

EVIDENCE FOR MOUSE Th1- AND Th2-LIKE HELPER T CELLS IN VIVO

Selective Reduction of Th1-like Cells after Total Lymphoid Irradiation

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Mouse Th cells are characterized by a distinct pattern of surface markers, including Thy-1, Lyt-1 (CD5), and L3T4 (CD4), but not Lyt-2 (CD8), antigens (1). These cells recognize class II MHC alloantigens and heterologous protein antigens in association with autologous class II MHC molecules on APC (2, 3). Although Th cells cannot be further subdivided phenotypically in the mouse using currently available subset-specific antibodies, they have shown a clear functional heterogeneity (4-11). Based on the analysis of a large group of mouse Th cell clones, at least two types of mouse Th clones have been identified that differ in function, and in their patterns of cytokine secretion (12, 13). Th1 clones synthesize IL-2, IFN- γ , and lymphotoxin, and mediate delayed-type hypersensitivity (DTH)¹ reactions in adoptive hosts (14, 15). Th1 clones can be cytotoxic for class II MHC-bearing target cells pulsed with specific antigens (16), and can suppress an in vitro IgE response, due to secretion of IFN- γ (17-19). Th2 clones secrete IL-4, IL-5, and IL-6, and augment B cell proliferation and antibody production (20-26). They are effective in T cell-dependent humoral immune responses, especially for helping IgE production, which is mediated by IL-4. Both types of Th clones synthesize GM-CSF, TNF, IL-3, and preproenkephalin (27). They also exhibit similar growth properties in vitro (27).

The goal of the present study was to compare the cell-mediated and humoral immune responses of CD4⁺ spleen cells of total lymphoid irradiation (TLI)-treated and normal BALB/c mice. TLI consists of fractionated irradiation delivered selectively to the major lymphoid tissues, including the spleen, thymus, and lymph nodes (28). Changes in the function of purified peripheral blood CD4⁺ (Leu-3⁺) lymphocytes in humans given TLI have been reported previously (29-31). In particular, these cells show a reduction in the proliferative responses to mitogens, alloantigens, and heterologous proteins in vitro as compared with CD4⁺ cells from normal individuals. Similarly, CD4⁺ spleen cells from TLI-treated mice showed a marked

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¹ *Abbreviations used in this paper:* BM, bone marrow; DTH, delayed-type hypersensitivity; GVHD, graft vs. host disease; KLH, keyhole limpet hemocyanin; TLI, total lymphoid irradiation; WBI, whole body irradiation.

deficit in the MLR that was associated with reduced IL-2 secretion and normal IL-2-R expression (Bass, H., and S. Strober, manuscript submitted for publication).

The results of the present study using TLI-treated BALB/c mice indicate that there are two or more functionally distinct mouse CD4 subsets *in vivo*. One subset of cells (Th1-like) secretes IL-2 and/or IFN- γ and contributes to cell-mediated immunity. The function of these cells recovers slowly after TLI (at \sim 3 mo) to the pretreatment levels. Another subset of cells (Th2-like) secretes IL-4 and/or IL-5 and plays a critical role in humoral immunity. The latter cells return more rapidly (at \sim 4–6 wk) after the irradiation. This results in a transient change in the proportion of these two subsets in the spleens of TLI-treated mice, such that Th1-like cells are selectively reduced after irradiation.

Materials and Methods

Animals. BALB/c (H-2^d), C57BL/Ka (H-2^b), and C3H/Km (H-2^k) mice were obtained from the specific pathogen-free colony in the Department of Radiology, Stanford University Medical School. During administration of irradiation, BALB/c mice were housed in conventional animal rooms. Tetracycline (0.5 μ g/ml) was added to the drinking water during the 3 wk of TLI treatment. CBA/J (H-2I^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in the Stanford Medical Center animal facility.

TLI. 4–6-mo-old male BALB/c mice were placed in an apparatus designed to irradiate the major lymphoid organs, including all major lymph nodes, spleen, and thymus, as previously described (18). The mice were given 250 rad/d, five times per week, for a total dose of 4,250 rad in 17 treatments. TLI was delivered from a single 250-Kv (15 A) source (Phillips Medical System Inc., Shelton, CT).

Whole Body Irradiation (WBI). The mice were placed in lucite containers and given a sublethal dose of 550 rad, or a lethal dose of 850-rad irradiation. WBI was delivered from a single 250-Kv (15 A) source (Phillips Medical System Inc.). The dose rate was 93 rad/min, using a 0.35-mm Cu filter at a 52-cm source-axis distance.

Preparation of Cells. Spleens were removed aseptically and a single cell suspension was prepared by gently pressing the spleen through a nylon fiber mesh (Tetko Inc., Elmsford, NY).

Immunofluorescent Staining and Analysis Using the FACS. The rat anti-mouse mAbs used for immunofluorescent staining included anti-Thy-1.2 (clone 30-H12, IgG2a; American Type Culture Collection [ATCC], Rockville, MD), anti-IgM (11B5, IgG1; gift of Dr. I. Weissman, Stanford University), anti-CD8 (Lyt-2 in mouse, clone 53-6.76, IgG2a; ATCC), anti-CD4 (L3T4 in mouse, GK 1.5, IgG2b; Becton Dickinson & Co., Mountain View, CA). Hamster anti-mouse IL-2-R mAb (M7/20) (IgM), was obtained from Dr. V. Kelley, Harvard University, Cambridge, MA. Fluoresceinated goat anti-rat Ig used as the second-stage antibody was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Phycoerythrin-conjugated anti-CD8 and CD4 antibodies were also obtained from Becton Dickinson & Co. A rat anti-mouse idiotype mAb was used as an irrelevant control for staining with rat-derived antibodies (Dr. R. Levy, Stanford University). The concentration of the staining reagents corresponded to their saturation points on the standard dilution curves.

Immunofluorescent staining procedures were as described previously (29). The stained cells were analyzed on a FACS II (Becton Dickinson & Co.). The negative controls contained cells stained with an irrelevant first-stage antibody. The percentage of positive cells was determined by thresholds described previously (29). Approximately 2–3% of the cells stained with irrelevant antibody were positive at this threshold point.

Purification of Ig⁺ Cells. A technique similar to that of Wysocki and Sato (32) was used. Petri dishes were coated with affinity-purified goat anti-rat IgG (Boehringer Mannheim Biochemicals) at 4°C for 10–12 h. Ammonium chloride potassium-treated spleen cells were first incubated on ice with rat anti-mouse Thy-1.2 mAb (clone 30-H12; Becton Dickinson & Co.) for 30 min. The cells were then washed and added to the coated petri dishes. 1 h later, the nonadherent cells were collected, washed, and resuspended in PBS.

