice to measure spleen colonies on day 8. Similar data were observed in another experiment where CFUs were measured on day 10. In Exp. 2, pooled marrow cells were analyzed by FACS® for expression of Thy 1.1<sup>+</sup> cells and Thy 1.2<sup>+</sup> cells using standard procedures (See Materials and Methods).

cells and other Ia<sup>-</sup> cells; Ia<sup>+</sup> cells were almost undetectable (data not shown). Host T cells were also evident in marrow (Table 4).

**Marrow Destruction by CD4<sup>+</sup> Cells Is Restricted to Host Cells.** As mentioned earlier, marrow aplasia induced by CD4<sup>+</sup> cells was mild or absent when the donor CD4<sup>+</sup> cells were supplemented with donor BM cells or a mixture of donor and host BM cells. The simplest explanation for this finding is that the donor CD4<sup>+</sup> cells destroyed host BM cells but spared donor BM cells. Alternatively, the presence of donor BM-derived cells might somehow have inhibited the destructive effects of the donor CD4<sup>+</sup> cells.

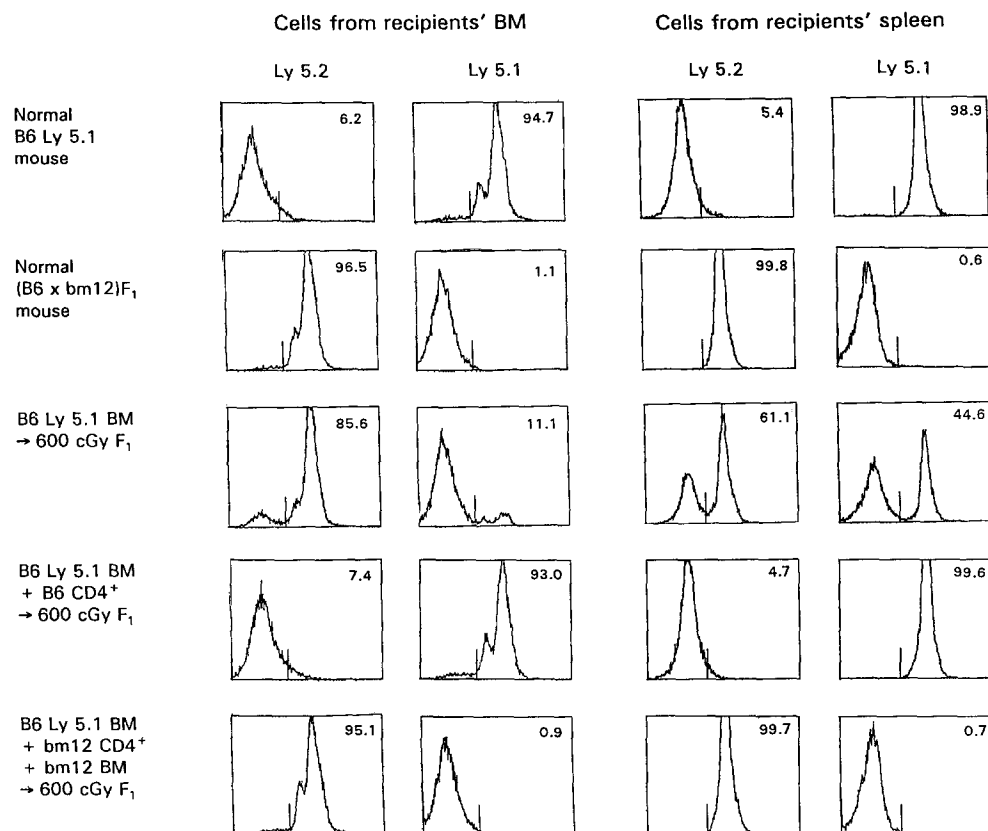
To distinguish between these two possibilities, 600 cGy (B6 × bm12)F<sub>1</sub> mice (Ly 5.2) were injected with BM cells from the B6-Ly 5.1 congenic line (Ly 5.1) with or without B6 CD4<sup>+</sup> cells (Ly 5.2). In terms of total cell counts in the marrow, both groups of recipients showed no evidence of marrow aplasia when tested 14–35 d after transfer. When the donor/host identity of the marrow cells was assessed by FACS<sup>®</sup> analysis, the recipients of B6-Ly 5.1 BM cells alone comprised a mixture of Ly 5.1<sup>+</sup> (donor) and Ly 5.2<sup>+</sup> (host) cells in both marrow and spleen. With injection of a mixture of B6-Ly 5.1 BM and B6 CD4<sup>+</sup> cells, by contrast, nearly all of the Thy 1<sup>-</sup> cells in the host marrow were Ly 5.1<sup>+</sup>.

