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CD38 Stimulation Lowers the Activation Threshold and Enhances the Alloreactivity of Cord Blood T Cells by Activating the Phosphatidylinositol 3-Kinase Pathway and Inducing CD73 Expression¹

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We have recently described in cord blood T cells (CBTC) a novel pathway linking CD38 and CD73, two signal transducers with ecto-enzyme activity. The aim of this study was 2-fold: first, to characterize the mechanisms by which CD38 regulates CD73 expression; and second, to determine whether surface-induced CD73 modulates CBTC responses. A marked increase in CD73 expression was observed in CD38⁺ cells after incubation with the appropriate CD38 mAbs. The induction of CD73 was blocked by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3-K). CD38 stimulation induced tyrosine phosphorylation of the p85 regulatory subunit of PI3-K and its association with other tyrosine-phosphorylated proteins. Surface-induced CD73 was as efficient in delivering activatory signals as the CD73 constitutively expressed on adult T cells. Highly CBTC, totally unresponsive to mitogenic concentrations of plastic-immobilized CD3 mAb, proliferated vigorously when exposed to the combination of plastic-immobilized CD3 and CD73 mAbs. The reactivity to allogeneic irradiated PBMC was also significantly enhanced by CD38 stimulation and was dependent on CD73 expression. Thus, CD38 stimulation lowers the activation threshold of CBTC by the CD3/TCR complex and enhances their reactivity to allogeneic cells via activation of the PI3-kinase pathway and CD73 expression. The Journal of Immunology, 1999, 162: 6238–6246.

C ord blood is gaining increasing attention in the setting of allogeneic transplantation due to its favorable hematopoietic progenitor cell potential and immune competence status (1). The lower risk of graft-vs-host-disease associated with its use has been attributed to the immature phenotype of its T and B cells (2–4); the lower density of adhesion molecules and cytokine receptors (4–7); the low production of cytokines such as IFN-γ, IL-4, IL-12, and IL-15 (8–12); and the decreased ability to generate alloreactive CTL, LAK cells, and NK activity (3, 8–10, 13, 14). However, cord blood lymphocytes are not immunologically frozen, and their immune reactivity is susceptible to modulation. For instance, exogenous cytokines, such IL-2, IL-12, and IL-15, rapidly reverse the cytotoxic defect of cord blood T and NK cells and turn them into potent effector cells (9–11, 14).

A distinct feature of these lymphocytes is their uniformly high expression of CD38 (2, 15). CD38 is a 46-kDa type II transmembrane glycoprotein with multicatalytic ecto-enzyme activities (NAD⁺ glycohydrolase, ADP-ribosyl cyclase, and cyclic ADP-ribose hydrolase) and signaling capacity in T and B cells (15–17). We have recently shown that its ligation specifically induces the expression of CD73 in cord blood T cells (CBTC)⁴ (18). CD73 is another cell surface differentiation Ag with ecto-enzyme activity (ecto-5’-nucleotidase, EC 3.1.3.5) and signaling ability (19, 20). It is involved in the generation of alloreactive CTL (21) and has agonistic activity toward CD3- and CD2-induced T cell activation (22, 23). Studies in B cells have been less extensive, but the clinical and experimental evidence correlates CD73 expression with functional maturation and IgG production (24–26). In CBTC, CD38 cross-linking induces a rapid export to the cell surface of preformed CD73 derived from an intracellular pool. This translocation is transient and lasts approximately 8 h, after which CD73 is removed from the cell surface by enzymatic cleavage (18). The aim of this study was 2-fold: first, to investigate the mechanisms by which CD38 regulates CD73 expression in cord blood lymphocytes; and second, to determine whether surface-induced CD73 regulates CBTC responses.

Materials and Methods

Abs and reagents

The production and characterization of the mAbs 1E9 (CD73, IgG3), 7G2 (CD73, IgG2a), B6 (CD38, IgG2b), and IB4 (CD38, IgG2a) have been previously described in detail (17, 27). Additional mAbs used for cell stimulation and phenotyping included T16 (CD38, IgG1; Immunotech, Marseille, France); AD2 (CD73, IgG1) and HB7 (CD38, IgG1); gifts from Dr. M. Cooper, University of Alabama, Birmingham, AL; OKT3 (CD3, IgG2a; American Type Culture Collection, Manassas, VA); T11₂ (CD2, 4 Abbreviations used in this paper: CBTC, cord blood T cells; mIg, mouse Ig; WN, wortmannin; PI-PLC, phosphatidylinositol-specific phospholipase C; CBMC, cord blood mononuclear cells; PI3-K, phosphatidylinositol 3-kinase; pTyr, phosphoryl-portion; GPL, glycosyl phosphatidylinositol.