After each panning procedure, the negatively selected cells were stained with rat anti-Thy-1.2 mAb (see above) or monoclonal rat anti-IgM (11B5), and analyzed on a FACS in order to check the purity of the cells. The Ig⁺ cells were enriched from a mean of 45.2% of the total spleen cells to a mean of 81.8% of the transferred "panned" cells. The mean contamination of the Thy-1.2⁺ cells after panning for B cells was 2.2%.

Purification of CD4⁺ Cells. To purify CD4⁺ cells, petri dishes were coated with affinity-purified goat anti-mouse IgG⁺ IgM (Tago Inc., Burlingame, CA) at 4°C for 10–12 h. After incubation at room temperature for 1 h, the nonadherent cells were collected, washed, and incubated on ice with rat anti-mouse CD8 mAb (clone 53-6.7; ATCC), for 30 min. The cells were then washed and added to affinity-purified goat anti-rat IgG (Boehringer Mannheim Biochemicals)-coated petri dishes. 1 h later, the nonadherent cells were collected, washed, and resuspended in PBS.

After each panning procedure, the purified CD4⁺ cells were stained with mAbs and analyzed using the FACS II in order to check the purity of the cells. After the panning procedure, the CD4⁺ cells were enriched from a mean of 24.3% of the unfractionated spleen cells to a mean of 80.4% of the purified (panned) cells. The mean contamination of Ig⁺ and CD8⁺ cells was 5.3% and 3.2%, respectively.

DTH. 24 h after sublethal whole body irradiation (550 rad), BALB/c mice were injected intravenously with syngeneic spleen cells and intraperitoneally with TNP keyhole limpet hemocyanin (TNP-KLH; 100 µg/mouse). KLH (purchased from Calbiochem-Behring Corp., La Jolla, CA) was conjugated with TNP according to the method of Little and Eisen (33). 3 wk later, the immunized mice were injected subcutaneously in the left footpad with 30 µl of TNP-KLH (15 µg/mouse) in PBS and in the right footpad with the same volume of PBS. Footpad thickness was measured both before and at different time points after the injection by using a dial-gauge caliper (7312; Mitutoyo). Footpad swelling is signified in the graph as the difference between the thickness of the experimental (TNP-KLH injected) and control (PBS injected) foot of each mouse. Data are presented in 10⁻³ cm.

Adoptive Cell Transfer Procedure. Adoptive hosts were given a single dose of whole body sublethal irradiation (550 rad). 24 later, graded numbers of nonfractionated syngeneic spleen cells or CD4⁺ cells plus IgG⁺ cells from normal or TLI-treated mice were injected into the hosts' tail vein. At the same time, the mice were immunized intraperitoneally with TNP-KLH. Each injection contained 100 µg TNP-KLH diluted with PBS in a total volume of 250 µl. The mice were bled from the retro-orbital veins both before immunization and at weekly intervals after immunization. The blood was allowed to clot at room temperature. Serum was separated by centrifugation and stored at -20°C for further testing.

Antibody Determination. Serum antibody to TNP was measured by the indirect ELISA as described previously (34). Flat-bottomed microtiter plates (96-well; Dynatech Laboratories, Inc., Alexandria, VA) were coated with TNP-BSA. Serial two-fold dilutions of the serum samples were added for 1 h at room temperature. The plates were washed and incubated at room temperature with 100 µl of a 1:3,000 dilution of peroxidase-conjugated goat anti-mouse IgG (γ and L chain specific, Tago Inc.). 1 h later, the plates were washed and 100 µl/well of substrate solution containing 15 µg/ml of 2,2-azino-Di-(3-ethylbenzthiazoline) sulfonic acid (Sigma Chemical Co., St. Louis, MO) and 0.3% H₂O₂ in citrate buffer (pH 4) were added. Antibody responses are represented in units, whereby, the number of units = (1/dilution of experimental serum [at a given OD])/(1/dilution of standard serum).

IgE-specific ELISA. The same reagents and techniques described by Coffman and Carty (18) were used. Briefly, polyvinyl chloride 96-well plates (Dynatech Laboratories, Inc.) were coated for 2 h with an anti-IgE mAb, EM 95, at a concentration of 4.0 µg/ml. The nonspecific binding sites were blocked with PBS plus 20% FCS. The plates were washed with PBS plus 0.04% Tween 20. The standard sample and experimental serum were added to wells at graded concentrations in 100 µl. Then the plates were incubated overnight at 4°C. The plates were then washed again with PBS plus 0.04% Tween 20. The second-step antibody, nitroiodophenyl acetic acid-conjugated purified anti-TNP-KLH, was added and incubated at room temperature for 4 h. An optimum concentration (1:3,000) of horseradish peroxidase-conjugated anti-NIP mAb was added after the plates were washed yet again. 1 h later, the plates were washed

and 100 μ l/well substrate solution was added. The plates were read on an ELISA machine (Dynatech Laboratories, Inc.).

Graft vs. Host Disease (GVHD) Assay. 24 h after C57BL/Ka (H-2^b) mice were lethally irradiated with a single dose of 850 rad, the recipients were injected intravenously with 2.5×10^6 allogeneic bone marrow (BM) cells obtained from the femurs and tibias of BALB/c (H-2^d) mice. Graded numbers of purified spleen CD4⁺ cells from either normal or TLI-treated BALB/c mice were injected intravenously together with BM cells. The control groups included lethally irradiated mice given no cells and mice injected with allogeneic BM cells alone. The mortality rate in all groups was recorded up to 100 d after cell transfer.

GVHD Suppression Assay. The experimental conditions were the same as those described above for the GVHD assay except that 2.5×10^6 putative suppressor cells (TLI CD4⁺ cells) were coinjected into one group of the recipients with an equal number of normal CD4⁺ cells and allogeneic BM cells. Mice that received CD4⁺ and BM cells from either normal or TLI donors were part of the control groups. The mortality rate was recorded up to 100 d after cell transfer.

