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Sustained Expression of CD154 (CD40L) and Proinflammatory Cytokine Production by Alloantigen-Stimulated Umbilical Cord Blood T Cells

Nick C. Matthews,* Meenu Wadhwa,† Chris Bird,‡ Francesc E. Borras,* and Cristina V. Navarrete2*‡

Recent data suggests that graft-versus-host disease (GVHD) is initiated by host APCs. Blockade of CD40:CD154 interactions between APCs and T cells in vivo induces T cell tolerance to host alloantigen and dramatically reduces GVHD. Because allogeneic cord blood (CB) transplantation results in a lower incidence and severity of acute GVHD compared with bone marrow transplantation, we have investigated whether CB T cells can express CD154 in response to stimulation by allogeneic monocyte-derived dendritic cells (MDDC) and have used 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling in combination with intracellular cytokine analysis to assess the proliferation and cytokine profiles of alloantigen-responsive cells. CB T cells stimulated with allogeneic MDDC showed stronger proliferation than adult blood T cells. Surface CD154 expression was detected in the actively dividing CFSElow populations of both the CD4+ and CD4− subsets and was brightest in cells that had divided the most. Assessment of supernatants from MDDC-stimulated CB and adult blood T cells showed no significant difference in the levels of either IFN-γ or TNF-α, but CB T cell supernatants did show a significant lack of detectable IL-2. Intracellular cytokine analysis revealed that dividing CB T cells had been primed to produce IFN-γ, TNF-α, and IL-2 on restimulation. Further phenotype analysis showed that 75% of CB T cells producing IFN-γ were CD8+ T cells. These data suggest that MDDC-stimulated CB T cells express functional CD154 and provide enough costimulation for dendritic cells to prime naive CD8+ CB T cells and induce type 1 cytokine production. The Journal of Immunology, 2000, 164: 6206–6212.

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llogeneic umbilical cord blood (CB) transplantation is increasingly being used for hemopoietic reconstitution. Clinical data suggests that CB transplantation has a lower incidence of severe graft-versus-host disease (GVHD) than bone marrow transplantation, even in the presence of multiple HLA mismatches (1, 2), and yet maintains sufficient graft-versus-leukemia effect to prevent leukemic relapse (3). The definitive mechanism for this advantageous feature of CB is unknown, but if understood it could well have implications for minimizing graft rejection in other transplant settings.

GVHD is initiated by the direct recognition of alloantigen by donor T cells. The pathophysiology of GVHD is mediated by donor cytotoxic T cells and proinflammatory cytokines that damage the epithelia and endothelium of the gut, liver, and skin and can ultimately be fatal (4). Studies comparing CB with adult blood (AB) T cells responses to alloantigen in the primary MLR suggest that although alloantigen-induced CB T cell proliferation is slightly less or equivalent to AB T cells, there is little or no generation of cytotoxic effectors (5, 6).

The induction of Ag-specific cytotoxic T cell responses is primarily dependent on T cell-APC CD154-CD40-mediated costimulation (7). CD154 is an inducible costimulatory molecule of the TNF/nerve growth factor family that is expressed by activated CD4+ T cells and by minor subsets of activated γδ T cells and CD8+ T cells (8, 9). Ligation of CD40 by CD154 induces B cell proliferation (10) and markedly enhances dendritic cell (DC) function by up-regulating the expression of costimulatory, adhesion, and Ag-presenting molecules and by inducing IL-12 production (11). Blockade of CD154:CD40 interactions in vitro blocks Th type 1 cytokine production in the primary MLR (12) and virtually abolishes cytotoxic T cell effector generation. Further, addition of anti-CD154 to ex vivo MLR cultures reduces GVHD lethality by 30-fold (13).

Various studies have shown that CB T cells express low levels of CD154 following PMA and ionomycin stimulation compared with AB T cells (14–16). This has been interpreted as a cellular defect, which might explain the diminished Ab production and poor anti-viral responses of neonates (15). Further, because CD154 interactions are necessary for cytotoxic T cell effector generation (17, 18), this might also explain the very low frequency of cytotoxic effectors generated in the primary and secondary CB MLR (6) and thus the low level of GVHD reported in CB transplantation. However, CB T cells can express high levels of functional CD154 on anti-CD3 stimulation, which is up-regulated further by IL-2 and IL-4 (16). Furthermore, CB T cells primed by PHA and IL-2 can express high levels of CD154 on subsequent PMA and ionomycin restimulation (16). CB T cells are essentially nearly all...
of the naive CD45RA+ phenotype and thus require optimal antigenic stimulation by DCs (19, 20). As conventional MLRs use PBMC as stimulators, which contain very few DCs, the full potential of CB Th type 1 and cytotoxic T cells generated by allostimulation may have been underestimated.

