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Alloantigen priming induces a state of unresponsiveness in human umbilical cord blood T cells

(tolerance/T lymphocytes)

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ABSTRACT **Induction of alloreactivity in human adult and umbilical cord blood T cells was evaluated in mixed leukocyte culture by exposure to an allogeneic lymphoblastoid line that expresses known costimulatory molecules. Initial exposure to alloantigen-presenting cells (allo-APC) induced strong proliferative responses in both adult and cord blood T cells. However, in contrast to adult T cells, cord blood T cells exhibited little proliferation after restimulation with donor APC. Primed cord blood T cells could respond to interleukin 2 (IL-2), but unresponsiveness to alloantigen was not overcome by addition of exogenous IL-2. Unresponsiveness was long-lasting and appeared to be maintained by a combination of induction of anergy and activity of $CD8^+$ suppressor cells. This information may contribute to use of human cord blood as an allogeneic source of transplantable stem cells.**

Experimental and clinical evidence has demonstrated the efficacy of human umbilical cord blood (CB) as a source of transplantable hematopoietic stem and progenitor cells (1–13). More than 60 CB transplants have been performed for treatment of either malignant or nonmalignant diseases in children. The majority have been HLA-matched sibling transplants. Several one-, two-, and three-antigen-mismatched sibling transplants and unrelated matched and partially mismatched transplants have also been performed and were characterized by a relatively low incidence of graft-versus-host disease (GVHD) (2–5, 7–13). These clinical observations led us to evaluate the alloreactive potential of CB T cells. We previously demonstrated that while little cytotoxic T-cell activity was generated after allogeneic stimulation of CB T cells, there was strong cellular proliferation in both adult and CB T cells (14). To determine if this proliferation results in similar qualitative changes in cellular activation/differentiation, we evaluated *in vitro* proliferative responsiveness of adult and CB T cells following primary alloantigen stimulation.

MATERIALS AND METHODS

Collection of Samples. Human CB was obtained via heparinized syringe from normal deliveries at Wishard Memorial Hospital in Indianapolis as approved by the Institutional Review Board of the Indiana University School of Medicine (1). Heparinized adult peripheral blood (PB) was obtained after informed consent from healthy volunteer donors. Mononuclear cells were obtained from all samples by centrifugation over Ficoll/Hypaque gradients (1).

T-Cell Purification. Purified T cells were obtained by incubating 15–25 $\times$ 10^6 mononuclear cells in Lympho-Kwik T (One Lambda, Los Angeles) for 20–30 min at 37°C or in Lympho-mAbs Kwik Th for 50 min at 37°C. This cocktail has monoclonal antibodies and complement that selectively lyses $HLA\text{-}DR^+$ cells, B cells, residual granulocytes, and $CD8^+$ cells (Lympho-Kwik Th). Cell purity was $\geq$ 90% $CD3^+$ and $\geq$ 93% $CD4^+$.

Mixed Leukocyte Cultures (MLC). Primary MLC were established in 75-cm^3 tissue culture flasks with 10–20 $\times$ 10^6 responding T cells and irradiated (5000 R; 1 R = 0.258 mC/kg) stimulators at a final density of 2 $\times$ 10^6 cells per ml in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and penicillin/streptomycin. Flasks were incubated at 37°C in an atmosphere containing 5% CO_2 for 5 days. Lymphoblastoid cell lines Raji and Daudi (American Type Culture Collection) served as sources of donor and third-party alloantigen stimulation, respectively. These stimulators were added at a responder/stimulator ratio of 10:1. Secondary MLC were generated from bulk primary MLC. Primed T cells were reisolated with Lympho-Kwik and recultured for 48 hr in culture medium. Viable T cells, as determined by trypan blue exclusion, were harvested and plated into 96-well plates with appropriate stimulators.

Proliferation Assays. T cells (5 $\times$ 10^4 per well) were cultured in medium alone or with donor or third party alloantigen-presenting cells (allo-APC). Restimulation of T cells with lymphoblastoid lines was done at a responder/stimulator ratio of 10:1. Thymidine incorporation was measured as described (14). Data represent the mean cpm of quadruplicate wells. In some experiments recombinant human interleukin 2 (IL-2; Immunex) was added to cultures. Addition of IL-2 (5–25 international units/ml) struck a balance in which enhancement of proliferation in experimental wells was observed without stimulating an undue amount of proliferation in control wells.