Establishment of Alloreactive Clones. Cells specific for alloantigens were cloned directly from the spleens of normal or TLI-treated BALB/c mice by limiting dilution with irradiated CBA/J stimulator cells (2×10^5 /well) (U-bottomed 96-well plate; Costar, Cambridge, MA) using the technique described previously by Mosmann et al. (12). The clones were grown on alternate cycles of alloantigen stimulation once every 2 wk, alternating with growth in the medium alone, which includes RPMI 1640, 10% FCS, 0.05 mM 2-ME, 50 μ g/ml gentamicin, and mouse rIL-2 (400–500 U/ml). Depending upon the density of cell growth in the wells, the cultured cells were expanded to another 96-well plate using the same medium.

Stimulation of CD4⁺ Clones and Spleen Cells for Cytokine Secretion. Alloreactive clones were washed twice and resuspended in IL-2-free medium containing 5 μ g/ml Con A, (Calbiochem-Behring Corp.). The supernatants were harvested 24 h later and analyzed using specific bioassays for the presence of IL-2 and IL-4 and in two-site sandwich ELISAs for the presence of IFN- γ and IL-5. Spleen cells from normal or TLI-treated BALB/c mice (10^7 /ml) were washed and resuspended in RPMI 1640 containing 10% FCS. These cells were stimulated with hamster anti-mouse T3 antibody (gift of Dr. J. Bluestone, University of Chicago) diluted to 1:200, or Con A (5 μ g/ml) plus PMA (10 ng/ml). The stimulation supernatants were harvested 8 and 24 h later and assayed for cytokine secretion.

Cytokine Determinations of IL-2 and IL-4. Samples were assayed for IL-2 and IL-4 using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (M2128; Sigma Chemical Co.) proliferation assays as described previously (35). Briefly, IL-2 was assayed on HT-2 cells in the presence of 11b11 anti-IL-4 mAb, and IL-4 was assayed on HT-2 cells in the presence of S4B6 anti-IL-2 mAb. Additional controls included testing samples on HT-2 cells alone or in the presence of both anti-IL-2 and anti-IL-4 antibodies. 1 U is defined as the amount of factor in 100 μ l that resulted in a signal in the MTT assay equal to 50% of maximum signal.

Cytokine Determinations of IFN- γ and IL-5. Samples were assayed for IFN- γ and IL-5 using two-site sandwich ELISAs (13, 36). Briefly, microtiter plates were coated with either mAb XMG 1.2 in PBS for IFN- γ or mAb TRFK5 in PBS for IL-5. After incubation with test samples and murine rIFN- γ and IL-5 standards, the plates were washed and incubated with affinity-purified rabbit anti-mouse IFN- γ for assaying IFN- γ or biotin-conjugated mAb TRFK2 for assaying IL-5. The plates were then washed and incubated with goat anti-rabbit/horseradish peroxidase conjugate (The Jackson Laboratory) for testing IFN- γ , and strept-avidin-conjugated horseradish peroxidase for testing IL-5. Cleavage of the substrate 2,2'-azino-Di (3-ethylbenzthiazoline sulfonic acid) (ABTS) was determined on a VMAX microplate reader (Molecular Services, Menlo Park, CA).

Phenotyping of Alloreactive Clones. The surface antigen phenotype of the clones was determined by a cytotoxicity assay using anti-CD8 or anti-CD4 mAbs (Cedarlane Laboratories, Hornby, Ontario, Canada) and complement (C'). Cytotoxicity was evaluated using the colorimetric MTT assay (35). The percentage of killing was calculated as: $100 \times [(\text{control wells (C' + medium)} - \text{assay wells (C' + antibodies)}) / \text{control wells}]$.

Statistical Analysis. Statistical analyses were performed by using student's *t* test (two tailed).

Results

Comparison of Adoptive Transfer of Spleen Cells from Normal and TLI-treated Mice: Antibody Production and DTH Response in the Same Recipients. 24 h after being given a single dose of whole body irradiation (550 rad), BALB/c mice were given TNP-KLH intraperitoneally and graded doses of syngeneic unfractionated spleen cells intravenously from normal or TLI-treated mice. In all instances, spleen cells were obtained from TLI-treated mice 4–6 wk after completion of irradiation. Previous experiments have shown that the percentage of CD4⁺ cells in the spleen was approximately normal at this time point after TLI (Bass, H., and S. Strober, manuscript submitted for publication). Serum was obtained before and at weekly intervals after cell transfer and immunization. 21 d after the primary immunization, host footpads were injected subcutaneously with TNP-KLH. Footpad swelling was measured before and at different time points after the local immunization. This experimental system assayed both the DTH reaction and antibody production in the same mouse.

Fig. 1 shows the results of DTH footpad swelling. As expected, in mice receiving normal spleen cells, the swelling peaked at 24 h after local immunization and started to decrease after 30 h. The control mice that received no cells but had antigen injected into their footpads showed no specific swelling. The footpad swelling increased with increasing numbers of cells transferred (6.25×10^6 to 10^8 cells) from normal mice. When 5×10^7 cells were transferred from TLI-treated mice, the response was less than that with 6.25×10^6 normal cells. Thus, the TLI cells were at least eight times less efficient than normal cells in causing footpad swelling in a DTH reaction. This pattern was observed in two similar experiments.

Fig. 2 shows the serum anti-TNP antibody response in the same groups of mice. The response peaked at days 14–21 after immunization. Background levels of antibody were seen in the control mice that did not receive cells. Again, the production of anti-TNP antibody increased substantially in the groups of mice receiving between 6.25×10^6 and 10^8 normal spleen cells. However, it was found that the anti-TNP antibody response transferred by 5×10^7 TLI spleen cells was similar to that with the same dose of normal spleen cells. The difference between these two groups was not statistically significant ($p \geq 0.05$). This pattern was observed in three other similar experiments.

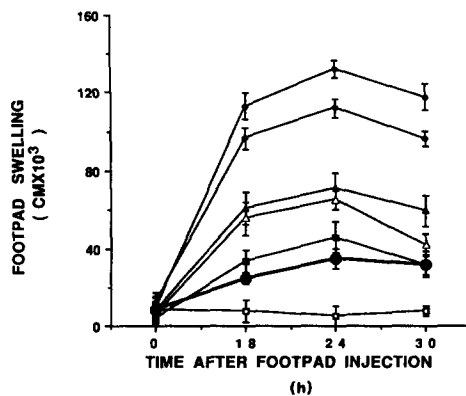


FIGURE 1. Comparison of DTH after adoptive transfer of normal and TLI spleen cells. 24 h after whole body sublethal irradiation (550 rad), BALB/c mice were injected intravenously with graded numbers of syngeneic spleen cells and intraperitoneally with TNP-KLH (100 μ g/mouse). 3 wk later, the immunized mice were injected subcutaneously in the footpad with TNP-KLH (15 μ g/mouse). The symbols in the graph represent no cells (□); 6.25×10^6 normal spleen cells (■); 1.25×10^7 normal spleen cells (△); 2.5×10^7 normal spleen cells (▲); 5×10^7 normal spleen cells (◇); 10^8 normal spleen cells (◆); and 5×10^7 TLI spleen cells (●). There were six mice in each group.