To address this, we have tried to induce CD154 expression with stimuli more physiologically relevant to GVHD by culturing CB and AB T cells with allogeneic monocyte-derived DCs (MDDC). The results show that CB T cells have stronger proliferative responses to allogeneic DCs than AB T cells and, in contrast to previous data, can generate CD8+ T cells producing levels of IFN-γ similar to those secreted by AB T cells. This pattern of differentiatated T cell generation reflects the similar ability of both CB and AB T cells to express CD154 on allostimulation and suggests that CB T cells, if optimally stimulated, may have the functional capacity to initiate cytotoxic immune responses. These data suggest that the phenomena of reduced GVHD in CB transplantation may reflect weak antigenic stimulation of naive CB T cells by host APC, rather than neonatal tolerance (21) or a specific cellular or cytokine deficit.

Materials and Methods

Cells and culture

All media components were supplied by Life Technologies (Paisley, U.K.) unless stated otherwise. All cytokines were obtained from R&D Systems Europe (Oxon, U.K.). Other chemicals were obtained from Sigma-Aldrich (Poole, U.K.).

T cell isolation and purification

Adult buffy coats were obtained from healthy volunteers. Umbilical CB from full-term neonates was supplied by the London Cord Blood Bank (following local Ethical Committee approval). CD3+ T cells were purified by negative selection (StemSep, Vancouver, Canada) from cryopreserved Ficoll Hypaque-separated peripheral blood and umbilical CB mononuclear cell samples. Purified T cells were routinely >95% CD3+.

MDDC

Adult peripheral blood monocytes were enriched by negative selection (StemSep) and differentiated to mature DCs as previously described (22). Briefly, 90% pure CD14+ monocytes were cultured at a density of 2 × 10^6/ml in 24-well plates (Corning Costar, High Wycombe, U.K.) for 7 days in media (RPMI 1640 medium containing 10% FCS, 250 μg/ml penicillin and streptomycin, and 25 mM HEPES) plus GM-CSF (70 ng/ml) and IL-4 (35 ng/ml). At day 7, nonadherent cells were removed by moderate pipetting and added to new wells with TNF-α (50 ng/ml). Fresh media and cytokines were added every 2 days. DCs were harvested on day 9 or 10.

Mixed lymphocyte DC reactions (MLDR)

CB or AB T cells were cultured with gamma-irradiated (25Gy) allogeneic MDDC at a ratio of 20:1 in complete media (RPMI 1640 medium containing 10% human AB serum, 2 mM l-glutamine, and 25 mM HEPES). Cell proliferation was assessed using the 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE)-based flow cytometry assay (23). Quantification of dividing T cells was achieved by membrane labeling before culture with CFSE (Molecular Probes Europe, Leiden, The Netherlands). Briefly, 5 × 10^3 per ml aliquots of isolated T cells resuspended in PBS were incubated at room temperature with an equal volume of 2.5 μM CFSE (a concentration that maximized intense fluorescence without compromising viability). After 5 min of gentle mixing unbound dye was washed twice in PBS before use in culture. Cell proliferation was indicated by the sequential loss of CFSE fluorescence upon cell division.

Flow cytometric analysis of CD154 expression

Alloantigen induced CD154 expression by CD3+, and CD4+ and CD8 subsets of CFSE-labeled CB and AB T cells was assessed by three-color flow cytometry. Briefly, aliquots of cells were stained for 30 min on ice with pretitrated aliquots of anti-CD154-PE (Coulter-Immunotech, Luton, U.K.) and anti-CD3-Cy-5 or anti-CD4-Cy-5 (Dako, Ely, U.K.) Conjugated isotype control Abs (Dako, Ely, U.K.) were used to determine the specificity of staining. Flow cytometry was performed with a Becton Dickinson FACSort cytometer using standard CellQuest acquisition software (Becton Dickinson, Mountain View, CA). All samples were gated using forward and side scatter to exclude dead cells. Fluorescence compensation was adjusted using samples of PBMC single stained with anti-CD4 Abs conjugated with each fluorochrome. FACS data was analyzed by WinMDI (Shareware from J. Trotter, The Scripps Research Institute, La Jolla, CA).

