T Cell Contact with Ia Antigens on Nonhemopoietic Cells In Vivo Can Lead to Immunity Rather than Tolerance

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Summary
Long-term H-2-heterozygous a → (a x b)F1 bone marrow (BM) chimeras prepared with supralethal irradiation (1,300 rad) are devoid of Ia⁺ host BM-derived antigen-presenting cells (APC), but show quite strong host Ia expression in germinal centers, probably on follicular dendritic cells (a class of nonhemopoietic stromal cells). To examine whether Ia expression on these non-BM-derived cells is capable of inducing post-thymic tolerance of T cells, thymectomized irradiated (a x b)F1 mice were reconstituted with parent a stem cells and then, 6 mo later, given parent a thymus grafts. As measured by primary mixed lymphocyte reactions and Vβ expression, the CD4⁺ cells differentiating in the thymus-grafted mice showed no detectable tolerance to the H-2 (Ia) antigens of the host. To examine whether the thymus-grafted mice contained immunologically significant quantities of host Ia antigens, long-term a → (a x b)F1 chimeras were injected with normal strain a CD4⁺ cells; the donor cells were recovered from thoracic duct lymph of the chimeras and tested for host reactivity in vitro. The results showed that Ia expression in the chimeras was sufficient to cause selective trapping of a substantial proportion of host-la-reactive CD4⁺ cells soon after transfer and, at later stages, to induce strong priming. Tolerance was not seen. The data place constraints on the view that T cell recognition of antigen expressed on cells other than typical BM-derived APC leads to tolerance induction.

T cell tolerance to MHC molecules occurs largely in the thymus (1, 2). Tolerance reflects deletion of immature T cells encountering MHC molecules displayed on marrow-derived APC, and, to a lesser extent, on epithelial cells. The possibility that T cells can also be tolerized to MHC antigens in the post-thymic environment is suggested by the finding that transgenic mice in which MHC molecules are expressed selectively in the β cells of the pancreas (3, 4) show marked tolerance to the transgenic MHC molecules. These and other findings (5) imply that contact of mature T cells with MHC molecules expressed on cell types other than typical APC can lead to tolerance rather than immunity.

To investigate this question, we constructed thymus-grafted mice under conditions designed to allow newly formed strain a CD4⁺ T cells to make post-thymic contact with strain b H-2 (Ia) antigens expressed selectively on non-bone marrow-derived (NBMD)¹ cells. In a second model, mature strain a CD4⁺ cells were exposed to Ia⁺ (a x b)F1; NBMD cells on adoptive transfer. No tolerance was seen in either model; indeed, conspicuous priming resulted in the second model. The data would thus appear to contradict the dogma that T cell contact with antigen on "nonprofessional" APC leads to tolerance.

Materials and Methods

Mice. Mice were bred at the breeding facility of the Research Institute of Scripps Clinic.

Preparation of Thymus-grafted Mice. (B6 × CBA/J)F1; mice were thymectomized (Tx) at 6-8 wk of age (6), exposed to heavy irradiation (1,300-1,400 rad) 2-3 wk later, and then, within 4 h, reconstituted intravenously with T-depleted bone marrow (BM) cells (~5 × 10⁶ BM cells treated with anti-Thy-1 mAb + C) (7): 5 wk or 6 mo later, the chimeras were grafted under the kidney capsule with 1-d-old donor vs. host thymuses (three per mouse) exposed to 1,100 rad in vitro (8). The mice were kept in positive-pressure isolators and given antibiotics in the drinking water. The mice were killed at 9-10 wk post-grafting to prepare T cells.

Monoclonal Antibodies. mAbs specific for Thy-1.2 (Hj, rat), heat-stable antigen (J11d, rat), CD4 (GK1.5, rat), CD8 (3.168.8, rat), Vβ11 (RR3-15, rat), Vβ8.1 + 8.2 (KJ16-133, rat), and I-Ak4.1.0

¹Abbreviations used in this paper: BM, bone marrow; BMC, bone marrow chimeras; BMR, bone marrow reconstituted; DC, dendritic cells; FDC, follicular dendritic cells; NBMD, non-bone marrow-derived; TDL, thoracic duct lymph; TG, thymus grafts; Tx, thymectomized.
in 0.05 M NaOAc pH 5.2) with 0.01% HzO;/ for 20 min. The ethylcarbazole (Sigma Chemical Co., St. Louis, MO) (0.1 IAg/ml 30 min, washed, and incubated with the substrate 3-amino-9-antibodies for 1 h. After washing, horseradish peroxidase-conjugated min, and incubated with optimal concentrations of biotinylated with parental strain BM cells (7).

IFN-γ Treatment In Vivo. Chimeras and control mice were given four intraperitoneal injections of murine rIFN-γ (80,000 U/injection) at daily intervals. Mice were used at day 4 after the first injection. IFN-γ was kindly provided by Ryuji Maekawa (Shionogi Research Laboratories, Osaka, Japan).

Blood-to-Lymph Recirculation of T Cells through Irradiated Hosts. Using a modification of a technique described elsewhere (12, 13), long-term chimeras and control mice were exposed to 100 rad and then, 4–6 h later, injected intravenously with a dose of ~8 × 10^6 purified B6 CD4^+ T cells. The above findings confirm that parent → F_1 bone marrow chimeras (BMC) prepared with heavy irradiation express significant levels of host Ia on stromal (NBMD) cells but lack host-derived BM-derived cells. The extent of host Ia expression in BMC is considered below.