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IgG2) and T11, (CD2, IgG3; gifts from Dr. E. Reinherz, Dana-Farber Cancer Center, Boston, MA); and OKT10 (CD38, IgG1; Ortho Diagnostic Systems, Milan, Italy). The mAbs used for cell separation and cell population phenotyping included Leu 4 (CD3, IgG1), Leu M3 (CD14, IgG2b), Leu 11a (CD16, IgG1), Leu 17 (CD38, IgG1), and Leu 19 (CD56, IgG1; Becton Dickinson Italia, Milan, Italy) and isotype-specific anti-mlg Abs (FITC-conjugated goat anti-mlgG3, anti-mlgG2a, anti-mlgG1; Caltag, Burlingame, CA).

Stock solutions of PMA, monensin, and wortmannin (WN; Sigma, Milan, Italy) were prepared in DMSO and were stored in small aliquots at −70°C until use. PHA, brefeldin A, and 1,10-phenanthroline were obtained from Sigma. Phosphatidylinositol-specific phospholipase C (PI-PLC) derived from Bacillus thuringiensis (gift from Dr. M. Low, Columbia University, New York, NY) was stored undiluted at −20°C until use.

Cell preparation

Cord blood was collected from the umbilical vein of neonates with a gestational age of 38–40 wk immediately after the termination of uncomplicated pregnancies. The neonates and their mothers had no signs of infection. Cord blood mononuclear cells (CBMC) were isolated by density gradient centrifugation and used, when indicated, without further purification. Purified CBTC were obtained by removal of monocytes by the plastic adherence method followed by carbonyl-iron treatment and further depletion with the lysosomotropic compound l-leucine methyl ester (22). T cells were then isolated by negative selection with CD19, CD16, and CD14 mAbs (Caltag) and Dynabeads magnetic particles conjugated with sheep anti-mlgG (Dynal, Oslo, Norway) or using an indirect panning technique with CD19, CD14, and CD56 mAbs and rabbit anti-mlgs (Dako, Copenhagen, Denmark) (23). The CBTC did not react to plastic-immobilized OKT3 mAb and PHA (see below). PBMC were obtained by density gradient centrifugation of peripheral blood from normal volunteers, processed by the local blood bank, and were used as allogeneic stimulators in MLR.

Cell stimulation with CD38 mAbs

Standard culture conditions were 1 × 10^6 cells/ml in complete medium for 2 h at 37°C in a humidified atmosphere of 5% CO2 in air. Cultures were supplemented with CD38 mAbs and reagents at various concentrations as indicated. Time-course experiments were performed as indicated in Results.

In some experiments CD37 was removed by PI-PLC (28). Briefly, CBMC were incubated for 1 h at 37°C in complete medium without FCS in the presence of PI-PLC at 0.3 U/ml, washed twice in RPMI and 10% FCS, and then cytofluorometrically analyzed for CD73 expression. Control cells were untreated and were washed twice before subsequent analysis. This treatment did not affect cell viability.

Flow cytometry

Cell surface Ags were detected by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). Direct staining was performed with FITC- or PE-conjugated mAbs when commercially available. To detect CD37 expression, indirect staining was performed with isotype-specific anti-mlg Abs from Caltag. The I9 mAb (IgG3) or 7G2 mAb (IgG2a) followed by the appropriate FITC-conjugated isotype-specific anti-mlg Abs were used to detect CD37 expression after incubation with HB7 (IgG1) or T16 (IgG1) mAbs. I9 mAb (IgG3) or AD2 mAb (IgG1) followed by the appropriate FITC-conjugated isotype-specific anti-mlg were used after incubation with Ib6 (IgG2b). No cross-reaction between CD38 and CD37 mAbs was observed, except when 7G2 mAb (IgG2a) and FITC-conjugated goat anti-mlgG2a were used to detect CD37 after incubation with Ib6 mAb (IgG2b). Five thousand events were accumulated and analyzed for each fluorescence. FITC- and PE-conjugated mouse myeloma proteins of the appropriate subclasses were used as negative controls.

Analysis of phosphatidylinositol 3-kinase (PI3-K) activation by immunoprecipitation, SDS-PAGE, and Western blotting

CBTC were resuspended in IMDM and were incubated for 30 min on ice in the presence of 80 nM WN or DMSO alone. After washing, incubation with T16 mAb was conducted at a concentration of 8 µg/10^6 cells for 10 min on ice. After removing the residual unbound mAbs by washing in cold IMDM, incubation with a F(ab')2, fraction of a goat anti-mlgG (3 µg/10^6 cells; Promega, Madison, WI) was conducted for 10 min on ice. Signals were then implemented by incubation at 37°C for 2.5 min, immediately followed by lysis with 1% Nonidet P-40 lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1 mM EGTA, 50 µM phenylarsine oxide, 10 mM iodoacetamide, 1 mM PMSF, and 2 µg/ml of antipain, chymostatin, leupeptin, and pepstatin) (29). After centrifugation, lysates were precleared by incubation with recombinant protein A-Sepharose beads (Repligen, Cambridge, MA) for 1 h and then supplemented with anti-p85 mAb (Transduction Laboratories, Lexington, KY) and recombinant protein A-Sepharose for overnight incubation. After extensive washings, immune complexes were eluted by boiling in reducing sample buffer and were run on 8% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes using a semidy transfer apparatus (Hoefer, San Francisco, CA) in Tris-glycine buffer containing 20% methanol. Filters were incubated in 1% BSA, probed with RC20 anti-pTyR mAb (Transduction Laboratories) and HRP-conjugated anti-mlgG (Promega) and were developed using enhanced chemiluminescence reagents (Amer sham, Aylesbury, U.K.). After stripping, membranes were blocked in 4% nonfat dry milk and reprobed with anti-p85 mAb and HRP-conjugated anti-mlgG.