Analysis of Suppressor Activity. Parallel autologous CB cultures containing $CD3^+$ T cells or $CD4^+$ ($CD8^-$) T cells were stimulated in primary MLC. After 5 days of culture $CD3^+$ cultures were harvested and stained with an anti-CD8 fluorescein isothiocyanate-tagged FITC mAb for sorting by flow cytometry. Sorted $CD8^+$ cells (>96% $CD8^+$) were cultured overnight in 10 units of IL-2 per ml. $CD4^+$ T cells were reisolated with Lympho-Kwik Th and rested overnight in culture medium. Recovered $CD8^+$ and $CD4^+$ populations were added to 96-well plates with 10 units of IL-2 per ml at a concentration of 25,000 cells per well in the presence of the appropriate allo-APC. Wells containing admixed populations contained 25,000 $CD4^+$ cells with 25,000 or 50,000 $CD8^+$ cells. Proliferative responses were measured on days 3 and 6.

Fluorescence-Activated Cell Sorting (FACS) Analysis. Phenotypic analyses were performed by one- and two-color flow cytometry with a Coulter EPICS cytometer as described (14).

RESULTS

In vitro proliferative responses of adult CB T cells were evaluated by: (*i*) activation of negatively selected T cells with

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Abbreviations: CB, cord blood; IL-2, interleukin 2; APC, antigen-presenting cells; MLC, mixed lymphocyte culture; PB, peripheral blood; MHC, major histocompatibility complex; TCR, T-cell antigen receptor; LFA, lymphocyte function-associated antigen.

lymphoblastoid B cells, Raji, at a responder/stimulator ratio of 10:1 for 5 days [Raji cells express known co-stimulatory molecules including B7 (15), B70 (15, 16), lymphocyte function-associated antigen 3 (LFA-3) (17), and intercellular cell-adhesion molecule 1 (ICAM-1) (17) and HLA class I and class II major histocompatibility complex (MHC) molecules (17)]; (*ii*) reisolation of T cells by negative selection; (*iii*) reculture of cells in medium for 48 hr; and (*iv*) restimulation with donor or third-party lymphoblastoid B cells at a responder/stimulator ratio of 10:1. Fig. 1 shows results of an experiment, representative of 9 adult and 18 CB samples, comparing proliferative responses of adult and CB T cells in a primary allogeneic reaction to either Raji or Daudi cells (which also express costimulating molecules) and in a secondary allogeneic reaction in which Raji primed cells are stimulated by either Raji (donor) cells or Daudi (third party) cells. Maximal proliferation of primed PB T cells to donor APC and to third-party APC was observed on day 3 of secondary cultures. Compared to the maximal primary responses, these responses to donor and third party were 350% and 117%, respectively. In contrast, restimulation of CB T cells by donor alloantigens resulted in proliferation $\leq$ 30% of the maximal primary response. The maximal response of primed CB T cells to third-party APC was 35% of the primary response to these allogeneic cells. These results demonstrate that interactions of CB but not adult T cells with allogeneic cells expressing class I and class II MHC antigens and costimulatory molecules result in cellular proliferation followed by prolonged unresponsiveness to subsequent exposure to priming allo-APC.

Down-regulation of antigen receptors or growth factor receptors after the initial proliferative response results in anergy (18). To evaluate this, T cells were cultured in primary MLC for 5 days, reisolated, and evaluated for CD3 expression (Table 1). No significant changes in the number of $CD3^+$ cells or surface antigen density were observed for adult or CB T cells. Expression of CD25, the IL-2 receptor, was evaluated to determine if initial encounter of adult and CB T cells with