Analysis of alloantigen-stimulated cytokine secretion

Supernatants from CB and AB T cells either unstimulated or stimulated by PHA or MLDCR were collected after 5 days and analyzed for IL-2 and TNF-α production by bioassay (using the CTLL-2 and KHY-M1 cell lines, respectively), as previously described (24, 25). Production of IFN-γ was measured by ELISA (BD Pharmingen, Oxford, U.K.). The relative ability of alloantigen-stimulated dividing and nondividing CB and AB T cells to produce type 1 cytokines was assessed by intracellular cytokine analysis of restimulated CFSE-labeled cells. Briefly, after 5 days culture, aliquots of 1 × 10^6 T cells were washed and cultured overnight in fresh complete media before stimulation for 6 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (1 μg/ml). To distinguish responder T cells from stimulator DCs, harvested T cells were washed and stained with anti-CD3-Cy5 before fixation and permeabilization with Permeafix (Ortho Clinical Diagnostics, Amersham, U.K.) and staining with 100-ng aliquots of PE-labeled Abs to TNF-α, IFN-γ, and IL-2 (PharMingen) or an equivalent amount of isotype control Ab. To assess cytokine production by CD8+ T cells, samples were stained with anti-CD8-Cy5 instead of anti-CD3-Cy5.

Statistical analysis

Comparisons between groups was analyzed by Mann-Whitney Rank Sum test for nonparametric data and by the unpaired t test for parametric data. A value of p < 0.05 was considered significant.

Results

Alloantigen induced T cell proliferation

The responsiveness of CFSE-labeled CB and AB T cells to alloantigen presented by MDDC was measured by flow cytometry after 5 days of stimulation (Fig. 1). Cells that have divided sequentially lost CFSE fluorescence directly in relation to the number of cell divisions undergone (Fig. 1, a and b). Cells that have not divided retained high CFSE fluorescence (Fig. 1, c and e). The results in Fig. 1 reflect the consistent difference between AB and CB in the proportion of T cells that were alloantigen responsive. CB MLDR cultures had twice the proportion of dividing cells compared with AB (66 ± 8% and 29 ± 14%, respectively; p < 0.001, n = 9). After 5 days of stimulation by MDDC, AB and CB T cells had similar levels of viability (as defined by forward and side light scatter characteristics of viable cells) (Fig. 1, d and f). While AB T cells cultured in media alone for 5 days showed little loss of viability (Fig. 1c), unstimulated CB T cells showed a dramatic decline in viability (Fig. 1e; a range of 8–30%). Both surviving CB and AB T cells had divided a maximum of eight times during the 5-day culture period, theoretically generating 128 daughter cells from one alloantigen-responsive precursor.

Induction of T cell CD154 expression by MDDC

Previous studies have shown that T cell proliferation induced by the MLR is dependent on CD154-CD40 interactions and that CB T cells have a deficiency in expressing CD154 upon PMA and ionomycin stimulation. To assess whether CB T cells stimulated with alloantigen showed defective CD154 expression, CB and AB T cells were stimulated with allogeneic MDDC for 5 days (Fig. 2, a and c). The proportion of cells expressing CD154 was variable between individuals and was restricted to the blast CFSElow cells in both AB and CB T cell cultures. In both AB and CB T cells, the intensity of CD154 expression was reciprocal to the number of cell divisions undergone. A greater proportion of CB T cells expressed CD154 than AB T cells, but this difference was due to the greater proportion of actively dividing CB T cells (Fig. 1). Phenotypic analysis of the CFSElow populations showed that in both AB and
CB T cell cultures CD154 was expressed by a similar proportion of dividing CD4 \(^+\) T cells (61 ± 6 % and 54 ± 17 %, respectively, \(n = 5\)) and CD4 \(^-\) T cells (23 ± 9 % and 16 ± 6 %, respectively) (Fig. 2, e and g). However, there was no difference in the intensity of surface CD154 expression by allogeneically stimulated CB and AB T cells. In some experiments, anti-CD40 was added to the MLDCR to prevent CD40-mediated CD154 down-regulation and thus obtain a clearer determination of the level of surface expression of CD154 by CB and AB T cells (Fig. 2, f and h). Anti-CD40 treatment had a slight enhancing effect on the level of viability of proliferation of both the CB and AB T cells; however, although the proportions of CD154 \(^+\) cells were marginally increased in both, the relative difference in the overall proportions of CD154 \(^+\) CB and AB T cells remained.