Host Ia Expression in a → (a × b)F_1 BM Chimeras. When (a × b)F_1 mice are exposed to suprathetal irradiation, e.g., 1,300 rad, and reconstituted with parental BM cells, Ia^+ (H-2 class II^+) host cells with APC function in vitro disappear rapidly (7). By 2 mo post-transfer, virtually all functional APC in suspensions of spleen and thymus are of donor origin. Host Ia expression in cryostat sections of spleen from a chimera tested at 6 mo post-reconstitution is shown in Fig. 1. Chimeras were constructed by transferring T-depleted B6.PL (H-2b) BM cells to (B6 × CBA)F_1 (H-2^b × H-2^k) mice exposed to 1,300 rad. Cells expressing a high density of host I-A^d or I-E^k molecules were not seen in the splenic red pulp and were conspicuously absent from the perivascular lymphoid sheaths (PALS) of the spleen and the marginal sinuses of the white pulp borders, i.e., the regions occupied by dendritic (interdigitating) cells (DC) and macrophages, respectively (Fig. 1, D–F). Likewise, high-density I-A^k+ cells were undetectable in LN, lung, liver, gut, and epidermis (data not shown). Nevertheless, moderately high I-A^d and I-E^k expression was observed in the central regions of primary follicles and germinal centers. Since a similar pattern of staining was observed in twice-irradiated chimeras given a total dose of 2,200 rad (see Materials and Methods), it is unlikely that the Ia^+ cells in primary follicles and germinal centers were BM derived. The staining in these sites was probably restricted to follicular dendritic cells (FDC) (15). These cells are not of hematogenous origin (16) and express significant though variable levels of Ia molecules (16, 17). FDC are not related to typical BM-derived DC.

In addition to FDC, a low but variable level of host Ia expression was evident on blood vessel endothelial cells of the chimeras, especially in the red pulp. Ia expression on these cells was markedly elevated in chimeras injected with rIFN-γ (80,000 U daily for 4 d) (Fig. 1, G and H).

The above findings confirm that parent → F_1 chimeras prepared with suprathetal irradiation are apparently devoid of host-derived Ia^+ BM-derived cells but do show significant Ia expression on certain NBMD cells, especially FDC and endothelial cells.

Antihost Reactivity of CD4^+ T Cells Developing in Tx (a × b)F_1 Mice Given a BM Cell and a TG. Tx (B6 × CBA)F_1 mice were exposed to heavy irradiation (1,300 rad) and reconstituted with T-depleted Thy-1.1^+ B6.PL (H-2^b) BM. After 5 wk, these Tx, irradiated, BM-reconstituted (Tx.Ir.BMR) mice received irradiated (1,100 rad) Thy-1.2^+ B6 vs. (B6 × CBA)F_1 neonatal TG. Primary MLR by B6.PL-derived (Thy-1.2^+) CD4^+ T cells prepared from these mice at 10 wk post-grafting are shown in Table 1. It can be seen that, both for LN cells and mature thymocytes, the H-2^b CD4^+ cells from mice given host-type (B6 × CBA)F_1 TG showed strong tolerance to host H-2^b APC but responded well to third-party bm12 APC (group 3). By contrast, CD4^+ cells from mice with H-2^k TG gave definite responses to H-2^k...
APC (group 4); though significant, these responses were nevertheless considerably lower than the control responses of normal H-2k CD4+ cells (group 1).

To examine whether the partial tolerance seen in the above mice reflected incomplete disappearance of host APC after irradiation, the interval between BMR and TG was extended from 5 wk (Table 1) to 6 mo (Table 2). Under these conditions, the LN CD4+ cells differentiating in H-2k TG showed no detectable tolerance to the H-2k antigens of the host (Table 2). The same findings applied to mature CD4+ (8-) cells prepared from the TG of the chimeras (Table 3). These data on TG mice contrasted with the strong tolerance seen in control parent → F1 chimeras, i.e., where the CD4+ cells differentiated in the endogenous thymus of the host (Table 2, bottom group).

Recently, we observed that CD4+ cells from B6 (I-E-) → (B6 × CBA)F1 (I-E+) chimeras show considerable (50-70%) deletion of Vβ11+ cells (7); in normal mice, Vβ11+ cells are selectively deleted in I-E+ mice. Vβ11 expression on T cells from the 6-mo TG mice discussed above are shown in Table 4. It can be seen that, in marked contrast to normal (B6 × CBA)F1 mice, Vβ11+ cells were not

Figure 1. Host Ia expression in spleens of (B6 × CBA)F1 and long-term B6.PL → F1 BMC. Cryostat sections of spleens from a normal (B6 × CBA)F1 mouse, and a 6-mo B6.PL → F1 BMC were stained for expression of I-Ak and I-Ek as described in Materials and Methods. (A) Low power (×40) view of normal F1 spleen stained for I-Ak; staining is evident throughout the spleen. (B) High power (×100) view of A: staining is evident in the red pulp (rp), the marginal zone (mz), and the follicles (f), and periarteriolar lymphocyte sheaths (pals) of the white pulp; the central arteriole (ca) is arrowed. (C) Low power (×40) view of B6 (I-Ak) spleen stained for I-Ak; except for the punctate staining of cells in the red pulp (indicative of cells with endogenous peroxidase activity), no staining is apparent. (D) Low power (×40) view of B6.PL → F1 BMC spleen stained for I-Ak; patchy staining in the white pulp and stellite staining of red pulp sinuoids is evident. (E) High power (×100) view of D; staining of the white pulp is limited to focal areas in follicles; there is no staining in the pals or mz. (F) High power (×200) view of spleen from B6 PL → F1 BMC stained for I-Ek; the pattern of staining is essentially the same as in E (note that the magnification is higher for F than for E, and that F and E involved different fields). (G) Low power (×40) view of spleen from B6.PL → F1 BMC pretreated with IFN-γ (Materials and Methods); strong staining is evident throughout the spleen. (H) High power (×100) view of G; strong staining of stromal cells (but not hemopoietic cells) is seen throughout the white pulp.
Table 1. Primary MLR by CD4$^+$ Cells Generated in Tx.Ir.BMR (H-2$^b$ x H-2$^k$)F$_1$ Mice Given H-2$^b$ Stem Cells Followed 5 wk Later by H-2$^k$ vs. F$_1$ Thymus Grafts

| Group | CD4$^+$-enriched responders (10$^6$) | Tissue source of responders | [H]$TdR$ incorporation with stimulators: |
|-------|------------------------------------|-----------------------------|----------------------------------------|
|       |                                    | LN                          | B6.PL (H-2$^b$)  | CBA (H-2$^k$)  | bm12 (H-2$^{bm12}$) | Response to H-2$^k$/response to H-2$^{bm12}$ |
| 1     | Normal B6.PL                        |                             | 1.5 146.5 80.8 1.8 |
|       |                                    | Thymus                      | 0.3 49.7 69.4 0.7 |
| 2     | Normal (B6 x CBA)F$_1$             | LN                          | 2.3 3.0 125.8 <0.1 |
|       |                                    | Thymus                      | 0.2 0.3 56.4 <0.1 |
| 3     | B6.PL BM → Tx.Ir (B6 x CBA)F$_1$   | LN                          | 0.2 3.1 60.0 <0.1 |
|       | with (B6 x CBA)F$_1$ TG             | Thymus                      | 0.2 0.4 35.2 <0.1 |
| 4     | B6.PL BM → Tx.Ir (B6 x CBA)F$_1$   | LN                          | 0.6 26.4 51.7 0.5 |
|       | with B6 TG                         | Thymus                      | 0.2 28.3 68.2 0.4 |