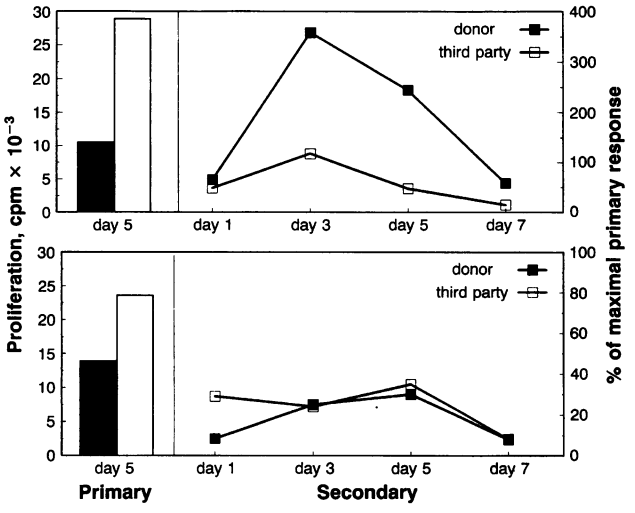

FIG. 1. Alloantigen stimulation primes adult T cells, but not CB T cells, for secondary alloantigen-specific responses. Adult (*Upper*) or CB (*Lower*) T cells were cocultured with irradiated allogeneic Raji (■) or Daudi (□) lymphoblastoid cells for 5 days for analysis of primary response. T cells were reisolated by monoclonal antibody and complement treatment and recultured 48 hr in medium. Kinetics of secondary responses of viable T cells were assessed following restimulation of Raji-primed cells with either Raji cells (as donor) or with Daudi cells (as third party). (*Upper* and *Lower Left*) Maximal primary responses are represented by the vertical bars. (*Upper* and *Lower Right*) Kinetics of secondary responses as a percentage of the maximal primary response. Note that the *y* axis (percent of maximal primary response) is different for PB and CB.

Table 1. Expression of CD3 and CD25 following alloantigen stimulation of T cells

T cells	$CD3^+$ cells,* %	Log MFI,* arbitrary units	$CD3^+$ cells expressing CD25,† %	Log MFI,† arbitrary units
Adult (n = 5)				
Preculture	93 ± 3	10 ± 8	7 ± 5	2 ± 0.3
Postculture	94 ± 2	13 ± 2	16 ± 7	6 ± 4
CB (n = 5–9‡)				
Preculture	93 ± 2	9 ± 2	7 ± 3	3 ± 1
Postculture	93 ± 2	11 ± 2	48 ± 16§	14 ± 9¶

*Surface expression of CD-3 on CB and adult T cells stained with FITC-conjugated OKT3 mAb (αCD-3) and analyzed by flow cytometry. MFI, mean fluorescence intensity. Data are presented as means ± SD.

†Surface expression of CD25 (IL-2 receptor) on adult and CB T cells stained with FITC-conjugated αCD25 and phycoerythrin-conjugated αCD3 mAbs. Cells were analyzed by two-color flow cytometry. Data are presented as the mean percentage (± SD) of $CD3^+$ T cells that coexpressed CD25. MFI, mean fluorescence intensity.

‡n = 5 for $CD3^+$ cells; n = 9 for $CD3^+$ cells expressing CD25.

§$P < 0.005$ compared with postculture adult blood; Student's t-test.

¶$P < 0.05$ compared with postculture adult blood.

allo-APC resulted in comparable states of activation (Table 1). At onset, CD25 expression on CB and adult T cells was similar, but alloantigen priming of CB T cells consistently resulted in greater increases in the number of CD25-expressing T cells and in the density of CD25. This increase in "activated" CB T cells following priming was supported by cell cycle analysis (four CB and three PB samples). After 5 days of allostimulation, primed CB T cells exhibited a greater percentage of cells in S phase or G_2/M phase (cells in G_0/G_1, S phase, and G_2/M phase, respectively, were 49%, 18%, and 14% for CB and 65%, 7%, and 6% for PB), suggesting that priming allo-APC provides signals to drive proliferation and activation of CB T cells.

Induction of nonresponsiveness *in vitro* to alloantigen is often accomplished by a decrease or block in the production of IL-2, but unresponsiveness can be overcome by exogenous IL-2. We examined whether restimulation of alloantigen-primed CB T cells in the presence of exogenous IL-2 would result in responsiveness. We compared secondary kinetics of proliferation of two CB and one PB following restimulation with donor APC in the presence of IL-2 at 25 units/ml (Fig. 2). Addition of IL-2 had little effect on overall response of

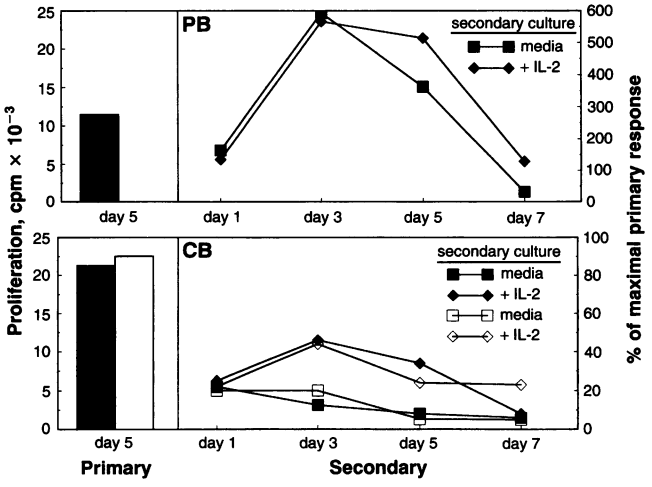

FIG. 2. IL-2 does not overcome the hyporesponsive state of primary CB T cells. Restimulation of alloantigen-primed adult and CB T cells was assessed in the presence or absence of 25 units of exogenous IL-2 per ml. (*Lower*) Data for two individual CB samples. Note that the *y* axis (percent of maximal primary response) is different for PB (*Upper*) and CB (*Lower*).