**Allogeneic MDDCs stimulate CB and AB T cells to make IFN-\(\gamma\), IL-2, and TNF-\(\alpha\)**

The production of IFN-\(\gamma\)-producing effectors from naive T cells is dependent on APC-derived IL-12. Because production of IL-12 by DCs is strongly induced by CD154-CD40 ligation, we investigated whether CB T cells stimulated by allogeneic MDDC produced IFN-\(\gamma\) and whether the cytokine profile generated was comparable to AB. Analysis of 5-day supernatants showed no significant difference in the levels of IFN-\(\gamma\) or TNF-\(\alpha\) generated by allostimulated CB and AB T cells (Table I). In contrast, the level of IL-2 in CB MLDCR supernatants was significantly less than that of AB T cells (as previously described). In comparison, mitogenic stimulation of unprimed T cells with PHA induced high levels of IFN-\(\gamma\) production by AB T cells but none by CB T cells. There was no significant difference in the amount of IL-2 or TNF-\(\alpha\) detected. Furthermore, no evidence of IL-4 production was found in either MDDC- or PHA-stimulated CB or AB T cell supernatants (data not shown).

Dividing MDDC-stimulated CB T cells but not resting CB T cells can produce IFN-\(\gamma\) on restimulation

The observation that MDDC-stimulated CB T cells secreted similar levels of IFN-\(\gamma\) to AB T cells, and that PHA-stimulated CB T

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**FIGURE 1.** AB T cells (a) and CB T cells (b) strongly proliferate to allogeneic MDDC and lose CFSE fluorescence on each cell division. Cells in media that have not divided maintained high fluorescence throughout the 5-day culture period (open histograms). AB T cells retained high viability after 5 days in complete media (c), whereas CB T cell viability was low (e). The loss of CFSE fluorescence by MDDC-stimulated AB and CB T cells (d and f, respectively) was proportional to a sequential increase in cell size. Surviving CB T cells had twice the proportion of allosreactive cells than AB T cells. This data is representative of nine pairs of MLDCRs.

**FIGURE 2.** Surface CD154 expression by AB and CB T cells after 5 days of allostimulation with MDDC. The upper panel (a–d) shows CD154 expression (y-axis) relative to loss of CFSE fluorescence (x-axis) through cell division in the presence (b and d) or absence (a and c) of anti-CD40 IgG by AB and CB T cells. The numbers shown in the upper panel indicate the percentage of CD154 \(^+\) cells as a proportion of total surviving CD3 \(^+\) T cells. The highest expression of CD154 is by T cells that have divided the most. The lower panel shows the expression of CD154 by gated dividing (CFSE\(^{low}\), gated in R1) AB (e and f) and CB (g and h) T cells relative to expression of CD4 (x-axis). The numbers shown in parentheses indicate the proportion of CD154 \(^+\) T cells in the CD4 \(^-\) (a) and CD4 \(^+\) (b) fractions. Anti-CD40 treatment increased the detection of CD154 expression by CB CD4 \(^+\) T cells but had little effect on AB T cells. Regions are drawn on the basis of background staining by isotype controls. The data shown are representative of five separate experiments.
cells produced none, suggested that the ability of CB T cells to produce IFN-γ was acquired upon cell division. To address this, the relationship between type 1 cytokine production and cell division was assessed by intracellular cytokine analysis of MDDC-stimulated CFSE-labeled T cells (Fig. 3). The overall proportion of alloantigen-stimulated CB and AB T cells secreting IFN-γ, although highly variable between individuals in both populations, was not significantly different (18.4 ± 8.3% and 24.6 ± 12.7%, respectively, n = 12). Similarly there was little difference in the proportion of CB and AB T cells producing IL-2 (38 ± 9% and 32 ± 14%, respectively, n = 6). In contrast, there was a substantial difference in the proportions of CB and AB T cells producing TNF-α (27 ± 12% and 50 ± 15%, respectively, p < 0.01, n = 7).

However, striking differences between the proportions of dividing and nondividing alloantigen-primed CB and AB T cells and cytokine secretion were observed. In CB T cells, virtually all IFN-γ, IL-2, and TNF-α production was confined to the dividing CFSElow population, whereas in AB T cells up to a third of IFN-γ production, and a half of IL-2 and TNF-α production, was from nondividing cells.