Tx.Ir. BMR mice were prepared as described in Materials and Methods and the text. To ensure that the T cells generated in the Tx.Ir.BMR mice were not contaminated with radioresistant cells present in the TG at the time of grafting, the mice received Thy-1.1$^+$ BM cells (B6.PL) and Thy-1.2$^+$ TG. Purified CD4$^+$ CD8$^-$ T cells depleted of Thy-1.2$^+$ cells were prepared from LN and the thymus of the hosts at 10 wk post-grafting and used as responder cells (10$^6$) in MLR with irradiated spleen cells (5 x 10$^6$) as stimulators. The data show mean responses for triplicate cultures harvested on day 4. Note that, to prevent responses to Mls$^+$ determinants, CBA/Ca rather than CBA/J stimulators were used.

Table 2. Primary MLR by CD4$^+$ Cells Generated in Tx.Ir.BMR (H-2$^b$ x H-2$^k$)F$_1$ Mice Given H-2$^k$ Stem Cells Followed 6 mo Later by H-2$^k$ Thymus Grafts: MLR by CD4$^+$ Cells from LN

| CD4$^+$-enriched responders (2 x 10$^6$) | Tissue source of responders | Day of MLR | B6.PL (H-2$^b$) | (B6 x CBA)F$_1$ (H-2$^{bm12}$) | bm12 (H-2$^{bm12}$) | Response to H-2$^k$/response to H-2$^{bm12}$ |
|-----------------------------------------|-----------------------------|------------|----------------|--------------------------------|---------------------|---------------------------------------------|
| Normal B6.PL                            | LN                          | 3          | 2.3 37.4 42.6 0.9 |
|                                         | 4                           | 4.4 76.4 64.5 1.2 |
| Normal (B6 x CBA)F$_1$                  | LN                          | 3          | 1.4 1.9 33.0 <0.1 |
|                                         | 4                           | 2.9 3.8 78.4 <0.1 |
| B6.PL BM → Tx.Ir (B6 x CBA)F$_1$ + B6 TG| LN                          | 3          | 2.5 73.6 61.9 1.2 |
|                                         | 4                           | 4.4 76.4 64.5 1.2 |
| B6.PL → Ir (B6 x CBA)F$_1$ (non-Tx)     | LN                          | 3          | 1.1 5.2 45.9 0.1 |
|                                         | 4                           | 2.3 15.4 95.5 0.1 |

Tx.Ir.BMR (B6 x CBA)F$_1$ mice were prepared as described in Table 1, except that thymus grafting was delayed for 6 mo after irradiation and BM reconstitution. Cell suspensions from LN were prepared at 9 wk after thymus grafting. Control parent → F$_1$ chimeras were made by exposing normal (euthymic) (B6 x CBA)F$_1$ mice to 1,300 rad and reconstituting these mice with B6 BM cells; 3 mo later, these mice received further irradiation (1,000 rad) followed by reconstitution with B6.PL BM; these mice are abbreviated "B6.PL → Ir (B6 x CBA)F$_1$" in the table. As in Table 1, all cell suspensions were treated with anti-Thy-1.2 mAb + C before use. Primary MLR were assayed as for Table 1, except that a higher dose of responder cells (2 x 10$^6$) was used.
Table 3. Primary MLR by CD4⁺ Cells Generated in Tx.Ir.BMR (H-2b x H-2k)F₁ Mice Given H-2b Stem Cells Followed 6 mo Later by H-2b Thymus Grafts: MLR by CD4⁺ Cells from LN vs. Thymus

| CD4⁺-enriched responders (2 x 10⁵) | Tissue source of responders | Day of MLR | B6.PL (H-2b) | CBA (H-2k) | bm12 (H-2bmtz) | Response to H-2b/ | Response to H-2bmtz |
|-----------------------------------|-----------------------------|-----------|-------------|------------|----------------|------------------|-------------------|
| Normal B6.PL                      | LN                          | 3         | 3.4        | 86.7       | 41.7           | 2.1              |
|                                   |                             | 4         | 4.1        | 219.0      | 89.3           | 2.5              |
|                                   | Thymus                      |           | 0.1        | 49.0       | 20.5           | 2.4              |
| Normal (B6 x CBA)F₁              | LN                          | 3         | 2.3        | 2.4        | 43.2           | < 0.1            |
|                                   |                             | 4         | 3.5        | 5.3        | 82.7           | < 0.1            |
|                                   | Thymus                      |           | 0.3        | 0.1        | 28.6           | < 0.1            |
| B6.PL BM → Tx.Ir (B6 x CBA)F₁ + B6 TG | Mouse no. 1 | LN        | 3         | 2.2        | 62.0           | 29.7             | 2.2              |
|                                   |                             | 4         | 3.3        | 176.9      | 72.0           | 2.5              |
|                                   | Mouse no. 2 | LN        | 3         | 2.7        | 112.4          | 32.4             | 3.7              |
|                                   |                             | 4         | 3.8        | 158.0      | 77.5           | 2.1              |
|                                   | Thymus                      |           | 0.1        | 104.6      | 44.6           | 2.4              |

As for Table 2, except that the CD4⁺ cells were prepared both from LN and the TG.