primed adult T cells. It substantially improved the proliferative response of CB T cells, but this was <50% of the initial primary response, suggesting that while primed CB T cells retain ability to respond to IL-2, hyporesponsiveness is not overcome. For both CB samples, third-party responses peaked at day 3 and were 10% of the maximal primary response to these alloantigens. IL-2 increased these responses to 20% and 14% of the maximal primary response, respectively (data not shown). Third-party responses of adult T cells peaked at day 5 of the secondary culture. These responses in the absence and presence of exogenous IL-2 were 68% and 74%, respectively, of the maximal primary response.

To evaluate cell death as a cause of unresponsiveness, the viability of T cells reisolated from primary MLC and cultured in medium for 4 days was assessed (Fig. 3 *Left*). While a more rapid decline in the viability of primed CB vs. PB T cells occurred, reculturing primed CB T cells with IL-2 (25 units/ml) during both the induction phase and reculture period maintained a level of viability similar to that of adult T cells.

Thus, with addition of IL-2, induction of unresponsiveness could be evaluated under conditions in which deletion was limited or absent. Fig. 3 *Right* presents proliferative responses of the CB and PB T-cell cultures evaluated in Fig. 3 *Left*. Primed CB T cells demonstrated a secondary response that was <25% of the initial response. Addition of IL-2 during the reculture period increased the peak secondary response. However, continuous culture of these CB T cells with IL-2 decreased the relative secondary response, in part because of increases in proliferation of cells in control wells containing medium and IL-2. The peak third-party response for PB was 64% of the maximal primary response and occurred on day 5. Peak CB third-party responses were 18%, 13%, and 20%, respectively, for cells restimulated in medium with exogenous IL-2 or cultured continuously with IL-2. Thus, in the absence of cell death, unresponsiveness of CB T cells is still maintained.

The above observations suggested that, in the absence of deletion, the unresponsive state was maintained by other mechanisms. To assess the role of $CD8^+$ CB T cells as suppressors of the secondary responses, we initiated the following "add-back" experiments. Since suppression of alloreactivity requires cooperation between $CD4^+$ and $CD8^+$ populations (19), $CD8^+$ T cells were sorted from bulk cultures of $CD3^+$ cells. To eliminate the potential of suppressor cell interactions on the $CD4^+$ responder populations, alloprimed $CD4^+$ T cells were obtained from parallel autologous culture initiated with only $CD4^+$ cells. Proliferation following co-culture of these primed populations in secondary MLC in the presence of IL-2 at 10 units/ml was assessed on days 3 and 6 (Fig. 4; one experiment). Suppression of $CD4^+$ responses occurred at both time points. In another experiment (not shown), suppression of $CD4^+$ proliferative response was only observed on day 6 of the secondary culture. In two other experiments, suppression could not be evaluated because restimulation induced little proliferation in the primed $CD4^+$ CB cells. This suggests that alloactivated $CD8^+$ CB T cells have suppressive activity in secondary MLC. Suppression of third-party responses suggests this function is not antigen specific.

DISCUSSION

This study confirms our previous observation (14) that encounter of adult and CB T cells with alloantigen results in comparable initial proliferative responses. However, in contrast to adult T cells, alloantigen priming renders CB T cells hyporesponsive to subsequent exposure to priming alloantigens. Use of negatively selected T-cell populations devoid of B cells and monocytes eliminated differences in accessory cell populations from consideration. Negative selection also precluded the possibility of antibody-induced perturbations during isolation of the T cells. Allogeneic stimulation was provided by a lymphoblastoid B-cell line expressing high levels of class I and class II MHC as well as a number of known costimulatory molecules. The use of these allogeneic APC is critical to this comparison, since allogeneic stimulation of adult T cells in the absence of costimulatory signals leads to induction of anergy (20–24). Our results with adult T cells are in agreement with a number of other investigators: restimulation of alloantigen-primed adult T cells results in accelerated secondary proliferative response, which peaks at day 3 (20–23, 25).