Further phenotype analysis showed that in both AB and CB T cell MLDCRs 70–80% of all IFN-γ-secreting cells (Fig. 4) were CD8±. Analysis of IFN-γ production in CD8-gated cells, relative to CFSE fluorescence, showed that nondividing CFSEhigh T cells accounted for 30% of IFN-γ-secreting cells in AB MLDCRs, but only 1–2% of CB MLDCRs. Approximately 50% of dividing CD8+ CB T cells had been primed to produce IFN-γ on restimulation by PMA and ionomycin.

### Discussion

The neonatal immune system is functionally and phenotypically naive but the immunocompetence of CB T cells is a matter of controversy. The poor anti-viral immunity of neonates and lack of ability of CB B cells to switch Ab isotype have prompted much work on the characteristics of CB T cell CD154 expression. PMA and ionomycin stimulation fails to induce high levels of CD154 expression by CB T cells (14–16), whereas OKT3 stimulation of CB T cells induces high levels of expression of functional CD154 (16). Using newly available conjugated anti-CD154 Abs on isolated T cells, we have recently found that cryopreserved CB CD4+ T cells can express very high levels of CD154 on PMA and ionomycin stimulation, but only at high doses of ionomycin (N. C. Matthews, M. Wadhwa, C. Bird, F. E. Borras, and C. V. Navarrete, manuscript in preparation). These data suggest that CB T cell CD154 expression is dependent on the mode as well as the strength of activation, rather than a particular intrinsic defect. However, to indirectly address the question as to whether donor CB T cells can express CD154 in vivo, we have used alloantigen presented by MDDC. New findings indicating that host APC initiate GVHD (26) suggest that this experimental model is physiologically far more relevant to understanding how CB T cells might respond to alloantigen in the initiation of GVHD.

CB T cells, being predominantly naive, are optimally stimulated by DCs in a primary immune response because only DCs express the high levels of adhesion, costimulatory, and Ag-presenting molecules needed to initiate a naive T cell immune response (19, 20).

![FIGURE 3](http://www.jimmunol.org/) The frequency of naive CB and AB T cells primed by allogeneic MDDC to secrete type 1 cytokines (TNF-α, IL-2, and IFN-γ) on restimulation was assessed by intracellular cytokine analysis. Cells were stained with anti-CD3-FITC, fixed and permeabilized, and stained with PE-labeled anti-IFN-γ, anti-IL-2, and anti-TNF-α mAbs. The two-dimensional histograms show the relationship of cytokine production by AB (upper panel) and CB T cells (lower panel) to the loss of CFSE fluorescence through cell division. The numbers in the upper left and upper right quadrant denote the proportion of CB and AB cytokine producers (cells that have divided at least once and cells that have not divided at all, respectively). The number in the lower left quadrant shows the proportion of cells that have divided but have not produced the respective cytokine.

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**Table I. Production of cytokines by CB and AB CD3+ T cells either unstimulated or stimulated by 1% PHA or allogeneic MDDC for 5 days**

<table>
<thead>
<tr>
<th></th>
<th>IL-2 (IU/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ PHA</td>
<td>0.2 ± 0.4</td>
<td>0</td>
<td>0.7 ± 0.9</td>
</tr>
<tr>
<td>Adult</td>
<td>1.0 ± 2.1</td>
<td>620 ± 988</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>+ MDDC</td>
<td>0.7 ± 1.0</td>
<td>191 ± 166</td>
<td>2.0 ± 2.1</td>
</tr>
<tr>
<td>Adult</td>
<td>4.6 ± 3.9</td>
<td>257 ± 130</td>
<td>1.9 ± 2.4</td>
</tr>
</tbody>
</table>

*Results shown are the mean and SD of eight samples (except CB T cells stimulated with PHA; n = 7). Alloantigen-stimulated CB T cells generated significantly less IL-2 than AB T cells (*, p < 0.03) and produced no detectable IFN-γ on PHA stimulation.*