Adherence of mAb to plastic

mAbs were bound to plastic as previously reported (22, 23). Briefly, they were diluted in PBS, and 200 µl of the appropriate concentration was dispensed into wells of 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). After 2-h incubation at room temperature, wells were washed twice with 200 µl of PBS. Residual binding sites were saturated by 1-h incubation at room temperature with 200 µl of complete medium.

Cell proliferation

CBMC or CBTC (2 × 10^5) were cultured in a final volume of 200 µl of flat-bottom microtiter plate (Costar) in triplicate wells in complete medium at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were stimulated with the appropriate mAbs and/or reagents as indicated in Results. Cultures were pulsed with 1 µCi of [3H]TdR (47 Ci/mmol; Amersham, Milan, Italy) and were harvested 4 h later with a multiple semiautomated sample harvester. The filters were counted in a liquid scintillation counter.

Mixed lymphocyte cultures

MLC were established in flat-bottom 24-well plates with CBMC or CBTC as responder cells mixed with irradiated (3000 rad) allogeneic PBMC (1/1) as stimulator cells at a final concentration of 1 × 10^6/ml in complete medium. MLC were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cell proliferation was evaluated by pulsing 200 µl of cells with 1 µCi of [3H]TdR (47 Ci/mmol; Amersham, Milan, Italy) and harvesting 4 h later with a semiautomated sample harvester.

Results

Induction of CD73 expression by CD38 ligation

On the average, freshly isolated, resting CBMC contained high and low proportions of CD38+ and CD37+ cells, respectively (CD38, 88 ± 20%; CD37, 10 ± 9%). CD33 and CD56+ cells accounted for >80% of CD38+ cells (CD33+, 54 ± 21%; CD56+, 27 ± 11%), whereas CD19+ and CD14+ cells made up only 12 ± 7 and 5 ± 2%, respectively. The large majority of CD33+ and CD56+ cells were CD38+ (CD33+ cells, 85 ± 10%; CD56+ cells, 92 ± 5%), whereas only a minority were CD37+ (CD33+ cells, 8 ± 10%; CD56+ cells, 5 ± 3%). We have previously reported that CD38 stimulation with appropriate mAbs induces CD37 expression in cord blood T cells and NK cells (18). As expected, a marked increase in the percentage of CD37+ cells was observed in CBMC (79 ± 21%) or purified CBTC (80 ± 12%) upon incubation for 2 h with 10 µg/ml soluble T16 mAb or, alternately, with 10 µg/ml soluble Ib6 mAb or 10 µg/ml soluble HB7 mAb (CD38). Depending on the isotype of CD38 mAbs, CD37 expression was detected with the I9, 7G2, or AD2 mAbs followed by the appropriate isotype-specific FITC- or PE-conjugated goat anti-mlgs (data not shown). The OKT10 mAb did not induce any CD37 expression either in the soluble form or after cross-linking as previously reported (18). Hereafter, soluble T16 mAb at 10 µg/ml was used as the standard reagent to ligate CD37, while I9 and FITC-conjugated goat anti-mlgG3 were used as the standard reagents for cytofluorimetric analyses. CD37 was detectable on the cell surface after 20 min and remained uniformly up-regulated for 6 h as previously reported in

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Role of PI3-K in the induction of CD73 expression

The next issue concerned the role of PI3-K in the kinetics of CD73 expression. PI3-K has a central role in the signaling events occurring in immature B cells after CD38 ligation (30, 31). Indeed, WN inhibits the protein serine/lipid kinase activity of the p85 catalytic subunit of PI3-K (32). WN completely blocked the induction of CD73 expression when added before or together with T16 mAb, but not when it was added 10 min after CBMC had been exposed to T16 mAb (data not shown).