Induction of unresponsiveness does not appear to involve surface molecules involved in antigen recognition or activa-

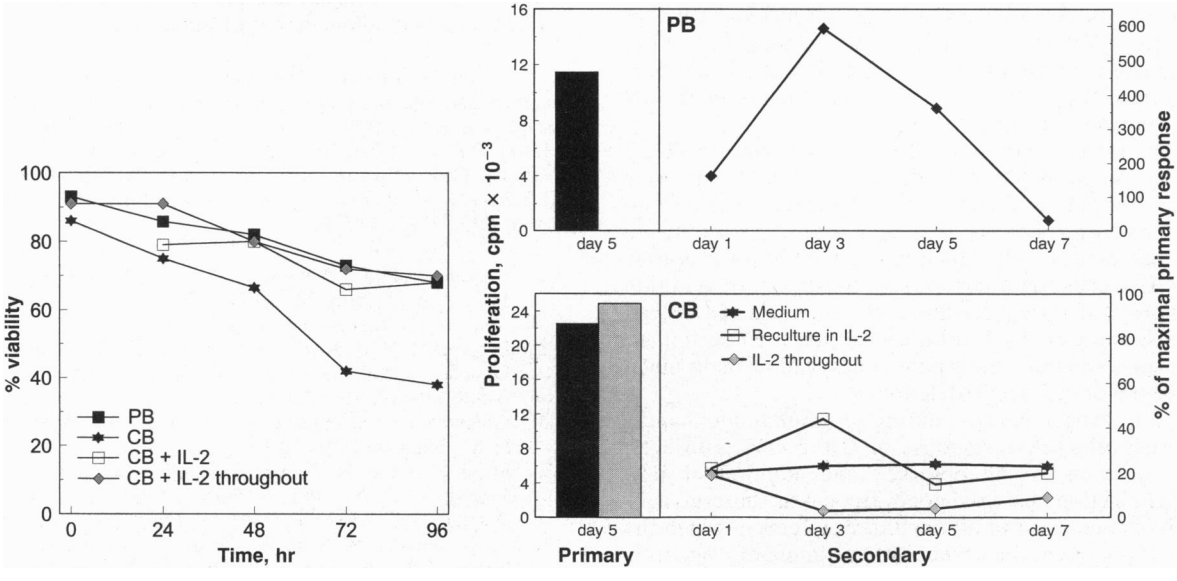

FIG. 3. IL-2 prevents cell death observed during reculture. IL-2 maintains cell viability of alloactivated CB cultures but does not prevent induction of unresponsiveness. (*Left*) The viability of CB T cells recultured in the presence or absence of 25 units of IL-2 per ml was evaluated during a 96-hr period. Viability of primed adult T cells recultured without exogenous IL-2 served as a positive control. (*Right*) Kinetics of secondary stimulation for the above CB and adult T-cell cultures. (*Upper*) Response of adult T cells recultured for 48 hr in medium alone. (*Lower*) Response of CB T cells recultured in medium alone or medium with IL-2 at 25 units/ml or primed, recultured, and restimulated in the presence of IL-2 at 25 units/ml. Note that the *y* axis of *Right* (percent of maximal primary response) is different for PB and CB.

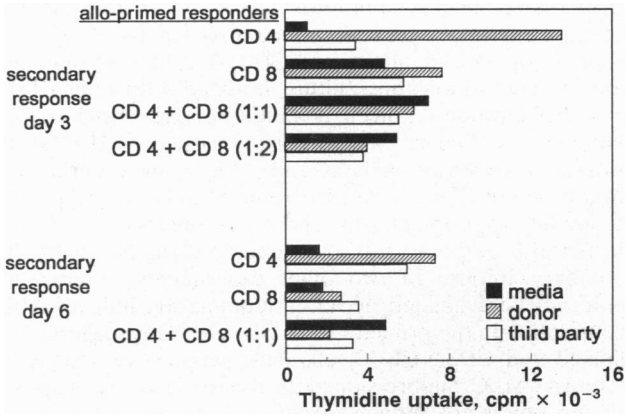

FIG. 4. Alloantigen priming induces suppressor function in $CD8^+$ CB T cells. Primed $CD4^+$ cells were isolated from $CD4^+$ ($CD8^-$) primary cultures. The response to donor or third-party cells of $CD4^+$ cells in the presence or absence of $CD8^+$ cells is presented. Secondary proliferative responses were measured in the presence of IL-2 at 10 units/ml on days 3 and 6. Data are presented as mean cpm of quadruplicate wells. Standard deviations for each group were ≤15% of the mean cpm.