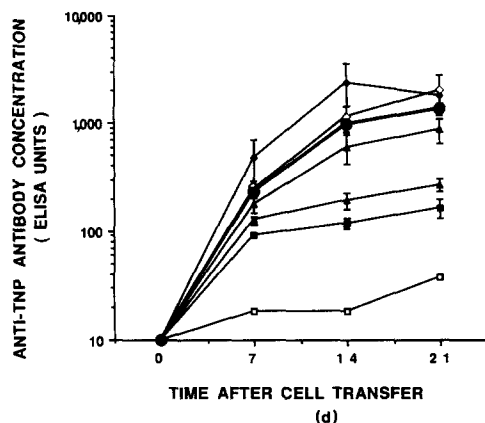


FIGURE 2. Comparison of antibody production after adoptive transfer of normal and TLI spleen cells. 24 h after sublethal irradiation (550 rad), BALB/c mice were immunized intraperitoneally with TNP-KLH (100 μ g/ml). Simultaneously, nonfractionated syngeneic spleen cells from normal or TLI mice were injected into the host tail veins. The mice were bled from retro-orbital veins before and at weekly intervals after immunization. The serum anti-TNP antibody concentration was measured by indirect ELISA. The symbols in the graph represents no cells (\square); 6.25×10^6 normal spleen cells (\blacksquare); 1.25×10^7 normal spleen cells (\triangle); 2.5×10^7 normal spleen cells (\blacktriangle); 5×10^7 normal spleen cells (\diamond); 10^8 normal spleen cells (\blacklozenge); and 5×10^7 TLI spleen cells (\bullet). There were six mice in each group.

Augmentation of Antibody Production by Normal Ig^+ Cells Using Normal and TLI Splenic $CD4^+$ Cells. To study the ability of TLI $CD4^+$ cells to assist normal Ig^+ cells in secreting antibodies, Ig^+ cells (5×10^6) and graded numbers of $CD4^+$ cells were injected together into sublethally irradiated syngeneic BALB/c mice. Each mouse also received TNP-KLH antigen intraperitoneally. The serum anti-TNP antibody concentration in ELISA units is shown in Fig. 3. The peak of the antibody response was on days 14–21. The negative control group, irradiated mice without transferred cells, showed background levels of specific antibody. The mice that received either only Ig^+ cells or $CD4^+$ cells showed low background antibody responses. Increasing doses of TLI or normal $CD4^+$ cells (5×10^5 to 5×10^6) with a constant number of Ig^+ cells produced increasing antibody responses. Although the TLI $CD4^+$ cells at day 7 produced lower levels of antibody than the normal control $CD4^+$ cells, the two groups showed no significant differences 14 and 21 d after cell transfer and immunization ($p \geq 0.05$). A similar pattern was observed in two other experiments.

Augmentation of Antigen-specific IgE Production by Normal Ig^+ Cells Using Normal and TLI Splenic $CD4^+$ Cells. It has been shown in other studies that IFN- γ and IL-4 regulate IgE antibody synthesis reciprocally; while IL-4 enhances IgE production >100 times in LPS-stimulated B cell cultures, a small amount of IFN- γ can completely inhibit such a response (18, 19). The difference in IgE production is one of the most important functional differences between Th1 and Th2 clones. Therefore, it was essential to determine the antigen-specific IgE levels in the serum samples collected from the mice receiving adoptive cell transfer as shown above. As shown in Table I, the pre-bleed group and the mice receiving only antigen, Ig^+ cells, or $CD4^+$ cells alone had minimal amounts of IgE antibody. The results also showed a clear dose-response curve when graded numbers of $CD4^+$ cells were added with a constant number of Ig^+ cells. The IgE antibody level in mice given $CD4^+$ and Ig^+ cells was higher on day 7 than day 14 or 21. $CD4^+$ cells from TLI mice were capable of helping normal Ig^+ cells to produce higher levels of specific IgE antibodies than the same number of $CD4^+$ cells from BALB/c control mice. The average percentage increase on day 7 in the IgE level using TLI vs. normal $CD4^+$ cells was 98% in the group given 5×10^5 , 101% in the group given 2×10^6 , and 62% in the group given 5×10^6 $CD4^+$ cells. These increases were significantly different for all groups ($p \leq 0.01$).

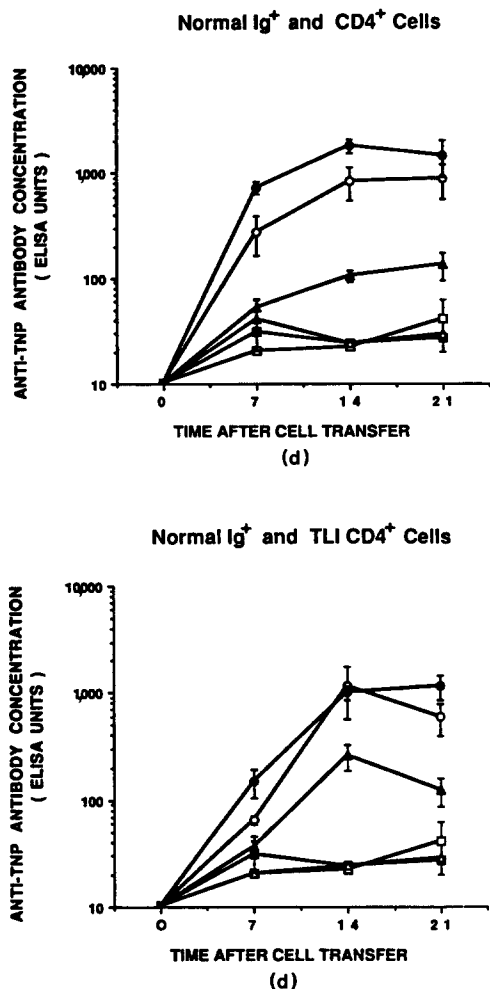


FIGURE 3. Comparison of antibody response transferred by normal Ig^+ cells and $CD4^+$ cells from either normal or TLI-treated mice. 24 h after sublethal irradiation, BALB/c mice were immunized intraperitoneally with $100 \mu g$ TNP-KLH (\blacksquare). Simultaneously, each mouse was injected intravenously with 5×10^6 syngeneic Ig^+ spleen cells (\square); 5×10^6 syngeneic $CD4^+$ cells (Δ); or the combination of Ig^+ cells with graded numbers of $CD4^+$ spleen cells; 5×10^5 (\blacktriangle); 2×10^6 (\circ); and 5×10^6 (\bullet). The mice were bled from retro-orbital veins before and at weekly intervals after immunization. The serum anti-TNP antibody concentration was measured by indirect ELISA. There were four mice in each group.