This is illustrated in the experiment shown in Fig. 4 in which a limiting dose of 2 × 10<sup>5</sup> B6-Ly 5.1 BM cells was

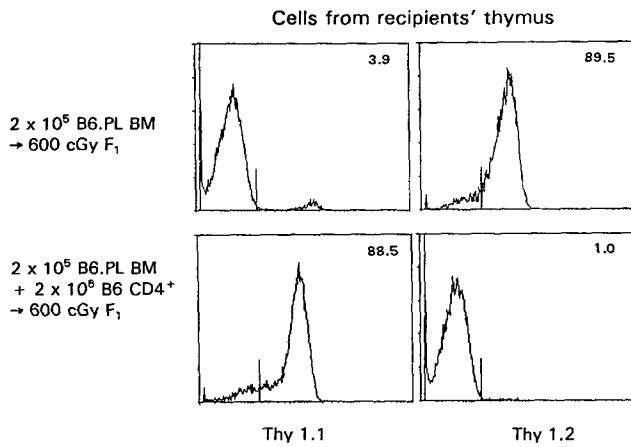
used for reconstitution. With this small dose of BM cells, reconstitution of the host marrow with donor Ly 5.1<sup>+</sup> cells was quite limited (11% Ly 5.1<sup>+</sup> cells 4 wk after transfer). In marked contrast, in recipients of a mixture of B6 CD4<sup>+</sup> cells and the same small dose of B6-Ly 5.1 BM cells, nearly all (93%) of the Thy 1<sup>-</sup> marrow cells were Ly 5.1<sup>+</sup> (compared with 95% staining of normal B6-Ly 5.1 BM). Reciprocal results were observed when the marrow from the two groups of mice were stained for host Ly 5.2 expression. Bearing in mind that the anti-Ly 5.2 mAb used caused weak (6%) cross-reactive staining of normal B6-Ly 5.1 marrow (Fig. 4), Thy 1<sup>-</sup> host Ly 5.2<sup>+</sup> cells were virtually absent in the mice given a mixture of B6 CD4<sup>+</sup> cells and B6-Ly 5.1 BM cells. Similar findings applied to the Thy 1<sup>-</sup> T cells in spleen (Fig. 4). As a control in this experiment, some of the mice were injected with B6-Ly 5.1 BM cells plus bm12 CD4<sup>+</sup> cells and bm12 BM cells (both Ly 5.2). The expectation here was that the bm12 CD4<sup>+</sup> cells would kill the allogeneic B6-Ly 5.1 BM cells (and also host F<sub>1</sub> BM cells) but spare the syngeneic bm12 BM cells, thus causing complete repopulation with Ly 5.2<sup>+</sup> cells, i.e., with bm12-derived cells. This was indeed the case (Fig. 4).

The data in Fig. 4 indicate therefore that, in the presence of a mixture of donor and host BM cells, donor CD4<sup>+</sup> cells selectively destroyed host BM-derived cells. The recipients thus showed complete repopulation with donor-derived cells.

**CD4<sup>+</sup> Cells Destroy Host Precursors of Thymocytes.** Thy-



**Figure 4.** Donor CD4<sup>+</sup> cells destroy host hemopoietic cells but spare donor cells. (B6 × bm12)F<sub>1</sub> mice (Ly 5.2) were exposed to 600 cGy and reconstituted with a low dose of 2 × 10<sup>5</sup> B6-Ly 5.1 BM cells with or without 2 × 10<sup>6</sup> B6 (Ly 5.2) CD4<sup>+</sup> cells. A control group of mice received B6-Ly 5.1 BM cells supplemented with bm12 CD4<sup>+</sup> cells and bm12 BM cells. Using cells pooled from two to three mice per group, marrow and spleen suspensions were prepared 4 wk after transfer, treated with anti-Thy 1 mAb plus C to remove mature T cells and stained for expression of Ly 5.1 and Ly 5.2 followed by FACS<sup>®</sup> analysis. The data are plotted on a log scale.