Table 4. V₈11 Expression on LN T Cells Generated in Tx.Ir.BMR (H-2b x H-2k)F₁ Mice Given H-2b Stem Cells Followed 6 mo Later by H-2b Thymus Grafts

| Mice tested                      | No. of mice tested | Percent of CD4⁺ cells expressing: | Percent of CD8⁺ cells expressing: |
|----------------------------------|--------------------|----------------------------------|----------------------------------|
|                                  |                    | V₈11    | V₈8    | V₈11    | V₈8    |
| Normal B6.PL                     | 3                  | 4.0     | 15.9   | 9.6     | 16.0   |
|                                  |                    | 4.3     | 15.4   | 9.0     | 17.6   |
|                                  |                    | 3.6     | 17.2   | 10.8    | 17.6   |
|                                  | Mean =             | 4.0     | 16.2   | 9.8     | 17.1   |
| Normal (B6 x CBA)F₁             | 3                  | 0.0     | 14.4   | 2.7     | 14.2   |
|                                  |                    | 0.5     | 15.8   | 2.8     | 15.7   |
|                                  |                    | 0.0     | 15.1   | 5.3     | 15.3   |
|                                  | Mean =             | 0.2     | 15.1   | 3.6     | 15.4   |
| B6.PL BM → Tx.Ir. (B6 x CBA)F₁  | 4                  | 7.6     | 25.6   | 12.5    | 22.1   |
| with B6 TG                      |                    | 5.2     | 20.0   | 13.6    | 27.3   |
|                                  |                    | 5.6     | 20.8   | 17.6    | 29.5   |
|                                  |                    | 3.9     | 23.0   | 14.2    | 24.4   |
|                                  | Mean =             | 5.6     | 22.4   | 14.5    | 25.9   |

LN T cells from normal mice and the TG mice used in Tables 2 and 3 were double stained for V₈11 (RR3-15) or V₈8.1 + 8.2 (KJ16 mAb) vs. CD4 or CD8 and analyzed by flow cytometry using two-channel immunofluorescence (Materials and Methods). For the TG mice, the data were calculated with respect to Thy-1.1⁺ cells.
cells was assessed by dual fluorescence and FACS® analysis (Materials and Methods). Mean values for individual mice (two to three mice/group) are shown. CD4+ cells in TDL were analyzed for expression of Vp11 (A), Vp8 (B), Vp5 (C), and for total numbers (D). Vp expression on CD4+ cells was assessed by dual fluorescence and FACS® analysis (Materials and Methods). Mean values for individual mice (two to three mice/group) are shown. It can be seen that B6 CD4+ cell transfer to B6.PL (I-E-) hosts led to no change in Vp ratios; cell outputs in TDL declined progressively and nearly all of the lymph-borne cells were donor-derived. B6 CD4+ cell transfer to normal F1 (I-E+) hosts led to an initial marked decline in Vp11+ and Vp5+ cells followed by an increase in these cells. This increase corresponded with the appearance of blast cells in the lymph; by day 60, nearly all of the lymph-borne cells were blasts. The decrease in Vp8+ cells at 47-68 h presumably means that these cells were underrepresented in the host-reactive early blast populations; the later rise in Vp8+ cells at 69-84 h may be an indication of the marked over-representation of Vp8+ cells seen at 2 wk post-transfer (see Fig. 6). With B6 CD4+ cell transfer to the chimeras, there was a minor decrease in Vp11+ and Vp5+ cells in early lymph collections and a slight increase in these cells in later collections; total cell outputs declined progressively, although blast cells were apparent in late lymph collections. In the particular experiment illustrated, all three groups of mice were treated with IFN-γ before T cell injection. Based on other experiments (see text), IFN-γ treatment did not enhance the degree of Vp selection seen in the chimeras or potentiate the production of blast cells. It should be noted that in all three groups of mice, the vast majority of the lymph-borne cells were donor-derived (Thy-1.2+, H-2d+) CD4+ cells.

Figure 2. Vp expression on thoracic duct cells collected from irradiated B6.PL mice (○), (B6 × CBA)F1 mice (●), and B6.PL → F1 BM chimeras (●) injected with normal B6 CD4+ cells. All mice were exposed to 900 rad 4 h before intravenous injection of 8 × 10⁷ B6 CD4+ cells (LN cells treated with J11d + anti-CD8 mAb + C); thoracic duct cannulas were inserted 14 h later, and lymph was collected continuously for the intervals shown. CD4+ cells in TDL were analyzed for expression of Vp11 (A), Vp8 (B), Vp5 (C), and for total numbers (D). Vp expression on CD4+ cells was assessed by dual fluorescence and FACS® analysis (Materials and Methods). Mean values for individual mice (two to three mice/group) are shown. It can be seen that B6 CD4+ cell transfer to B6.PL (I-E-) hosts led to no change in Vp ratios; cell outputs in TDL declined progressively and nearly all of the lymph-borne cells were small lymphocytes. B6 CD4+ cell transfer to normal F1 (I-E+) hosts led to an initial marked decline in Vp11+ and Vp5+ cells followed by an increase in these cells. This increase corresponded with the appearance of blast cells in the lymph; by day 60, nearly all of the lymph-borne cells were blasts. The decrease in Vp8+ cells at 47-68 h presumably means that these cells were underrepresented in the host-reactive early blast populations; the later rise in Vp8+ cells at 69-84 h may be an indication of the marked over-representation of Vp8+ cells seen at 2 wk post-transfer (see Fig. 6). With B6 CD4+ cell transfer to the chimeras, there was a minor decrease in Vp11+ and Vp5+ cells in early lymph collections and a slight increase in these cells in later collections; total cell outputs declined progressively, although blast cells were apparent in late lymph collections. In the particular experiment illustrated, all three groups of mice were treated with IFN-γ before T cell injection. Based on other experiments (see text), IFN-γ treatment did not enhance the degree of Vp selection seen in the chimeras or potentiate the production of blast cells. It should be noted that in all three groups of mice, the vast majority of the lymph-borne cells were donor-derived (Thy-1.2+, H-2d+) CD4+ cells.

deleted in Tx.Ir.BMR (B6 × CBA)F1 hosts given H-2b BM plus H-2k TG.