TABLE I
Comparison of the Adoptive IgE Response Using $CD4^+$ Cells from Normal or TLI-treated Mice

Antigen	Ig^+ cells	$CD4^+$ cells	Day 0	Day 7	Day 14	Day 21	
+	-	-	<1	13.3 ± 0.8	9.8 ± 0.5	17.9 ± 2.5	
+	5×10^6	-	<1	15.6 ± 2.8	13.5 ± 0.4	25.3 ± 2.4	
+	-	Normal	5×10^6	<1	20.6 ± 2.6	11.6 ± 0.9	14.1 ± 0.9
+	5×10^6	Normal	5×10^7	<1	144.0 ± 5.5	155.0 ± 24.7	70.3 ± 8.8
+	5×10^6	Normal	2×10^6	<1	85.3 ± 11.4	176.0 ± 19.6	115.0 ± 59.5
+	5×10^6	Normal	5×10^6	<1	302.0 ± 18.2	214.0 ± 32.6	186.0 ± 13.0
+	-	TLI	5×10^6	<1	64.8 ± 7.1	9.9 ± 0.8	25.0 ± 1.2
+	5×10^6	TLI	5×10^7	<1	263.8 ± 27.3	156.0 ± 16.6	218.0 ± 21.4
+	5×10^6	TLI	2×10^6	<1	378.0 ± 41.1	332.0 ± 27.7	244.0 ± 57.0
+	5×10^6	TLI	5×10^6	<1	489.0 ± 42.3	375.0 ± 75.0	279.0 ± 29.7

Sublethally irradiated BALB/c mice were immunized intraperitoneally with TNP-KLH ($100 \mu g/mouse$). The mice were also injected intravenously with syngeneic Ig^+ cells and/or $CD4^+$ cells from either normal or TLI-treated mice. The serum IgE anti-TNP-KLH antibody concentration was measured by indirect ELISA. Values shown are in units calculated as: $[1/dilution \text{ of experimental serum (at a given OD)}] / [1/dilution \text{ of standard serum}]$. See Materials and Methods for details.

Comparison of GVHD Mediated by Normal and TLI CD4⁺ Cells. 24 h after lethal whole body irradiation, C57BL/Ka (H-2^b) mice received an intravenous injection of 2.5×10^6 normal BALB/c (H-2^d) BM cells and purified CD4⁺ cells from either normal or TLI-treated mice. The clinical signs of GVHD and mortality rate of all groups were recorded up to 100 d after the cell transfer. Fig. 4 shows that there was a close correlation between the dose of transferred CD4⁺ cells and the survival rate in both groups of mice. All six C57BL/Ka mice given allogeneic bone marrow alone survived >100 d. None of the irradiated mice without bone marrow cell transfer lived >14 d. When 2.5×10^6 normal CD4⁺ cells were injected, five of six mice died by 100 d after cell transfer. The same dose of TLI CD4⁺ cells only resulted in one death within six mice. Similarly, 5×10^6 TLI CD4⁺ cells caused a higher mortality rate (two of the six mice died) than 5×10^5 normal CD4⁺ cells (one of six mice died) but a lower mortality rate than 10^6 normal CD4⁺ cells (three of six mice died). From these results, the TLI CD4⁺ cells were estimated to be about seven to eight times less effective in inducing lethal GVHD than the same dose of CD4⁺ cells from

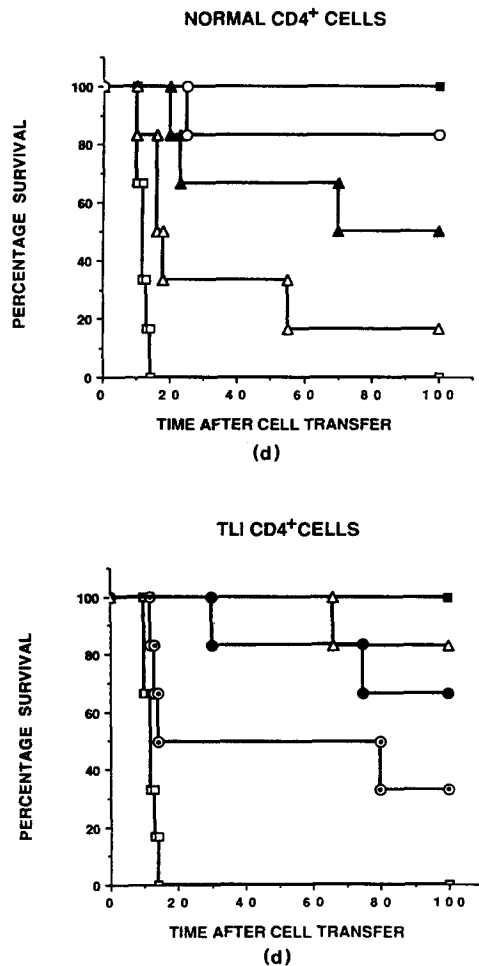


FIGURE 4. Comparison of GVHD mediated by CD4⁺ cells from normal and TLI-treated mice. 24 h after lethal whole body irradiation (850 rad; □), each C57BL/Ka mouse received intravenously 2.5×10^6 allogeneic BALB/c bone marrow cells (■). Simultaneously, graded doses of CD4⁺ spleen cells from normal BALB/c mice; 5×10^5 (○), 10^6 (▲), and 2.5×10^6 (△) or from TLI-treated BALB/c mice; 2.5×10^6 (△), 5×10^6 (●), and 10^7 (⊙) were injected intravenously into each host. The mortality rate was recorded up to 100 d after the cell transfer. There were six mice in each group.

normal mice. The markedly reduced capacity of TLI CD4⁺ cells to induce GVHD was observed in three other experiments.