**Figure 5.** Donor CD4<sup>+</sup> cells destroy host precursors of thymocytes. (B6 × bm12)F<sub>1</sub> mice exposed to 600 cGy were injected with a low dose of 2 × 10<sup>5</sup> B6.PL (Thy 1.1) BM cells with or without 2 × 10<sup>6</sup> B6 (Thy 1.2) CD4<sup>+</sup> cells. Pooled thymocytes from both groups of mice (40–60 × 10<sup>6</sup>/mouse) were stained for expression of Thy 1.1 vs. Thy 1.2 4 wk after transfer and then analyzed with a FACS<sup>®</sup>. The data are plotted on a log scale.

mocyte repopulation in irradiated mice is known to depend upon a constant influx of stem cells, probably pluripotential stem cells, from the marrow (18). To examine whether thymic cells can act as targets for CD4<sup>+</sup> cells, 600 cGy (B6 × bm12)F<sub>1</sub> mice (Thy 1.2) were reconstituted with a low dose of 2 × 10<sup>5</sup> B6-Thy 1.1 BM cells ± B6 CD4<sup>+</sup> cells (Thy 1.2). When thymocytes from the two groups of mice were analyzed by FACS<sup>®</sup> 4 wk later, thymocytes from the control group given B6-Thy 1.1 BM cells alone contained very few donor BM-derived cells, i.e., 4% Thy 1.1<sup>+</sup> cells (Fig. 5). In marked contrast, donor-derived Thy 1.1<sup>+</sup> cells accounted for virtually all of the thymocytes recovered from recipients of a mixture of B6-Thy 1.1 BM and B6 CD4<sup>+</sup> cells. This finding implies that the injected B6 CD4<sup>+</sup> cells selectively destroyed host stem cells, thereby allowing the thymus to be repopulated solely by donor stem cells.

**Resistance of Ia<sup>-</sup> BM Cells to Destruction by CD4<sup>+</sup> Cells.** The above data on phenotyping of marrow cells and thymocytes indicate that the destructive function of CD4<sup>+</sup> cells is strongly Ia restricted and is directed, at least in part, to stem cells. The key problem with this scenario is that despite ex-

**Table 5.** Resistance of B6 Ia<sup>o</sup> BM Cells to Destruction by bm12 CD4<sup>+</sup> Cells

Exp. No.	Cells transferred to 600 cGy hosts		Spleen/BM recovered from hosts at days 14–16	
	T cells	BM cells	No. of nucleated cells in both tibiae (× 10 <sup>-6</sup> ) (SD)	Spleen weight (mg) (SD)
<i>(B6 × bm12)F<sub>1</sub> hosts</i>				
1	bm12 CD4 <sup>+</sup>	bm12	19.7 (1.0)	166 (32)
	bm12 CD4 <sup>+</sup>	B6	0.8 (0.1)	80 (2)
	bm12 CD4 <sup>+</sup>	B6 Ia <sup>o</sup>	23.1 (6.8)	220 (19)
	B6 CD4 <sup>+</sup>	bm12	1.1 (0.1)	69 (5)
	B6 CD4 <sup>+</sup>	B6	22.5 (5.8)	230 (32)
	B6 CD4 <sup>+</sup>	B6 Ia <sup>o</sup>	23.3 (6.0)	204 (64)
2	bm12 CD4 <sup>+</sup>	bm12	19.8 (7.4)	126 (8)
	bm12 CD4 <sup>+</sup>	B6	0.1 (<0.1)	38 (3)
	bm12 CD4 <sup>+</sup>	B6 Ia <sup>o</sup>	20.2 (2.7)	183 (110)
	–	B6	20.1 (2.4)	76 (2)
	–	B6 Ia <sup>o</sup>	24.3 (0.6)	72 (6)
<i>(B6 × bm1)F<sub>1</sub> hosts</i>				
3	bml CD8 <sup>+</sup>	B6	1.2 (0.2)	43 (9)
	bml CD8 <sup>+</sup>	B6 Ia <sup>o</sup>	0.3 (0.2)	50 (12)
	–	B6 Ia <sup>o</sup>	21.4 (4.5)	77 (1)