We have recently shown in Jurkat cells that the disappearance of CD73 is prevented by 1,10-phenanthroline, an effective inhibitor of metalloproteases and phospholipases specific for the GPI anchor (33, 34). CD73 was determined cytofluorometrically on CBMC after incubation for 2 h with 10 µg/ml soluble T16 mAb (CD38) alone (A) or with 20 nM WN (B), 20 µM 1,10-phenanthroline (C), 10 µM monensin (D), and 5 µg/ml brefeldin A (E). In the release phase experiments, CBMC were cultured for 4 h with 10 µg/ml soluble T16 mAb to induce full CD73 expression. At this point, different inhibitors were added, and CD73 expression was reanalyzed 8 h later. As expected, CD73 expression completely disappeared in the control cells (F) as well as in CBMC cultured with WN (G), monensin (I), and brefeldin A (J). By contrast, CD73 expression remained almost unaltered in CBMC cultured with 1,10-phenanthroline (H). Boldface histograms represent cells stained with 1E9 mAb (CD73) and FITC-conjugated goat anti-mouse IgG3; light histograms represent the same cells stained with isotype-matched control mAb and FITC-conjugated goat anti-mouse IgG3. The results are from one experiment that is representative of seven (WN) or two (monensin) experiments.

CD73 cross-linking transduces activatory signals

To examine the signaling ability of surface-induced signals, CBMC were incubated for 2 h with 10 µg/ml soluble T16 mAb, washed in medium alone, and transferred into flat-bottom microwells where three CD73 mAbs (1E9, AD2, and 7G2) or monoclonal mIgs (FLOPC-21) had previously been immobilized on plastic. CBMC were also stimulated with PHA or OKT3 mAb as a control (Fig. 3). Multivalent CD73 cross-linking with CD73 mAbs induced a vigorous CBMC proliferation, whereas plastic-immobilized FLOPC-21 had no effect. Proliferative responses to PHA and OKT3 mAb were not influenced by CD38 ligation. Kinetic analysis showed that proliferation peaked on day 3 (data not shown).

FIGURE 1. Role of PI3-K activity in the kinetics of CD73 expression induced by CD38 ligation. Effects of WN, 1,10-phenanthroline, monensin, and brefeldin A on the induction (A–E) and the release phase (F–J) of CD73 expression in CBMC. CD73 was determined cytofluorometrically on CBMC after incubation for 2 h with 10 µg/ml T16 mAb (CD38) alone (A) or with 20 nM WN (B), 20 µM 1,10-phenanthroline (C), 10 µM monensin (D), and 5 µg/ml brefeldin A (E). In the release phase experiments, CBMC were cultured for 4 h with 10 µg/ml soluble T16 mAb to induce full CD73 expression. At this point, different inhibitors were added, and CD73 expression was reanalyzed 8 h later. As expected, CD73 expression completely disappeared in the control cells (F) as well as in CBMC cultured with WN (G), monensin (I), and brefeldin A (J). By contrast, CD73 expression remained almost unaltered in CBMC cultured with 1,10-phenanthroline (H). Boldface histograms represent cells stained with 1E9 mAb (CD73) and FITC-conjugated goat anti-mouse IgG3; light histograms represent the same cells stained with isotype-matched control mAb and FITC-conjugated goat anti-mouse IgG3. The results are from one experiment that is representative of seven (WN) or two (monensin) experiments.
Extensive removal of monocytes and other FcR⁺ cells was conducted in some experiments to determine their influence on the vigorous proliferation observed in CBMC upon CD73 stimulation (Fig. 3B). These highly purified CBTC were totally unresponsive to plastic-immobilized OKT3 and PHA, indicating very effective monocyte depletion. They also became unresponsive to plastic-immobilized CD73 mAbs alone (Fig. 3B). However, when they were exposed to the combination of plastic-immobilized CD3 and

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**FIGURE 2.** Tyrosine phosphorylation of the p85 regulatory subunit upon CD38 ligation in purified CBTC. Highly purified CBTC were incubated in medium alone (NS lane) or were stimulated with T16 mAb (CD38) in the absence (T16 lane) or the presence (T16 + WN lane) of WN (see Materials and Methods). Cell lysates were prepared, and p85 immunoprecipitates or whole cell lysates were probed with anti-pTyr mAb (A) and then stripped and reprobed with anti-p85 mAb (B). The arrow points to the band identified as the p85 subunit of PI3-K. A p85⁻ control lysate is also included in B.