Effect of Adding TLI CD4⁺ Cells to Normal CD4⁺ Cells in the GVHD Assay. To identify whether the reduced efficiency of TLI CD4⁺ cells in inducing GVHD was due to suppressor cells that have been observed in the spleen after TLI (37-39), an experiment was done to test the ability of CD4⁺ cells from TLI mice to suppress normal GVHD. 2.5×10^6 TLI BALB/c CD4⁺ cells were injected intravenously into lethally irradiated C57BL/Ka mice in combination with 2.5×10^6 each of normal BALB/c BM cells and CD4⁺ cells. The mortality rate is shown in Fig. 5.

All mice that received irradiation without BM cells died within 2 wk. None of the irradiated mice given allogeneic BM cells died within 100 d. One of the six mice receiving TLI CD4⁺ cells died 64 d after cell transfer; in comparison, all mice given the same number of normal CD4⁺ cells survived <3 wk. When 2.5×10^6 TLI CD4⁺ cells were coinjected with normal CD4⁺ cells, the TLI CD4⁺ cells failed to suppress the lethal GVHD. All six mice died rapidly after the two inocula of CD4⁺ cells were coinjected. Thus, the high survival rate of mice injected with TLI CD4⁺ cells was more likely due to the lack of GVHD-inducing cells rather than to the presence of suppressor cells in the cell inoculum.

Comparison of Cytokine Secretion by Normal and TLI Spleen Cells. The results described above demonstrated that unfractionated or CD4⁺ spleen cells from TLI-treated mice had a relatively normal ability to help B cells in the adoptive anti-TNP antibody response, but had an impaired capacity to transfer DTH or to induce GVHD. Since IL-4 and IL-5 are important cytokines in augmenting the antibody responses of B cells, and IL-2 and IFN- γ are considered more crucial for the function of cell-mediated immunity, the levels of cytokine secretion of spleen cells from normal or TLI-treated BALB/c mice were analyzed.

Spleen cells (10^7 /ml) obtained from both normal and TLI-treated BALB/c mice (12 mice/group) were stimulated with either hamster anti-mouse T3 mAb or Con A plus PMA in vitro. Supernatants were harvested after 8 and 24 h and assayed for cytokine secretion.

Table II shows the levels of four cytokines from the spleen cell supernatants 24 h after stimulation. Con A plus PMA, in general, gave a much stronger stimulation

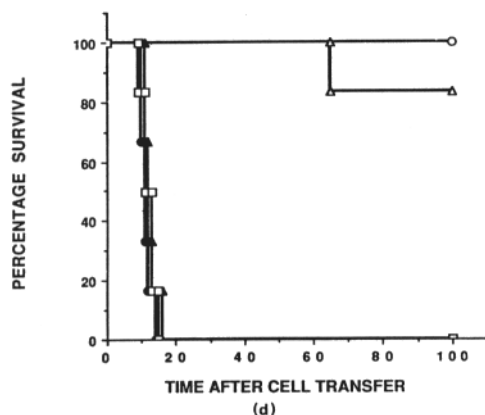


FIGURE 5. Effect of adding CD4⁺ spleen cells obtained from TLI-treated mice to normal CD4⁺ cells in the GVHD assay. 24 h after lethal irradiation (850 rad; ●), each C57BL/Ka mouse was injected intravenously with 2.5×10^6 allogeneic BALB/c bone marrow cells (O) and 2.5×10^6 CD4⁺ spleen cells from normal BALB/c mice (▲) or TLI-treated BALB/c mice (□). Another group of mice received 2.5×10^6 allogeneic BALB/c bone marrow cells, 2.5×10^6 CD4⁺ spleen cells from normal BALB/c mice, and the same number of CD4⁺ spleen cells from TLI-treated mice (□). There were six mice in each group.

TABLE II
Profiles of Cytokine Production from Unfractionated Spleen Cells 24 h after Stimulation

	IL-2	IFN- γ	IL-4	IL-5
	U/ml	ng/ml	U/ml	ng/ml
BALB/c Control*	<1	<0.2	<1	<0.3
BALB/c + anti-T3	1,343 \pm 156	8.6 \pm 1.2	26 \pm 5	21.0 \pm 2.0
TLI + anti-T3	63 \pm 7	0.6 \pm 0.4	15 \pm 3	34.3 \pm 4.5
BALB/c + Con A/PMA	2,865 \pm 264	13.9 \pm 2.6	55 \pm 6	29.7 \pm 2.2
TLI + Con A/PMA	268 \pm 58	2.7 \pm 0.8	48 \pm 4	30.3 \pm 1.9

10^7 /ml unfractionated spleen cells were stimulated with either anti-T3 antibody (final concentration 1:200) or Con A (5 μ g/ml) plus PMA (10 ng/ml). The supernatants were harvested 24 h later. The levels of IL-2 or IL-4 were measured by colorimetric MTT bio-assays, and the levels of IL-5 or IFN- γ were measured by ELISAs.

* There were 12 mice in each group. Data is represented by mean \pm SE.

than anti-T3 antibody with both normal and TLI spleen cells, but the cytokine secretion pattern was consistent in the two groups. TLI spleen cells secreted mean levels of IL-5 at least as high as those of normal spleen cells, and 59–88% of the mean normal level of IL-4. The same cells only secreted 5–9% of the mean normal level of IL-2 and 6–20% of the mean normal level of IFN- γ . No detectable cytokines were found in the supernatants lacking anti-T3 antibody or Con A plus PMA. The supernatants harvested at 8 h after stimulation showed a lower cytokine concentration, but a similar pattern (data not shown).

Characterization of Alloreactive Clones from Spleen Cells of Normal and TLI-treated Mice. Alloantigen-specific clones from both normal and TLI mice were established by limiting dilution. The clones were stimulated with alternate cycles of irradiated allogeneic spleen cells followed by growth in a medium containing rIL-2. Some clones were lost in the early cycles of stimulation. After four stimulations over a period of 2 mo, most clones stabilized and secreted sufficient cytokines to be detected. Three cloning experiments have been carried out from the spleen cells of normal and TLI-treated mice.

10 clones obtained from normal BALB/c spleen cells in cloning experiment no. 1 were selected in a blind manner (Table III). The quantitative results in different clones could not be compared, because the cell numbers in each harvested well differed at the time of the assay. 8 of the 10 clones had clear cytokine secretion patterns characteristic of either Th1 (IL-2 and IFN- γ detected without IL-4 and IL-5) or Th2 (IL-4 and IL-5 detected without IL-2 and IFN- γ) clones. Clones 1 and 10 were considered mixed cytokine clones because they secreted cytokines characteristic of both Th1 and Th2 cells. The surface phenotypes of these clones are also shown in Table III. 9 of the 10 clones had at least 72% cell lysis with anti-CD4 antibody and complement. Clone numbers 7 and 8 had some degree (8.5 and 15.9%) of cell lysis with the anti-CD8 antibody.