Recognition of Host Ia Antigen in Parent → F1 Chimeras by Normal T Cells. One explanation for the lack of functional tolerance in the TG mice is that host Ia expression in these mice was simply too low to be relevant. To address this ques-

tion, we used the technique of blood-to-lymph recirculation of normal T cells through irradiated hosts (13, 18-21). This technique traps host-reactive T cells in the lymphoid tissues, presumably as a manifestation of T cell binding to host H-2 bearing cells. Trapping is manifested by a transient disappearance of host-reactive T cells from TDL. In normal immunogenic donor/host combinations, this stage of negative selection is maximal at 1-2 d post-transfer. Thereafter, the progeny of the trapped cells proliferate extensively and re-enter TDL in large numbers as blast cells: the stage of positive selection.

For the experiments discussed below, B6.PL → 1,300 rad (B6 × CBA)F1 (F1) chimera was exposed to 900 rad at 6 h post-transfer and injected 2-4 h later with a large dose (~8 × 10⁷) of purified CD4+ cells prepared from normal B6 mice; as controls, B6 CD4+ cells were also transferred to irradiated normal F1 and B6.PL mice. In some of the experiments, the host mice were pretreated with IFN-γ for 3 d before irradiation and T cell transfer. Thoracic duct cannulation was performed ~16 h after T cell injection, and TDL were collected continuously over the next 4-5 d. The vast majority (>90%) of the lymph-borne cells were of donor (Thy-1.2+) origin.

Vp Expression. With I-E- → I-E+ strain combinations, negative and positive selection of host-I-E-reactive T cells is demonstrable by monitoring Vp11 expression on the donor T cells entering TDL (13). As shown in Fig. 2, negative and positive selection of Vp11+ cells and also Vp5+ cells was conspicuous when B6 CD4+ cells were transferred to normal F1 hosts. The proportions of Vp11+ and Vp5+ cells were approximately eightfold below normal in early lymph collections (16-34 h) but then rose to two- to threefold above normal levels in later collections (47-68 h) before declining; Vp8+ cells, which are not I-E reactive, showed reciprocal kinetics. No alteration in Vp expression occurred when B6 CD4+ cells were transferred to syngeneic B6.PL hosts; here, the proportions of Vp11+, Vp5+, and Vp8+ cells in the lymph remained constant throughout the 4-d drainage period. In contrast to normal F1 hosts, negative and positive selection of Vp11+ and Vp5+ cells was quite limited when B6 CD4+ cells were transferred to the chimeras. In a total of four experiments, including two with mice pretreated with IFN-γ, the proportions of Vp11+ and Vp5+ cells in lymph of the chimeras were reduced by 10-20% in early collections and increased by 10-30% in late collections. The best evidence for selection is shown in the experiment illustrated in Fig. 2 in which the hosts were IFN-γ pretreated. Based on other experiments, however, it did not appear that IFN-γ pretreatment enhanced selection. The point to emphasize is that negative and positive selection of Vp11+ and Vp5+ (but not Vp8+) cells was reproducibly demonstrated in the chimeras but was clearly much less marked than in normal F1 hosts.

Activation Markers on Lymph-borne Cells. With transfer of B6 CD4+ cells to normal F1 hosts, the re-appearance of Vp11+ and Vp5+ cells in TDL after the stage of negative selection was associated with a marked (~10-fold) increase in total numbers of cells in the lymph (Fig. 2 D). By day 3, nearly all of these cells were blast cells. With T cell transfer
to the chimeras, by contrast, the cell content of the lymph was no higher than in the control B6 CD4+ \(\rightarrow\) B6.PL group. This applied for the first 3 d post-transfer. Thereafter, there was a slight (but reproducible) increase in TDL cell numbers in the chimeras. This increase was associated with the appearance of small numbers of blast cells; by day 4, blast cells accounted for 20–40% of the lymph-borne cells (compared with 10–20% for the B6 \(\rightarrow\) B6.PL group).

Fig. 3 shows the expression of activation markers on the lymph-borne cells collected at 69–84 h from the mice tested in Fig. 2; the cells were double stained for V\(\alpha\)11 vs. 3G11, a marker for virgin (resting) T cells (11). Early lymph collections from all three groups of mice consisted almost entirely of small 3G11- cells (data not shown). For later lymph collections (Fig. 3), the cells from the B6 \(\rightarrow\) B6.PL group remained 3G11+. By contrast, theblast cells (including V\(\alpha\)11+ blasts) generated in the B6 \(\rightarrow\) B6.PL group were nearly all 3G11-, indicative of activated T cells. With the B6 \(\rightarrow\) chimera group, 3G11- cells began to appear in the lymph at the end of day 3 and reached 60% of total cells by day 4–5. The proportion of these cells was not significantly higher in IFN-\(\gamma\)-pretreated chimeras. Essentially similar findings applied when the cells were stained for other markers that distinguished resting and activated T cells, e.g., Pgp-1 (data not shown).

**T Cell Selection Measured by MLR.** The experiments in Fig. 4 were designed to examine whether acute blood to lymph recirculation of B6 CD4+ cells through the B6.PL \(\rightarrow\) F1 chimera was able to cause trapping of functional T cells, i.e., T cells responsive to normal F1 stimulator cells in vitro. The donor cells were collected from the lymph at 20–40 h post-injection and used as responder cells for MLR. The results of three experiments are shown in Fig. 4 (A and B, C–E, F–H); in one of the experiments (F–H), the chimeras (and controls) were pretreated with IFN-\(\gamma\). As expected, B6 CD4+ cells filtered through control B6.PL mice gave high MLR to normal (B6 \(\times\) CBA)F1 stimulators (I-A + I-E difference) and weaker responses to B10.A(5R) stimulators (I-E difference alone). By contrast, B6 CD4+ cells filtered through normal F1 hosts were completely unresponsive to F1 and 5R stimulators; responses to third-party bm12 stimulators were retained. The significant finding was that filtration of B6 CD4+ cells through the chimeras caused a partial reduction in the response to F1 and 5R stimulators. This reduction was specific because responses to bm12 remained largely unchanged. Based on the magnitude of the MLR mediated by graded doses of responder cells, the specific reduction in the anti-F1 and anti-5R MLR by CD4+ cells filtered through the chimeras vs. control B6.PL hosts amounted to \(~50\%\) (see Fig. 4 legend). This also applied to chimeras pretreated with IFN-\(\gamma\).