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**Figure 3.** The frequency of naive CB and AB T cells primed by allogeneic MDDC to secrete type 1 cytokines (TNF-α, IL-2, and IFN-γ) on restimulation was assessed by intracellular cytokine analysis. Cells were stained with anti-CD3-FITC, fixed and permeabilized, and stained with PE-labeled anti-IFN-γ, anti-IL-2, and anti-TNF-α mAbs. The two-dimensional histograms show the relationship of cytokine production by AB (upper panel) and CB T cells (lower panel) to the loss of CFSE fluorescence through cell division. The numbers in the upper left and upper right quadrant denote the proportion of CB and AB cytokine producers (cells that have divided at least once and cells that have not divided at all, respectively). The number in the lower left quadrant shows the proportion of cells that have divided but have not produced the respective cytokine.
Previous studies of CB immune responsiveness show a normal MLR but no cytotoxic T cell induction (5, 6). If naive T cells can only be optimally activated by DCs, we hypothesized that the lack of Th1 and cytotoxic effectors could be due to the lack of DCs in the stimulator population and that, if stimulated with mature DCs, CB T cells would express functional CD154, which would prime DCs to give cytotoxic T cell help (17, 18).

The results above show that CB T cells responded strongly to allogeneic MDDC stimulation and generated a higher proportion of dividing cells than AB T cells. CB T cells have a characteristic feature that unless they are stimulated or rescued by anti-apoptotic cytokines (27) they die by spontaneous apoptosis within 4–5 days. The low level of cell death of allostimulated dividing CB T cells, relative to the high level of cell death of CB T cells cultured in media alone, illustrates the rescue effect of allostimulation on CB T cell survival in vitro. CB T cells either made sufficient levels of cytokines or induced the secretion of anti-apoptotic or growth-promoting cytokines by DCs.

After 3 days of MDDC stimulation, it was noted that CD154 could be detected on large dividing T cell blasts but not on resting T cells. After 5 days of MDDC stimulation, and with the expansion of proliferating T cell blasts, it was possible to study the CD154 expression by alloantigen-responsive AB and CB T cells. Although a greater proportion of CB T cells expressed CD154 overall, this was because CB MLDCR had twice the proportion of dividing cells than AB MLDCRs. There was no substantial difference between CB T cells and AB T cells in the proportion of dividing CFSElow cells expressing CD154. The predicted effect of CD154 expression in this model would be to rescue DCs from apoptosis, further enhance stimulatory capacity, and induce the production of proinflammatory cytokines (28). Crucially, CD40 ligation stimulates the secretion of IL-12, which induces IFN-γ production and promotes Th1 T cell and cytotoxic T cell differentiation (11, 29). In feedback, IL-12 up-regulates and sustains CD154 expression (30, 31). CD154 is an early activation gene, detectable an hour after PMA and ionomycin stimulation and maximal after 8 h (32). The fact that CD154 was still detectable in our system after 96–120 h suggests that the surface expression of CD154 can be sustained by AB and CB T cells through continual stimulation by DCs (33), which are reciprocally costimulated through CD40 and CD80 by alloresponsive T cells. This would delay the death of both DCs and activated T cell effectors and promote rapid T cell clonal expansion (34).

Our data are consistent with studies where primary naive CD4+ T cells from either CB or AB both expressed low levels of CD154 on stimulation with allogeneic mature Langerhans cells and staphylococcal superantigen. The detection of CD154 was dramatically enhanced by the addition of anti-CD40, because the intensity of expression of CD154 by activated T cells is dependent on the rate of production, down-regulation, and internalization of CD154 elicited by CD40 binding (33). The relatively low intensity of CD154 (compared with PMA plus ionomycin stimulation) measured on CB and AB T cells could be an indication of ongoing CD154 down-regulation by CD40 ligation, or a reflection of the much lower precursor frequency of alloreactive T cells compared with the combination of allostrogen- and superantigen-responsive cells. The addition of anti-CD40 increased the detection of CD154-positive T cells in both the AB and CB MLDCR but had little effect on the intensity of detectable surface CD154 expression. Furthermore, our data are consistent with recent reports indicating that 60% of allospecific CD4+ T cell lines express CD154 on restimulation with allogeneic APC (35). An unexpected finding was that up to a third of AB and a fifth of CB CFSElow CD4 T cells also expressed CD154. A subset of CD8 T cells and γδ T cells express CD154 on stimulation (8, 9) and can induce isotype switching in B cells, but these cells have the characteristics of Th2 T cells (36) and no IL-4 was found in this system.