Comparison of Th1 vs. Th2 Clone Ratios in Spleen Cells from Normal and TLI Mice. A summary of three cloning experiments is shown in Table IV. The cloning frequency for both normal and TLI-treated BALB/c mice was essentially equivalent (0.42–1.15% for normal BALB/c mice; 0.14–1.48% for mice 1 mo after TLI treatment; 0.83–0.95% for mice 3 mo after TLI treatment). A minority of clones secreted only one of the

TABLE III
Examples of Cytokine Production from CD4⁺ Clones

Clone	IL-2	IFN- γ	IL-4	IL-5	CD4 ⁺ cells*	CD8 ⁺ cells	Clone type
	U/ml	ng/ml	U/ml	ng/ml	%	%	
1	<1.2	4.3	342.1	13.1	41.7	- 4.2	Mixed
2	339.4	11.4	<0.1	<0.3	85.9	3.9	Th1
3	38.2	11.4	<0.1	<0.3	77.6	7.2	Th1
4	121.5	14.9	<0.1	<0.3	92.5	- 8.9	Th1
5	<1.2	<0.2	770.2	27.8	90.8	2.5	Th2
6	<1.2	<0.2	439.2	43.1	72.8	3.3	Th2
7	55.3	36.1	<0.1	<0.3	95.7	15.9	Th1
8	<1.2	<0.2	151.2	7.4	88.8	8.1	Th2
9	77.2	23.2	<0.1	<0.3	84.1	- 4.8	Th1
10	469.8	13.1	<0.1	1.1	94.3	- 2.3	Mixed

10 clones obtained from normal BALB/c spleen cells were selected in a blind manner.

* Surface phenotype of the clone was determined by cytotoxicity assay.

TABLE IV
Summary of Cloning Experiments

Exp.		Cells/well	Positive wells	Frequency*	Cytokine pattern		
					Th1	Th2	Mixed
				%			
1	Normal	10	4	0.42	12	6	2
		30	16	0.56			
	TLI	30	4	0.14	1	3	0
2	TLI	3	14	0.97	5	62	4
		10	57	1.48			
3	Normal	3	9	0.78	21	15	17
		10	44	1.15			
	TLI	3	9	0.78	6	26	6
		10	29	0.76			
	TLI (3 mo)	3	11	0.95	17	17	9
		10	32	0.83			

* Frequency (%) = $100 \times (\text{number of positive wells}) / (\text{number of cells per well} \times \text{number of wells})$.

two cytokines in a subset: IL-2 or IFN- γ for Th1 clones; IL-4 or IL-5 for Th2 clones. These clones were still categorized as either Th1 or Th2 in Tables IV and V, but could be more appropriately considered as Th1- or Th2-like clones.

The ratios of Th1 and Th2 clones in both normal and TLI-treated BALB/c mice are shown in Table V. In 73 clones obtained from normal BALB/c mice, there were more Th1 than Th2 clones. However, in 113 clones established from the spleens of TLI mice 1 mo after irradiation, there were seven times more Th2 than Th1 clones. In a similar experiment, there were 43 clones obtained from BALB/c mice 3 mo after TLI treatment. Previous studies have shown a normalization of the MLR at that time point (31). The ratio of Th1 to Th2 clones was 1:1 in the latter mice. Out

TABLE V
Comparison of Th1/Th2 Ratio of Normal and TLI-treated BALB/c Mice

	BALB/c	TLI (1 mo)	TLI (3 mo)
Positive wells	73	113	43
Th1	33	12	17
Th2	21	91	17
Th1/Th2	1:0.64	1:7.58	1:1
Mixed cytokine clones	19	10	9

of a total of 229 clones gathered in three experiments, 38 mixed cytokine clones were observed.

Discussion

The present study demonstrated a functional dichotomy of unfractionated spleen cells obtained from mice 4–6 wk after TLI. The spleen cells transferred a weak DTH reaction but a normal serum anti-TNP antibody response in the same recipients. To relate the results obtained from unfractionated spleen cells to changes in the CD4⁺ subset, purified CD4⁺ cells were used to study helper function in the antibody response, and cell-mediated immunity (GVHD) in adoptive cell transfer systems. The data showed that CD4⁺ cells from TLI-treated BALB/c mice were able to provide normal helper function for adoptive serum anti-TNP antibody responses with normal B cells. The same cells also amplified the IgE anti-TNP-KLH antibody response of normal B cells at least as well as CD4⁺ cells from normal BALB/c mice. On the other hand, these CD4⁺ cells were seven to eight times less efficient than normal CD4⁺ cells in inducing lethal GVHD in irradiated C57BL/Ka mice. This result was not likely to be explained by the existence of suppressor cells in the spleen of TLI mice, since coinjection of TLI CD4⁺ spleen cells with normal CD4⁺ spleen cells did not alter the GVHD-inducing capacity of the latter cells.

Experiments using two different polyclonal T cell stimulators, anti-T3 antibody and Con A plus PMA, further demonstrated that both IL-2 and IFN- γ secretion in vitro were profoundly depressed in spleen cells obtained from TLI-treated mice. However, IL-4 and IL-5 secretion in vitro were close to normal. Since the biological activities of cytokines in stimulated cell supernatants have been shown to correlate fairly well with their mRNA levels (13), it is reasonable to expect that secreted cytokines of spleen cells from TLI mice could also reflect their level of transcription activity.

The above studies indicated that spleen cells obtained from mice 4–6 wk after TLI showed several characteristics that suggested a selective deficiency of Th1-like cell functions. The cells had a reduced ability to adoptively transfer DTH and GVHD (cell-mediated immunity), and synthesized reduced amounts of IL-2 and IFN- γ in response to polyclonal stimulation. In contrast, the Th2-like functions of TLI-derived CD4⁺ cells were similar to those of normal BALB/c mice. B cell helper function in an adoptive transfer system was normal, and the synthesis of IL-4 and IL-5 by spleen cells from TLI-treated mice was close to normal values.