tion. Down-modulation of the CD3–T-cell antigen receptor (TCR) complex was not observed on either adult or CB T cells after priming, and expression of CD28, CD2, and LFA-1 on resting CB T cells is equivalent to that of adult T cells (26, 27). These surface molecules are the respective counterreceptors for the costimulatory molecules B7, LFA-3, and intercellular adhesion molecule 1 found on the donor allo-APC used in these experiments.

Alloantigen stimulation did result in a greater number of "activated" $CD3/CD25^+$ T cells in CB cultures. Given the nonclonal nature of our bulk cultures, this may result from the activation of both antigen-specific and -nonspecific T cells, raising the possibility that lack of responsiveness is influenced by an expansion of antigen-nonspecific T cells, which results in a dilution of the antigen-specific responding populations. The weak responses obtained against third-party alloantigens do not support this notion. Another possibility is that several levels of cellular activation exist in T-cell populations. While activation of CB T cells by exposure to alloantigens is sufficient to induce proliferation, the thresholds for the induction of full effector function may not be exceeded. Failure to reach these thresholds results in cells entering a default pathway characterized by unresponsiveness.

Decreases in the viability of CB T cells during reculture suggested a role for deletion in maintaining the unresponsive state. Death of activated lymphocytes typically involves apoptosis. When using a sensitive *in situ* detection system, no consistent differences were noted in the numbers of apoptotic adult or CB T cells (data not shown). Furthermore, addition of exogenous IL-2 during reculture prevented significant cell death in CB cultures, yet had little effect on their secondary response, suggesting that unresponsiveness can be maintained in the absence of cell death/deletion.

Addition of exogenous IL-2 during restimulation enhances the relative proliferative response of CB T cells. However, hyporesponsiveness is still observed and addition of IL-2 during the induction phase did not overcome the unresponsive state. This is in contrast to other culture systems in which the induction of anergy in the absence of costimulatory signals is broken by IL-2 (21). These data, together with the high level of CD25 expression on activated CB T cells, suggest that the induction/maintenance of the unresponsive state is not due to an inability of CB T cells to respond to IL-2.

Nonresponsiveness may be secondary to suppression. Results of our "add-back" experiments suggest that activated $CD8^+$ CB T cells can suppress the proliferative response of primed $CD4^+$ cells. However, in some experiments little proliferation is observed following rechallenge of cultures containing only primed $CD4^+$ CB cells (data not shown). Thus, suppression may act as a "fail-safe" mechanism and may be observed only when anergy is not fully operative.

It should be noted that the weak response of CB T cells to third-party alloantigens does not necessarily indicate a lack of specificity in this system. The magnitude of response to third-party alloantigens is likely controlled by the presence of nonspecific suppressive influences and frequency of cells capable of responding to third-party alloantigens. A decrease in this frequency may result from the initial expansion of donor-reactive CB T cells during the primary stimulation period.

Significant qualitative differences exist in the activation state of adult and CB T cells following priming with alloantigens. This may be related to differences in the activation requirements of memory and naive T cells (17). For a given population of T cells, it may be assumed that two pathways of activation/differentiation are available, one leading to effector function, the other to tolerance or unresponsiveness. In this case, differences in the cellular response most likely result from intracellular signaling events that follow TCR–MHC and costimulatory ligand–receptor interactions. It is of interest then that no qualitative differences in the ability of naive CB and adult primed or memory T cells to express effector lymphokine genes following repeated αCD3 stimulation have been observed (28), suggesting that signaling pathways of CB T cells, activated by crosslinking the CD3–TCR complex, are intact and fully functional.

A goal of transplantation immunology is to develop approaches that avoid graft-versus-host disease while retaining immunity to opportunistic infections. We present evidence here that the priming of CB, but not adult T cells, with APC-expressing allogeneic MHC and costimulatory molecules induces an initial proliferative response followed by a sustained state of unresponsiveness, complimenting our previous findings that repeated stimulation of CB T cells fails to generate significant antigen-specific cytotoxic T-lymphocyte activity against allogeneic phytohemagglutinin-stimulated blasts (14). This information may be of relevance for the use of human CB in allogeneic transplantation.

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