In each experiment, T cells and BM cells were injected in a dose of 2 × 10<sup>6</sup> cells/mouse. The mice were analyzed at 14–16 d after transfer. The data represent the mean of three mice per group.



haustive attempts we were unable to demonstrate Ia expression on stem cells. Thus, even after incubation with lymphokines in vitro, treating marrow cells with anti-Ia mAb plus C and/or removing Ia<sup>+</sup> cells by panning failed to cause any significant reduction in CFUs or remove pluripotential stem cells (data not shown). Since the above methods might not be sufficiently sensitive to detect limited (but physiologically significant) Ia expression on stem cells, we examined whether CD4<sup>+</sup> cells could destroy BM cells taken from Ia "knockout" (Ia<sup>o</sup>) mice; the Ia<sup>o</sup> line used was backcrossed to the B6 background (12).

As shown in Table 5, BM cells from this line were completely resistant to the cytotoxic effects of bm12 CD4<sup>+</sup> cells. Thus, whereas bm12<sup>+</sup> CD4<sup>+</sup> cells caused marked marrow and spleen atrophy when transferred to 600 cGy (B6 × bm12)F<sub>1</sub> mice with B6 BM cells, transferring bm12 CD4<sup>+</sup> cells with B6 Ia<sup>o</sup> BM cells caused no sign of marrow atrophy and led to splenomegaly (Table 5, Exp. 1 and 2). Control experiments indicated that the B6 Ia<sup>o</sup> BM cells retained sensitivity to marrow aplasia directed to class I antigens. Thus, as with B6 BM cells, transferring B6 Ia<sup>o</sup> BM cells to 600 cGy (B6 × bm1)F<sub>1</sub> hosts in the presence of bm1 CD8<sup>+</sup> cells led to marked marrow atrophy (Table 5, Exp. 3).

*A Search for Nonspecific Destruction of Donor BM Cells by CD4<sup>+</sup> Cells.* To assess the possibility that the destruction of BM cells by CD4<sup>+</sup> cells reflected a subtle bystander reaction, we reconstituted 600 cGy (B6 × bm12)F<sub>1</sub> mice with B6-Ly 5.1 BM cells ± B6 CD4<sup>+</sup> cells and then studied the kinetics of the appearance of the donor Ly 5.1<sup>+</sup> BM cells in the host marrow by FACS<sup>®</sup> analysis. If the destruction of the host marrow by CD4<sup>+</sup> cells reflected a bystander reaction rather than direct CTL activity, this reaction would be expected to impede engraftment by the donor B6-Ly 5.1 BM cells. However, counting total numbers of Ly 5.1<sup>+</sup> cells in the host long bones during the period of rapid engraftment (days 6–15) showed little or no evidence that the presence of B6 CD4<sup>+</sup> cells impaired engraftment with B6-Ly 5.1 BM cells.

## Discussion

This article documents that small doses of normal CD4<sup>+</sup> cells transferred to lightly irradiated Ia-different hosts caused profound atrophy of host hemopoietic cells 2–3 wk after transfer. By all parameters studied, the destruction of hemopoietic cells by CD4<sup>+</sup> cells was limited to Ia<sup>+</sup> host cells. Evidence for bystander damage of donor BM-derived cells or Ia<sup>-</sup> host cells was conspicuously absent. The key question is whether the injected CD4<sup>+</sup> cells destroyed hemopoietic cells via direct Ia-restricted CTL activity.