Since these results were consistent with selective depletion of Th1-like cells from the spleen of TLI-treated mice, we attempted to directly determine whether there

was a selective deficiency of precursor cells of Th1 clones after TLI. Spleen cells from TLI and normal mice were stimulated *in vitro* with alloantigens, cloned by limiting dilution, and their cytokine secretion patterns were analyzed to identify Th1 and Th2 clones. A total of 229 clones were established from spleen cells from both normal and TLI-treated BALB/c mice. Clones that secreted IL-2 or IFN- γ without IL-4 and IL-5 can be considered Th1-like, and those that secreted IL-4 or IL-5 without IL-2 and IFN- γ can be considered Th2-like. Spleen cells from normal BALB/c mice generated more Th1 and Th2 clones (ratio 1:0.6). Spleen cells from TLI-treated mice generated a total of 113 clones with more Th2- than Th1-like clones, thus inverting the Th1/Th2 ratio to \sim 1:7.6. Furthermore, 3 mo after TLI, a time when the MLR recovers to normal levels (31), the ratio of Th1- to Th2-like clones (1:1) was closer to that of normal mice.

The results with TLI mice can be explained by a selective decrease in the number of precursor or mature Th1 as compared with Th2-like cells in the spleen 4–6 wk after TLI. Since most of the evidence showing normal Th2 but reduced Th1-like cell numbers and function was derived from cloning or adoptive transfer experiments, which would allow time for differentiation of precursor cells, it is likely that the differences observed in the TLI spleen are due to changes in the frequencies of Th1-like precursor cells. This deficiency may be due to an inability of the thymus of TLI-treated mice to produce or export T cells capable of differentiating into Th1-like cells. Alternatively, the export of such cells may be normal, but post-thymus maturation of Th1-like cells may be selectively impaired.

It is of interest that all Th1-like functions were reduced after TLI, but Th2-like functions remained at normal levels, with the exception of IgE secretion, which was somewhat elevated. This may be related to the ability of IFN- γ (from Th1 cells) to inhibit IL-4-induced IgE secretion, and the release of this inhibition in TLI mice.

Two lines of evidence suggest that there are other Th cell cytokine secretion patterns in addition to the prototypical Th1 and Th2 patterns. First, the Pgp-1⁻ subset of CD4⁺ spleen cells secretes substantial levels of IL-2 but very little IFN- γ (40, 41), and normal spleen cell populations often secrete IL-2 but little or no detectable IL-4 or IL-5 (42, 43). These results are not consistent with the pattern expected if CD4⁺ spleen cell populations consisted only of prototypic Th1 and Th2 cells. Further evidence for additional heterogeneity of Th cells was obtained in the present study, since there were 38 mixed cytokine clones in a total of 229 clones analyzed in the above experiments. This could be accounted for by multiple clones (Th1 and Th2) coexisting in a single well. However, it was unlikely that these wells contained multiple clones because of the low frequency of positive wells. Many clones of mixed cytokine secretion phenotype have also been isolated in additional studies (Street, N., and T. Mosmann, unpublished results), and in some cases, the mixed pattern has remained stable on subcloning. These mixed cytokine clones might constitute another type of CD4⁺ cells that combines the cytokine secretion and functions of both Th1 and Th2 cells. They may also represent different stages of the CD4⁺ cell differentiation pathway. Regardless of the extent of heterogeneity within the Th population, the present study shows that several immune functions are selectively and coordinately reduced in TLI mice, and all of these correspond to functions of *in vitro* Th1-like clones. Conversely, all functions predicted to be mediated by Th2-like cells are preserved. Thus, this study demonstrates a dichotomy of immune function

in vivo that parallels the dichotomy of immune functions mediated by Th1 and Th2 clones in vitro.

The previous studies of TLI mice showed a diminished primary antibody response, but a vigorous secondary antibody response to SRBC (44). The primary antibody response to DNP-KLH in CFA was also decreased (45). The current study shows a normal ability of TLI spleen cells to adoptively transfer the primary antibody response to TNP-KLH in aqueous solution, and appears inconsistent with the previous results. However, differences in activation of Th1- or Th2-like cells might be determined by the type of antigen or adjuvant used. In recent cloning experiments, it was found that some antigens appear to preferentially favor the activation and growth of certain clones. For example, CFA injected into mice favors the activation/differentiation of Th1 cells (Street, N., and T. Mosmann, unpublished results). Thus, in mice given TLI, antibody responses that depend heavily on Th1-like cells might be substantially reduced.

Several previous studies from patients given TLI support the current conclusions. Patients with Hodgkin's disease lost their DTH skin reaction to dinitro-chlorobenzene for many months after radiation therapy (30). In addition, purified CD4⁺ cells from TLI-treated patients had markedly reduced proliferative responses to alloantigens and mitogens in vitro (29). However, the CD4⁺ cells can help the same patients' unirradiated B cells to produce normal levels of Ig in vitro in response to PWM (Field, E., and S. Strober, unpublished observations). The incidence of severe bacterial infection among TLI patients was <1% (31). In addition, there are no significant changes in total or specific IgE levels in patients after TLI (46). Thus, the dichotomy of Th functional deficits observed in mice is consistent with the results in humans.

Summary

Purified CD4⁺ BALB/c spleen T cells obtained 4–6 wk after total lymphoid irradiation (TLI) helped normal syngeneic B cells to produce a vigorous antibody response to TNP keyhole limpet hemocyanin in adoptive cell transfer experiments. However, the same cells failed to transfer delayed-type hypersensitivity to the adoptive hosts as measured by a foot pad swelling assay. In addition, purified CD4⁺ cells from TLI-treated mice were unable to induce graft vs. host disease in lethally irradiated allogeneic C57BL/Ka recipient mice. In response to mitogen stimulation, unfractionated spleen cells obtained from TLI mice secreted normal levels of IL-4 and IL-5, but markedly reduced levels of IL-2 and INF- γ . A total of 229 CD4⁺ clones from spleen cells of both normal and TLI-treated mice were established, and the cytokine secretion pattern from each clone was analyzed. The results demonstrate that the ratio of Th1- and Th2-like clones in the spleens of normal BALB/c mice is 1:0.6, whereas the ratio in TLI mice is \sim 1:7.

These results suggest that Th2-like cells recover rapidly (at \sim 4–6 wk) after TLI treatment and account for the early return of antibody helper activity and secretion of IL-4 and IL-5, but Th1-like cells recover more slowly (in \sim 3 mo) after irradiation, and this accounts for the deficit in cell-mediated immunity and the reduced amount of IL-2 and IFN- γ secretion.

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