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**FIGURE 3.** Proliferation of T16-treated CBMC and CBTC induced by CD73 cross-linking. A, CBMC were treated with 10 μg/ml soluble T16 mAb (CD38) for 2 h in complete medium. CBMC (2 × 10⁵) were washed and stimulated with three plastic-immobilized CD73 mAbs (1E9, 7G2, and AD2), mouse myeloma protein (FLOPC-21), 5 μg/ml of PHA, or plastic-immobilized OKT3 mAb (CD3; solid bars). Untreated CBMC were plated under the same culture conditions as controls (open bars). After 3 days of incubation, CBMC were pulsed with 1 μCi of [³H]TdR/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from 16 (1E9 mAb) or two (FLOPC-21) experiments, each performed in triplicate. B, Highly purified CBTC were incubated with 10 μg/ml soluble T16 mAb (CD38) for 2 h in complete medium (solid bars). CBTC cells (2 × 10⁵) were washed and stimulated with three plastic-immobilized CD73 mAbs (1E9, 7G2, and AD2), 5 μg/ml of PHA, plastic-immobilized OKT3 mAb (CD3), or the combination of plastic-immobilized OKT3 (CD3) and 1E9 (CD73) mAbs. Untreated cells were plated under the same culture conditions as controls (open bars). After 3 days of incubation, CBTC were pulsed with 1 μCi of [³H]TdR/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from three experiments, each performed in triplicate.
FIGURE 4. CD38 stimulation enhances the alloreactivity of CBMC and CBTC. A, CBMC were incubated for 2 h with the following reagents: complete medium, 10 μg/ml soluble T16 mAb (CD38), 10 μg/ml soluble IB4 mAb (CD38), 10 μg/ml soluble OKT10 mAb (CD38), and 10 μg/ml soluble mIgs. CBMC were washed, and MLC were established with irradiated allogeneic PBMC (3000 rad) at a responder to stimulator cell ratio of 1:1 (solid bars). Controls were not mixed with irradiated allogeneic PBMC (open bars). Cultures were incubated at 37°C in 5% CO2 for 3 days. Triplicate wells were pulsed with 1 μCi of [3H]TdR/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from one experiment that is representative of seven (T16 mAb) or two (IB4 mAb) experiments, each performed in triplicate. B, Highly purified CBTC were incubated for 2 h in complete medium or in the presence of 10 μg/ml soluble T16 mAb (CD38). MLC were established with irradiated allogeneic PBMC (3000 rad) at a responder to stimulator cell ratio of 1:1 (solid bars). MLC conditions were T16-treated CBTC and allogeneic PBMC (T16); T16-treated CBTC, allogeneic PBMC, and mIgs (T16 + mIgs); and T16-treated CBTC mixed with allogeneic PBMC preincubated with mIgs (T16 + PBMC/mIgs). Controls were not mixed with irradiated allogeneic PBMC (open bars). Cultures were incubated at 37°C in 5% CO2 for 3 days. Triplicate wells were pulsed with 1 μCi of [3H]TdR/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from three experiments, each performed in triplicate.

Enhancement of alloreactivity by CD38 ligation

After incubation for 2 h with T16 mAb, CBMC cells were washed twice and mixed with irradiated allogeneic PBMC. Controls were incubated for 2 h with OKT10 and IB4 mAbs (that do not induce CD37 expression) and mIgs. Proliferation was determined by [3H]TdR incorporation at different time points. The results presented in Fig. 4A indicate that only T16-treated CBMC strongly reacted against allogeneic PBMC. Kinetics analysis showed that proliferation peaked on day 3 (data not shown).

To rule out the possibility that enhancement of alloreactivity was mediated by nonspecific cross-linking of T16 mAb, a series of MLC was set up in which highly purified CBTC were used as responders (Fig. 4B). These cell preparations were unresponsive to PHA and CD3 stimulation, showing that they were extensively depleted of monocytes and/or other FcR+ cells. T16 treatment greatly enhanced the alloreactivity of CBTC (Fig. 4B). To rule out the possibility that cross-linking was mediated by allogeneic PBMC used as stimulators, the latter were preincubated with mIgs before mixing with responders, or, alternatively, mIgs were added to the MLC. The enhanced alloreactivity of CBTC was not altered (Fig. 4B).

The high proliferative response of T16-treated CBTC against allogeneic PBMC was associated with the formation of large clusters of cell aggregates (Fig. 5A) undetectable in T16-untreated cells (Fig. 5B). Thus, transient CD37 expression during the recognition phase of MLC strongly amplifies the activatory signals delivered by allogeneic stimulators.

Enhancement of alloreactivity is mediated by CD37

Three lines of experiments were pursued to determine the role of CD37 in the CBMC alloreactivity triggered by T16 treatment (Fig. 6). The first strategy exploited the GPI anchor used by CD37 to bind the cell membrane. T16-treated CBMC were exposed to PI-PLC to remove CD37 from the cell surface and then were incubated with irradiated allogeneic PBMC. As a control, the effect of PI-PLC treatment was evaluated on the mitogenic effect induced by OKT3 mAb and PHA. PI-PLC completely abrogated the alloreactivity induced by T16, whereas it did not influence the responses to PHA and OKT3 mAb (data not shown). The second strategy exploited the kinetics of CD37 expression induced by CD38 ligation. CBMC were mixed with allogeneic irradiated PBMC after 12-h incubation with T16 mAb, i.e., after CD37 had completely disappeared from the surface. No alloreactivity was triggered. The third strategy was to incubate T16-treated CBMC, fully expressing CD37 on the cell surface, with different mAbs to CD37 in the soluble form. CBMC were then washed and mixed with irradiated allogeneic PBMC. The results indicate that this blockade completely prevented the triggering of alloreactivity.