The notion that the injected CD4<sup>+</sup> cells killed host hemopoietic cells via direct CTL activity strains credulity when it is borne in mind that complete marrow atrophy in the B6/bm12 combination was induced by as few as 7 × 10<sup>4</sup> CD4<sup>+</sup> cells. Thus, taking into consideration the extensive size of the host marrow, it is extremely difficult to envisage how the antigen-reactive progeny of such a conspicuously small dose of CD4<sup>+</sup> cells caused virtually complete destruc-

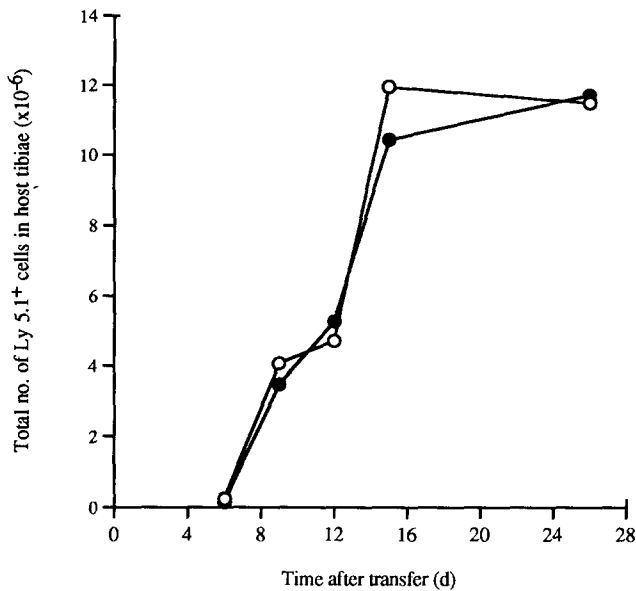
tion of host marrow cells in a brief period, i.e., during the second week after transfer. Surely one has to argue in terms of cell destruction by humoral factors?

In this respect, perhaps the simplest possibility is that the host cells were destroyed through the production of anti-Ia antibody, i.e., by donor-derived B cells. There are three crucial problems with this idea. First, given that the injected CD4<sup>+</sup> cells were effective in very small doses and were highly purified, the number of contaminating B cells in the injected CD4<sup>+</sup> cells must have been extremely low. Moreover, the purification method used to prepare the CD4<sup>+</sup> cells included treatment with anti-Ia mAb plus C in doses sufficient to kill >95% of spleen B cells. Second, to our knowledge the antigenic difference between I-A and I-A<sup>bm12</sup> is serologically undetectable. Third, and most importantly, we have been unable to detect antibody activity in the serum of irradiated (B6 × bm12)F<sub>1</sub> hosts injected with B6 CD4<sup>+</sup> cells (data not shown). For these reasons, it is very difficult to sustain the argument that host hemopoietic cells were destroyed via anti-Ia antibody. It is also worth noting that treating normal or lymphokine-induced BM cells with high concentrations of anti-Ia mAb plus C failed to impair stem cell activity. Yet stem cells appeared to be one of the main targets of attack by CD4<sup>+</sup> cells (see below).

Another possibility is that hemopoietic cells were destroyed via local production of toxic cytokines such as TNF. This notion deserves serious consideration because anti-TNF-α mAb is reported to be highly effective in preventing some of the in vivo effector functions of CD4<sup>+</sup> cells, e.g., gut damage associated with GVHD (19). The chief problem with the idea that tissue destruction reflected local production of toxic cytokines is that bystander damage by CD4<sup>+</sup> cells appeared to be remarkably limited. Thus the disappearance of host hemopoietic cells in the marrow did not seem to injure host stromal cells or cause significant impairment of donor BM-derived repopulation of marrow and thymus. These findings, plus the failure of CD4<sup>+</sup> cells to destroy residual radioresistant host T cells or host-type BM cells from Ia<sup>-</sup> deficient mice, strongly suggest that the destruction of host cells was directed exclusively to host Ia<sup>+</sup> cells.

For the reasons cited above, it is very difficult to explain the destruction of hemopoietic cells by CD4<sup>+</sup> cells in terms of humoral factors. By exclusion, one is thus forced to consider the possibility that the injected CD4<sup>+</sup> cells killed host hemopoietic cells via direct Ia-restricted CTL activity. All of the available data are consistent with this possibility. The following model can be considered. During the first week after transfer, the intravenously injected CD4<sup>+</sup> cells homed to the spleen and became sensitized to host Ia antigens expressed on dendritic cells. The host-reactive CD4<sup>+</sup> cells then underwent considerable clonal expansion, differentiated into effector cells, and percolated throughout the body, including the BM (where >50% of the cells from aplastic marrow were donor CD4<sup>+</sup> cells). Through direct CTL activity, the effector cells then destroyed Ia<sup>+</sup> cells, leaving Ia<sup>-</sup> host cells and Ia<sup>+</sup> donor cells untouched.