Priming of CB and AB T cells with allogeneic MDDC induced the endogenous secretion of type 1 cytokines. In this system, all cytokine production is endogenous. Analysis of CB and AB supernatants generated during MDDC priming showed moderate levels of TNF-α and IFN-γ and a significant difference in the level of IL-2. This might reflect a lack of IL-2 production by CB T cells or a greater rate of IL-2 usage by proliferating CB T cells than AB T cells. The disparity between the high proportion of CB T cells primed to make IL-2 on restimulation, and the amount of IL-2 present in the supernatant after 5 days, is most likely due to the fact that two different systems of IL-2 detection were used. The IL-2 present in the MLDCR supernatant is the sum of alloantigen-induced production minus consumption because the CTL assay employed detects only bioactive IL-2 whereas the intracellular staining indicates the proportion of cells that produce and accumulate IL-2 protein intracellularly on restimulation by PMA plus ionomycin. We favor the explanation that CB MLDCR supernatants probably have low amounts of detectable IL-2 because CB T cells are highly activated (high levels of CD25), strongly proliferating, and therefore have a greater level of IL-2 consumption than AB T cells. However, to clearly state how dependent CB T cells are on endogenous IL-2 for cell growth, additional experiments are required. Highly purified CB CD45RA CD4+ T cells have been reported to produce low levels of IL-2 and express low levels of CD25 in response to anti-CD2 and anti-CD28 stimulation (37). In contrast, the combination of anti-CD3 and anti-CD28 induces the production of high levels of IL-2 (38). The level and duration of IL-2 production by MDDC-stimulated CB T cells would have a critical limiting effect on clonal expansion in acute GVHD, particularly of CD8+ CTL. These effectors are dependent on IL-2, which can only be provided by CD154+ alloreactive CD4+ T cells (39). A deficiency of CD154 leads to an abortive CD8+ CTL alloresponse (39). Data from intracellular staining showed that virtually all IL-2, IFN-γ, and TNF-α production by
CB T cells was restricted to the dividing blast population. This suggests that CB T cells in an MLDCR have to proliferate to generate effectors that produce proinflammatory cytokines. After 5 days of stimulation, about half of CB effectors are primed to produce IL-2, and a third produce IFN-γ and TNF-α on restimulation. Further analysis showed that three-quarters of CB T cells primed to produce IFN-γ on restimulation were CD8+ CD45RA+ CD27+. Cord and adult naive CD8 T cells are CD45RA+ CD27+, produce very little IFN-γ before priming, and have very little cytolytic ability. In our system, CB T cells generated as much IFN-γ in the MLDCR as AB T cells. Although no specific cytotoxicity assays were conducted, this induction of high IFN-γ production by CB CD8+ T cells is strongly suggestive of the generation of the CD45RA+ CD27+ effector CTL subset, which has potent cytotoxic function and possesses high levels of perforin and granzyme B (40). How long this type 1 pattern of cytokine production can be sustained is unclear and will be the subject of future experimentation.

The expression of CD154, and other TNF-related molecules, is dependent on intracellular calcium mobilization (41, 42). However, the high dose of ionomycin needed to induce CD154 expression by CB T cells (N. C. Matthews, M. Wadhw, C. Bird, F. E. Borras, and C. V. Navarrete, manuscript in preparation) suggests that a higher level of calcium flux is needed in CB T cells for complete TCR-mediated cell activation, which although provided by DCs would probably not be provided by less potent APC (43, 44). If CB T cells are not activated or receive exogenous anti-apoptotic cytokines, they apoptose (27). It is of note that patients with CB transplantation had a greater proportion of apoptotic T cells in the peripheral blood than patients who had received bone marrow or mobilized peripheral blood stem cells (45) and that although CB transplantation has a lower incidence of severe GVHD, it is not absent and can be fatal (3). If the level of proinflammatory cytokines generated by CD8+ CB T cells stimulated by allogeneic MDDC in vitro was replicated and sustained in vivo, this could potentially initiate GVHD. Our data suggest that donor CB T cells that encounter alloantigen presented by mature host DCs in vivo would be strongly activated and could provide sufficient CD154:CD40 costimulation for APC priming of naive cytotoxic T cells and for enhancing T cell clonal expansion. Additional experiments are now underway to assess the cytotoxic activity of these effectors. As the incidence of severe GVHD following CB transplantation is low, this would indicate that donor CB T cells either fail to activate and die or that host APC are lacking in stimulatory capacity and fail to prime naive cytotoxic T cells. Because MDDC-stimulated CB T cells have some of the functional characteristics of mature T cells, the reduced GVHD observed following CB transplantation could be the result of the failure of host APCs to strongly stimulate CB T cells in vivo rather than to an innate inability of CB T cells to effectively respond to alloantigenic stimulation.

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References