As expected, large cell aggregates were observed only in T16-treated CBMC. They were not detectable in cultures containing T16-treated CBMC subsequently exposed to PI-PLC, IB4-treated CBMC, OKT10-treated CBMC, or T16-treated CBMC incubated with soluble 1E9 mAb (CD37) or soluble AD2 mAb (CD73) (data not shown).
Discussion

We have recently shown that CD38 ligation induces CD73 expression in CBTC (18). The pathway is specific, since CD73 cannot be induced by stimulation with PHA, PMA, or mAbs to CD3, CD2, and CD28. Similarly, CD38 ligation does not modify the expression of a number of other cell surface molecules (18).

The kinetics of CD73 expression paralleled those observed in Jurkat and Raji cell lines (18) and consisted of an induction phase, which occurs rapidly after CD38 ligation, and a release phase, which begins after approximately 6 h of continuous CD73 expression. We have already shown that protein tyrosine kinase activity is essential in the induction phase (18). Previous observations in normal and malignant immature B cells have shown that CD38 ligation also involves the PI3-K pathway (30, 31). PI3-K is a heterodimer consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit that possesses both protein serine and lipid kinase activity (37). The PI3-K pathway is very flexible and adaptable. It is coupled to a variety of other signal transducers and may trigger different signaling cascades. In particular, the p85 subunit acts as an adaptor-like molecule that extends signaling to other pathways quite unrelated to the catalytic activity of the p110 subunit. WN is a specific inhibitor of PI3-K when used at 2–20 nM (30–32) and, thus, can be exploited as a pharmacological probe to assess its involvement in different signaling pathways. WN blocked CD73 induction in T16-treated CBMC, suggesting that PI3-K was crucial in the signaling pathway leading from CD38 ligation to CD73 expression. CD73 induction was blocked when CBMC were exposed to WN before or during CD38 ligation, but not when WN was added as early as 10 min after CD38 ligation (data not shown). These data are consistent with the ideas that WN is an irreversible inhibitor of PI3-K (32) and that the bulk of PI3-K activation occurs within the first 10 min after CD38 ligation (30, 31). Cell viability was not affected by WN; CBMC fully expressed CD73 when WN was added after CD38 ligation, and functional data (see below) further confirmed that WN did not merely exert a general cell toxicity. Although suggestive, these data did not formally prove the involvement of PI3-K in the signaling cascade leading from CD38 stimulation to CD73 expression. Indeed, there are some limitations in this use of WN as a probe mainly because it does not affect the multiple adaptor-like functions of the p85 regulatory subunit. One mechanism for activation of PI3-K consists of tyrosine phosphorylation of the p85 subunit (37), leading to the recruitment of other tyrosine-phosphorylated proteins (30, 31).
Triplicate wells were pulsed with 1 μg/ml T16-treated CBMC incubated with 10 μg/ml CD73 mAbs (1E9, T16, 7G2) and then mixed with allogeneic PBMC (3000 rad) at a responder to stimulator cell ratio of 1:1 (solid bars). MLC conditions were T16-treated CBMC and allogeneic PBMC (T16), T16-treated CBMC incubated with PI-PLC and then mixed with allogeneic PBMC (T16 + PI-PLC), T16-treated CBMC mixed with allogeneic PBMC after CD73 had disappeared from the cell surface (T16 (after 24 h)), and T16-treated CBMC incubated with 10 μg/ml soluble CD73 mAbs (1E9, AD2, 7G2) and then mixed with allogeneic PBMC (T16 + 1E9, T16 + AD2, T16 + 7G2). Controls were not mixed with irradiated allogeneic PBMC (open bars). Cultures were incubated at 37°C in 5% CO2 for 3 days. Triplicate wells were pulsed with 1 μCi of [3H]-Tdr/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from one of two experiments, each performed in triplicate.

FIGURE 6. Enhancement of alloreactivity induced by CD38 stimulation depends on CD73 expression. CBMC were incubated for 2 h with 10 μg/ml soluble T16 mAb (CD38). After washing, CBMC were divided into aliquots, and different MLC were established with irradiated allogeneic PBMC (3000 rad) at a responder to stimulator cell ratio of 1:1 (solid bars). MLC conditions were T16-treated CBMC and allogeneic PBMC (T16), T16-treated CBMC incubated with PI-PLC and then mixed with allogeneic PBMC (T16 + PI-PLC), T16-treated CBMC mixed with allogeneic PBMC after CD73 had disappeared from the cell surface (T16 (after 24 h)), and T16-treated CBMC incubated with 10 μg/ml soluble CD73 mAbs (1E9, AD2, 7G2) and then mixed with allogeneic PBMC (T16 + 1E9, T16 + AD2, T16 + 7G2). Controls were not mixed with irradiated allogeneic PBMC (open bars). Cultures were incubated at 37°C in 5% CO2 for 3 days. Triplicate wells were pulsed with 1 μCi of [3H]-Tdr/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from one of two experiments, each performed in triplicate.