MLR by the lymph-borne cells collected from the above three groups of mice at a later stage, i.e., 48–62 h post-injection, are shown in Fig. 5 and Table 5. At this stage, most of the cells in the lymph of the B6 CD4+ \(\rightarrow\) normal F1 group were blast cells (see above). These cells gave a low but significant MLR to F1 stimulators, which peaked on day 2–3 and then declined to low levels by day 4 (Table 5). This is a typical response of recently activated T cells (21). Quite different results were seen with CD4+ cells collected from the chimeras. As with cells from the control B6 \(\rightarrow\) B6.PL group, the lymph-borne cells from the chimeras gave typical primary responses to F1 stimulators with higher responses on day 4 than on day 3 (Table 5). Significantly, in contrast to earlier lymph collections, there was no reduction in the response to F1 (Fig. 5) or 5R (data not shown) stimulators. This was seen in three separate experiments, including one experiment with IFN-\(\gamma\)-pretreated mice (Fig. 5, C and D).

The above findings apply to cells recovered from the chimeras at 50–60 h post-transfer, i.e., the early stage of positive
Figure 4. Acute recirculation of B6 CD4+ cells through B6.PL → F1 chimeras causes partial depletion of cells responsive to host-type F1 and 5R (host I-E only) spleen cells in vitro. Doses of 8 × 10^7 normal B6 CD4+ cells were transferred intravenously to normal B6.PL mice (O), normal F1 mice (.), or B6.PL → F1 BMC (■) exposed to 900 rad 4 h before (Fig. 2); the donor cells were recovered from TDL at 20–40 h post-injection. Various doses of these cells were used as responder cells for MLR using F1, 5R, and bm12 irradiated spleen cells as stimulators (■) (Materials and Methods); MLR were harvested on day 4 or 5. The results of three different experiments are shown (A and B, C and D, E and F). In the third experiment (F-H), the mice were pretreated with IFN-γ before T-cell injection. Each point represents the mean of triplicate cultures. It can be seen that filtration of B6 CD4+ cells through normal F1 hosts reduced the MLR to F1 and 5R by close to 100% but had little effect on the response to third-party bm12. In contrast to early lymph collections (Fig. 4), it can be seen that the anti-F1 MLR mediated by CD4+ cells collected from the chimeras at 48–62 h post-injection (■) was no lower than the response by CD4+ cells filtered through control B6.PL mice (O); note that, for the cells passed through the chimeras, the higher response to F1 (■) in the second experiment (C) was associated with an equivalent increase in the response to bm12 (■). Since activated (3G11- ) cells were rare in the lymph at this time (3G11- cells appeared in TDL of the chimeras only after 70 h, see above), it was important to study the host reactivity of cells recovered at later stages. Testing the MLR of cells collected from the chimeras at >60 h was difficult because of low yields of cells. Instead, the following approach was used. Groups of chimeras and control normal F1 mice were exposed to sublethal irradiation (500 rad), injected with a large dose of B6 CD4+ cells, and then left for 16 d before thoracic duct cannulation. As shown in Fig. 6, purified donor B6 CD4+ cells recovered from TDL of the chimeras showed clear evidence of specific priming. MLR to third-party (bm12) stimulators were unchanged (relative to normal B6 CD4+ responders), but responses to F1 and 5R were markedly elevated and easily detectable with a low dose of 5 × 10^4 responder cells (G, H, and I); with higher doses of responders, anti-F1 MLR were prominent as early as day 2 of culture (D and E). The selective priming to F1 selection.
Table 5. MLR by B6 CD4+ Cells Filtered through B6 → F1 BMC and Recovered during Early Stage of Positive Selection

| Filtration hosts (900 rad) for B6 CD4+ cells* | No. of responders for MLR (10^5) | B6 | (B6 x CBA)F1 | bm12 |
|---------------------------------------------|---------------------------------|----|-------------|------|
| B6.PL                                       | 1                              | 0.7| 8.1         | 9.0  |
|                                             | 2                              | 2.2| 27.4        | 23.2 |
| B6.PL → F1 BMC                              | 1                              | 0.8| 7.0         | 6.4  |
|                                             | 2                              | 2.7| 31.0        | 23.8 |
| (B6 x CBA)F1                                 | 1                              | 1.0| 12.1        | 33.9 |
|                                             | 2                              | 2.4| 11.6        | 52.4 |

* Cells recovered from lymph at 48-62 h post-injection.

and 5R stimulators was not associated with a significant alteration in $V_\beta$ ratios ($F$). Passing B6 CD4+ cells through the control normal F1 mice gave somewhat different results. These cells gave primed responses to F1 and 5R stimulators, but this was associated with reduced responses to bm12 (relative to control B6 CD4+ responders) (Fig. 6, A and B, and data not shown). Significantly, priming in normal F1 mice led to a marked alteration in $V_\beta$ ratios: proportions of $V_\beta 11^+$ and $V_\beta 5^+$ cells were considerably reduced whereas $V_\beta 8^+$ cells were elevated (C), i.e., the converse of the ratios observed during early positive selection (see Fig. 2).

Discussion

In previous studies, we reported that the environment of parent → F1 BM chimeras prepared with supralethal irradiation (1,300 rad) was strongly tolerogenic for newly formed T cells (7). The donor-derived CD4+ cells generated in the host thymus gave only weak MLR to normal host-type APC (spleen) in vitro and showed 50-70% depletion of host-I-E-reactive $V_\beta 11^+$ T cells. Since spleen and thymocyte suspensions from the chimeras appeared to be completely depleted of host-type APC, it was argued that tolerance to host la antigens was induced intrathymically, presumably by thymic epithelium. The possibility that tolerance was induced extrathymically, however, could not be excluded. As shown here, host la expression in the spleen of long-term chimeras was not seen in sites occupied by BM-derived APC but was clearly demonstrable in germinal centers, probably on follicular dendritic cells (which are reported to be non-BM derived [16]); in IFN-γ-treated chimeras, host la expression was also prominent on vascular endothelium. Based on the findings of others (see Introduction), one might expect such la expression on “nonprofessional APC” to be highly tolerogenic, especially for newly formed T cells.