At least two objections can be raised against this scenario.



**Figure 6.** Donor CD4<sup>+</sup> cells fail to impede marrow engraftment of donor BM cells. (B6 × bm12)F<sub>1</sub> mice exposed to 600 cGy were injected with 2 × 10<sup>6</sup> B6-Ly 5.1 BM cells alone (O) or with a mixture of 2 × 10<sup>6</sup> B6-Ly 5.1 BM cells and 2 × 10<sup>6</sup> B6 CD4<sup>+</sup> cells (●). At the intervals shown, BM cells recovered from both tibiae of two mice per group were counted and then stained for Ly 5.1 expression followed by FACS<sup>®</sup> analysis. The data shown were calculated from the percentage of Ly 5.1<sup>+</sup> cells and the total BM counts.

First, one has to explain how typical BM cells, most of which are largely Ia<sup>-</sup>, could be destroyed en masse by CD4<sup>+</sup> cells. The explanation we favor here is that the CTL activity of CD4<sup>+</sup> cells is directed predominantly to various types of stem cells and/or their immediate progeny. In the absence of stem cells, the short-lived, terminally differentiated descendants of these cells rapidly disappear and hemopoietic failure results. In support of this idea, we observed a marked paucity of stem cells in the atrophic marrow of mice given CD4<sup>+</sup> cells. It is disturbing, however, that we failed to find evidence of Ia expression on stem cells by antibody plus C treatment or by panning. Moreover, studies in both humans (20) and mice (Spangrude, J., and I. Weissman, personal com-

munication) have found that purified pluripotential stem cells (nonlymphokine induced) are Ia<sup>-</sup> by FACS<sup>®</sup> analysis. Despite these findings, it is striking that the destruction of B6 BM cells by bm12 CD4<sup>+</sup> cells did not apply to B6 BM cells from Ia-deficient mice. This finding strongly suggests that Ia expression on stem cells is physiologically significant and sufficient to act as a target for CD4<sup>+</sup> cells. Since the stem cell targets for CD4<sup>+</sup> cells in irradiated hosts are probably cycling and exposed to various cytokines, it would seem likely that Ia expression on stem cells has to be induced. In favor of this possibility, it has been found that Ia expression on human stem cells can be upregulated by lymphokines (20). We presume the same applies to mouse stem cells but this remains to be proved.

The second problem with the notion that CD4<sup>+</sup> cells destroy hemopoietic cells via direct CTL activity is that we have had little success in demonstrating more than minimal CTL activity by CD4<sup>+</sup> cells in vitro. In several experiments we observed significant class II-restricted lysis of tumor cells and LPS blasts by in vivo-sensitized CD4<sup>+</sup> cells in the B6/bm12 combination (our unpublished data). However, the level of killing was quite low—far lower than for anti-class I killing by CD8<sup>+</sup> cells—unless the effector cells were restimulated with antigen in vitro. Yet Ia-restricted tissue destruction by CD4<sup>+</sup> cells in vivo was as potent, or more potent, than the destruction mediated by CD8<sup>+</sup> cells responding to a class I difference. In view of this paradox, the existing techniques for demonstrating CTL activity by CD4<sup>+</sup> cells in vitro may be a poor model for the in vivo functions of these cells (6).

Whatever the explanation for the mechanism of tissue destruction, the finding that very small doses of CD4<sup>+</sup> cells were capable of mediating massive Ia-restricted destruction of hemopoietic cells in irradiated hosts with no evidence of an overt bystander reaction indicates that the CTL activity of CD4<sup>+</sup> cells in vivo is extremely potent. Direct evidence on whether the CTL activity of CD4<sup>+</sup> cells in vivo reflects direct lysis or the local release of humoral factors will have to await further investigation. As a footnote it may be mentioned that destruction of stem cells by CD4<sup>+</sup> CTL could explain the finding that BM engraftment across Ia barriers is enhanced when the recipients are depleted of CD4<sup>+</sup> cells (21).

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