and activation of the p110 catalytic subunit. CD38 stimulation induced tyrosine phosphorylation of the p85 subunit and a number of other substrates coprecipitated by p85 mAb. Interestingly, neither CD38-induced tyrosine phosphorylation nor p85 recruitment was inhibited by WN. These data are in agreement with a previous report in progenitor B cells showing that tyrosine phosphorylation triggered by CD38 stimulation is not inhibited by WN (31). This appears to be the first demonstration that CD38 ligation directly leads to activation of PI3-K in normal T cells. These data also indicate that the induction of CD73 depends on the catalytic activity of the p110 subunit (which is indeed the target of WN) other than the regulatory activity of the p85 subunit. This finding may explain the apparent discrepancy observed between IB4 and T16 mAbs in terms of their common ability to activate the PI3-K pathway, but not to induce CD73 expression. Indeed, the agonistic IB4, T16, and HB7 mAbs all induce tyrosine phosphorylation of the p85 subunit and its association with other tyrosine-phosphorylated proteins (30, 31, 38), whereas only T16 and HB7 mAbs induce CD73 expression. IB4 mAbs may not be as efficient as the other agonistic mAbs in activating all the adaptor-like functions of p85 and extend CD38 signaling to other signal transducers, including the catalytic p110 subunit. Indeed, differences in the magnitude of tyrosine phosphorylation have been reported in THP-1 cells stimulated with HB7, T16, and IB4 mAbs, the latter being much less effective (38). In addition, the epitopes recognized by T16, HB7, and IB4 mAbs are not identical; they are discontinuous and are located not only on the carboxyl-terminal sequence of 273–285, but also on a sequence(s) starting from the amino acid residue of 219 (38). Thus, it is not surprising that different agonistic mAbs activate the same transduction pathway and share the same early signaling events, but not the same array of final events.

We have recently shown in Jurkat cells that the release phase is prevented by 1,10-phenanthroline, an effective inhibitor of metalloproteases and phospholipases, specific for the GPI anchor (33, 34). It also prevented CD73 release in T16-treated CBMC, indicating that the same mechanism is active in immature normal lymphocytes. The release phase was not inhibited by WN, even though WN has been claimed to inhibit phospholipases (39, 40). Thus, WN does not affect phospholipases at concentrations inhibitory for PI3-K, and PI3-K is not involved in the CD73 release phase. The release mechanism could involve internalization of CD73, followed by cleavage from the GPI anchor and exocytosis, as previously reported for other GPI-linked molecules such as CD14 in monocytes (41). To examine this possibility, experiments were conducted in the presence of brefeldin A and monensin. Neither inhibitor blocked CD73 release when added to CBMC after 4 h of incubation with T16 mAb. These data indicate that CD73 is released by a cleavage reaction directly from the cell surface and not in vesicular form. The former mechanism is more common among GPI-anchored proteins (42, 43).

The next issue concerned the functional competence of CD73 induced by T16 treatment. CD73 delivers potent agonist signals to mature adult peripheral blood T cells (22, 23, 44). T16-treated CBMC responded very vigorously to CD73 stimulation with three independent plastic-immobilized CD73 mAbs, whereas their reactivity to PHA, a mitogenic combination of soluble CD2 mAbs, and soluble or plastic-immobilized CD3 mAb was not modified. Thus, reactivity to CD73 stimulation was specifically related to CD73 expression and not to a general state of hyper-reactivity induced by T16 mAb. As expected, WN, by blunting CD73 induction, abrogated the ability of T16-treated CBMC to proliferate in response to plastic-immobilized CD73 mAbs. WN-treated CBMC, however, were still able to react to plastic-immobilized OKT3. These data confirmed that WN, at these concentrations, does not exert a general toxicity on CBMC, and that the CD3 signaling pathway, unlike the CD38 signaling pathway, is resistant to PI3-K inhibition (45).

A series of findings ruled out the possibility that the proliferation of T16-treated CBMC was merely due to nonspecific cross-linking of CD38 and/or CD73 mAbs. First, isotype-specific mouse Iggs (FLOPC-21) did not induce the proliferation of CBMC (even though they have surface-bound, CD73-inducing CD38 mAbs); second, proliferation was not observed after incubation with CD38 mAbs not inducing CD73 expression (these cells have surface-bound CD38 mAbs and are exposed to plastic-immobilized CD73 mAbs). The agonistic activity of CD73 was also evaluated in purified CBTC after extensive removal of monocytes and other FcR+ cells (i.e., CD19+ and CD56+ cells). CBTC were totally unresponsive to plastic-immobilized OKT3 and PHA and became unresponsive to plastic-immobilized CD73 mAbs alone as well. However, when these cells were exposed to the combination of plastic-immobilized CD3 and CD73 mAbs, a vigorous proliferation was observed in the absence of any other activatory signal. These data indicate that CD73 transduces activatory signals in CBTC and lowers their activation threshold by the CD3/TCR complex. This is the first time that such an agonistic signaling is documented as a rapid and transient event related to the de novo surface-induced CD73.