The thymus grafting experiments reported here failed to verify this prediction. Thus, when strain a stem cells differentiated in strain a TG placed in long-term Tx.Ir.BMR (a × b)F1 mice, the a-derived CD4+ cells gave high MLR to normal host-type (a × b)F1 spleen cells in vitro and showed no depletion of host I-E-reactive $V_\beta 11^+$ cells; this contrasted with the strong tolerance seen when the chimeras received (a × b)F1 rather than a TG, i.e., a situation where host la was encountered on thymic epithelium. These results provide an alternative explanation for the TG experiments of Bradley et al. (22). In contrast to the present study, Bradley et al. (22) observed strong host tolerance when Tx.Ir.BMR (a × b)F1 mice were given parent a stem cells and a parent a TG. The authors concluded from this finding that T cells are susceptible to prethymic tolerance. Since the TG were applied before irradiation, however, we suggest that the TG were rapidly permeated with host BM-derived cells and that these cells induced intrathymic tolerance of newly formed T cells. We avoided this problem by leaving the Tx.Ir.BMR (a × b)F1 mice for a prolonged period, i.e., 6 mo, before applying the TG. Grafting after a shorter interval, i.e., 5 wk, led to partial tolerance.

A trivial explanation for the lack of tolerance seen in our long-term TG mice is that host la expression in these mice was simply too low to be relevant. Alternatively, the CD4+ cells in the TG mice might have been selectively tolerized to unique self-peptide/la complexes expressed only on NBMD cells (23). These possibilities were assessed by injecting long-term parent → F1 chimeras with normal parental-strain CD4+ cells. The expectation here was that, if host la expression in the chimeras was insignificant or tissue-specific, the donor CD4+ cells would maintain their normal pattern of blood-to-lymph recirculation and show unimpaired reactivity to the host la antigens expressed on normal F1 spleen cells in vitro.
In practice, recognition of host Ia antigens in the chimeras appeared to be considerable. Thus, when the donor CD4+ cells were recovered from TDL of the chimeras 1–2 d post-transfer and tested in vitro, the MLR to normal host-type spleen cells in vitro was specifically reduced by ~50%. A priori, one might expect this selective trapping of host-reactive CD4+ cells by antigen expressed on NBMD cells to be followed by tolerance induction. This was not found. Instead, the host-reactive CD4+ cells reappeared in the lymph at day 3 post-transfer. Interestingly, the lymph-borne cells collected at this time (50–60 h) showed no obvious sign of activation. This was in sharp contrast to CD4+ cells transferred to normal F1 hosts; here, the lymph at day 3 contained large numbers of blast cells that responded to host-type APC in vitro with accelerated kinetics. The implication, therefore, is that Ia expression in the chimeras was not overtly immunogenic but simply slowed the rate at which the Ia-reactive cells moved through the lymphoid tissues: after a transient period of trapping in the spleen and other organs, most of the host-reactive cells re-entered the circulation still in a resting state. This explanation is in line with the report of Ford et al. (24) that T cell trapping in vivo does not necessarily result in cell activation. In parent → normal F1 combinations, these workers found that up to 50% of the (selectively) trapped cells failed to enter cell cycle. The subsequent fate of these cells was not studied.

Host Ia expression in the chimeras was clearly immunogenic for a small proportion of the injected CD4+ cells. Activated (3G11+CD4+) cells were rare in the chimeras before day 3 post-transfer but were readily detectable at later stages and accounted for 40–60% of the lymph-borne cells by day 4–5 (compared with <20% for syngeneic transfers). Progressive expansion of these cells presumably accounted for the striking degree of priming observed when CD4+ cells were left in the chimeras for a 2-wk period. At this stage, the lymph-borne CD4+ cells from the chimeras gave very powerful and specific responses to host Ia antigens in vitro. Indeed, the level of priming in the chimeras was as high or higher than when CD4+ cells were transferred to normal F1 hosts.

Although the kinetics and duration of CD4+ cell priming in the chimeras vs. normal F1 hosts has yet to be examined in detail, it is notable that Vg ratios in the two groups of primed cells were quite different. With the I-E<sup>-</sup> → I-E<sup>+</sup> combinations used, transfer of CD4+ cells to normal F1 hosts induced strong negative selection (trapping) and positive selection (blast cell production) of host-I-E-reactive Vg11<sup>+</sup> and Vg5<sup>+</sup> cells but no obvious selection of Vg8<sup>+</sup> cells (which are not I-E reactive). Paradoxically, despite the marked enrichment for Vg11<sup>+</sup> and Vg5<sup>+</sup> cells in the blast population generated at day 3 post-transfer, the primed cells collected at 2 wk post-transfer showed a specific reduction in

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Figure 6. B6 CD4+ cells transferred to B6.PL → F1 chimeras for 2 wk exhibit marked sensitization to the host antigens. Groups of B6.PL → F1 chimeras and normal F1 mice were exposed to light irradiation (500 rad) and injected with a dose of 8 × 10⁷ B6 CD4+ cells. The two groups of mice were set up separately and were thus tested at different times. At day 16 post-transfer, the mice were cannulated to obtain TDL, and the lymph-borne cells (pooled from two to three mice/group) were used as responder cells for MLR as in Figs. 4 and 5, and also tested for Vβ expression; >90% of the lymph-borne cells were donor-derived CD4+ cells. The two groups of mice were exposed to light irradiation (500 rad) and injected with a dose of 8 × 10⁷ B6 CD4+ cells. The two groups of mice were set up separately and were thus tested at different times. At day 16 post-transfer, the mice were cannulated to obtain TDL, and the lymph-borne cells (pooled from two to three mice/group) were used as responder cells for MLR as in Figs. 4 and 5, and also tested for Vβ expression; >90% of the lymph-borne cells were donor-derived CD4+ cells.