CD38 ligation significantly enhanced the ability of CBMC to proliferate in response to allogeneic PBMC in primary MLC. The alloreactive capacity of cord blood lymphocytes depends on MLC conditions (i.e., primary vs secondary or tertiary MLC) and the cells used as stimulators (i.e., tumor cell lines vs allogeneic PBMC) or responders (i.e., CBMC vs purified CBTC) (3, 8, 9, 13, 46, 47). Enhancement of alloreactivity was not mediated by nonspecific FcR-mediated cross-linking, since it was not observed in
MLC set up in the presence of mIgs or in MLC set up in the presence of CD38 mAbs that do not induce CD73 expression. Moreover, removal of CD73 by PI-PLC treatment abrogated enhancement of alloreactivity even though CBMC had potentially cross-linkable surface-bound CD38 mAbs (able to induce CD73 expression). Lastly, masking of CD73 on the cell surface by the addition of soluble CD73 mAbs also abrogated enhancement. Under these conditions, CBMC have surface-bound CD38 and CD73 mAbs (reacting with the CD73-induced molecule). Thus, they offer almost twice the amount of cross-linkable mAbs to FcγR cells present in the responders or stimulators, but enhancement was abrogated rather than further increased. Alloreactivity was also enhanced in MLC set up with highly purified CBTC as responders. These cell preparations were unresponsive to PHA and CD3 stimulation and, thus, were extensively depleted of monocytes and/or other FcγR cells. Furthermore, preincubation of allogeneic stimulators with mIgs or addition of mIgs to MLC did not alter the enhancement.

The CD73 induced on the cell surface by CD38 ligation was essential to enhance alloreactivity. In the first place, alloreactivity was not enhanced when T16-treated CBMC were incubated with PI-PLC, which removes CD73 from the cell surface by cleaving the GPI anchor (28). The effect mediated by PI-PLC was not due to a general cell toxicity, since the ability of CBMC to respond to PHA and OKT3 was unchanged. Similar data have been reported in adult peripheral blood T cells, which were still able to respond to PHA, PMA, and CD3 mAb after PI-PLC, but not to polyclonal Abs to CD73 and PMA (44). Although suggestive, these data did not formally prove the role of CD73 in the enhanced alloreactivity. PI-PLC treatment is not selective for CD73 and may have released other GPI-linked molecules. In the second place, the kinetics of CD73 expression paralleled the enhanced ability of CBMC to react to allogeneic PBMC. When T16-treated CBMC were mixed with PBMC after CD73 had disappeared, i.e., about 12 h after CD38 ligation, enhancement also disappeared. CD38 ligation has already generated a number of late signals at this stage. None of them, however, confer the enhanced ability to respond to allogeneic PBMC. Thus, enhancement of alloreactivity is an early event induced by CD38 ligation and is restricted to the few hours coincidental with CD73 expression. Another piece of evidence came from the lack of enhancement in CBMC treated with OKT10 and IB4 mAbs (CD38). Neither mAb induces CD73 expression in CBMC or in other CD3+ CD73+ cells (18), with a major difference between their signaling properties: OKT10 mAb is usually inactive, whereas IB4 mAb transduces activating signals in many experimental models (17, 38). Thus, two conditions are required to enhance alloreactivity: CD38 must be ligated by mAbs that both transduce activating signals and induce CD73 expression. Finally, enhancement was abrogated when T16-treated CBMC, fully expressing CD73 on the cell surface, were incubated with soluble mAbs to CD73.

Enhancement of alloreactivity was uniformly associated with the presence of large clusters of cell aggregates in the cultures, except when CD73 expression was absent because it was not induced or it was blocked. These data suggest that CD73 promotes cell-to-cell adhesion by interacting with a natural counter-receptor, currently unknown, expressed by PBMC. The ability to promote cellular interactions is a recognized function of CD73 in the lymphoid system. Airas et al. have shown that CD73 mediates lymphocyte adhesion to cultured endothelial cells (48) and B cell adhesion to follicular dendritic cells (49).

In conclusion, we have shown that the CD38/CD73 pathway regulates the reactivity of CBTC to CD3 stimulation and allogeneic cells. Thus, the CD38/CD73 pathway may have important implications in the development of new strategies for the regulation of graft-vs-host disease in the transplantation setting. Interestingly, it has recently been shown that WN prevents the development of alloantigen-specific T cell responses and induces an IL-2-reversible, alloantigen-specific tolerance (50).

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References