(A and B) Early (day 2) MLR by normal B6 CD4+ cells (○) vs. B6 CD4+ cells passed through the control normal F1 hosts (□); (C) Vβ expression on the passaged cells (hatched) vs. control normal B6 CD4+ cells (open). The lymph-borne cells gave elevated responses to F1 (relative to normal B6 CD4+ cells) and reduced responses to bm12; Vs11+ and Vg5+ cells were reduced whereas Vg8+ cells were elevated. Panels (D–F) Day 2 MLR and Vβ expression for normal B6 CD4+ cells (○, open bars) vs. B6 CD4+ cells passed through the chimeras (□, closed bars). Relative to the control responder cells, the response to F1 was markedly elevated whereas the response to bm12 was near normal; Vβ ratios remained unchanged. (G–I) A time response for the MLR by cells passed through the chimeras, using 3 × 10⁴ responder cells and F1, 5R, and bm12. With this small (limiting) dose of responder cells, the responses to F1 and 5R were far higher than with the control responders. These responses reached a peak on day 3–4 (F1) or day 4–5 (5R), whereas the response to bm12 peaked on day 5.
V\textsubscript{p}11\textsuperscript{+} and V\textsubscript{p}5\textsuperscript{+} cells and a reciprocal increase in V\textsubscript{p}8\textsuperscript{+} cells. CD4\textsuperscript{+} cell transfer to the chimeras, by contrast, resulted in only minimal changes in the frequency of V\textsubscript{p}11\textsuperscript{+} and V\textsubscript{p}5\textsuperscript{+} cells (even in IFN-\gamma-treated hosts), despite the fact that the CD4\textsuperscript{+} cells recovered at 2 wk post-transfer showed priming to host I-E (5R) antigens. Although a full interpretation of these findings will need further experimentation, two points can be made. First, the data indicate that, in addition to causing substantial trapping, Ia expression in the chimeras was clearly immunogenic for a proportion of normal parental strain CD4\textsuperscript{+} cells. Second, the data on V\textsubscript{p} ratios imply that priming in the chimeras vs. normal F\textsubscript{1} hosts was qualitatively different, which argues against the possibility that the chimeras contained residual BM-derived host APC.

At face value, the strong T cell priming seen in the chimeras at 2 wk post-transfer would seem to challenge the view that T cell priming is under the control of specialized BM-derived APC. The point to stress here is that, in terms of total blast cell production, proliferation of the donor CD4\textsuperscript{+} cells in the chimeras was far less prominent than in normal F\textsubscript{1} hosts (where proliferation was presumably driven by radioreistant host APC). As suggested above, T cell contact with follicular dendritic cells (or other NBMD cells) in the chimeras was presumably overtly immunogenic for only a small proportion of the donor CD4\textsuperscript{+} cells, perhaps a subset of very high affinity cells. These cells eventually underwent substantial expansion, but only after a prolonged (2 wk) period and under conditions where competition with T cells responding to antigen on typical APC was avoided. These "optimal" conditions in vivo might be difficult to reproduce in vitro. The precise specificity of the CD4\textsuperscript{+} cells primed in the chimeras is still unclear. The possibility that these cells were selectively primed to tissue-specific Ia antigens seems unlikely since normal spleen cells were used to detect priming.

Since the environment of the chimeras proved to be immunogenic for normal CD4\textsuperscript{+} cells (albeit a small proportion of these cells), one might expect to see comparable evidence for immunogenicity in the TG mice. Two points should be made here. First, it may be noted that for several of the TG mice tested the LN CD4\textsuperscript{+} cells tended to give slightly above normal MLR to host-type APC for early (day 3) responses (Table 3, see ratios on far right). Second, it is interesting that the TG mice eventually became ill and showed lymphocytic infiltration in the liver suggestive of graft-vs.-host disease (our unpublished data). Alternatively, the pathology seen in the TG mice might have been mediated by host class I-reactive cells; the issue of tolerance/immunity at the level of CD8\textsuperscript{+} cells is still under study.

The data in this paper are difficult to reconcile with the popular view that antigen presented on cells other than typical BM-derived APC is tolerogenic (25). Although this notion is well supported by in vitro experiments, evidence that antigen on "nonprofessional APC" induces tolerance in vivo is quite sparse. Indeed, given the evidence that in vitro tolerance induced by chemically fixed APC can be blocked by bystander APC (25), it is difficult to envisage how this mechanism of tolerance could operate in vivo (where bystander APC are presumably numerous). In the case of solid tissue allografts, it is well accepted that depleting grafts of "passenger leukocytes" impairs rejection (26). Such grafts rarely induce true tolerance, however, and the lack of rejection can be attributed to sequestration of the graft from the host's immune system. Perhaps the best evidence that antigen on nonprofessional APC can be tolerogenic in vivo has come from the finding that some (3, 27), though not all (28-30), transgenic lines expressing foreign Ia antigens selectively in the pancreas show tolerance at the level of CD4\textsuperscript{+} cells. A puzzling feature of this model is that tolerance is evident in the thymus as well as in the periphery, a clear contrast to the TG mice reported here where neither thymus nor LN showed evidence of tolerance induction. The site and mechanism of tolerance induction in the transgenic mice is still unclear. With regard to the present data, it is of particular interest that immunity rather than tolerance occurred when the above transgenic lines were injected with normal wild-type T cells (although only when the host was irradiated or T depleted) (27). This finding does not fit easily with the notion that tolerance in the transgenic lines is simply a reflection of defective antigen presentation. However, these data from transgenic mice do agree closely with the current observation that transfer of CD4\textsuperscript{+} cells to long-term chimeras led to immunity rather than tolerance.

The factors controlling tolerance induction of mature T cells are clearly still poorly understood. Recent work has suggested that the distinction between tolerance and immunity can be quite subtle. Thus, under certain conditions, T cell tolerance can be the end result of a powerful immune response (31). In light of this and the above findings, the notion that immunity and tolerance simply reflect different modes of antigen presentation will need to be re-evaluated.

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