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 Expression

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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 Expression

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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.

Cord 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 as 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 multicalytic 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) (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), Ib6 (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, IgG2a; American Type Culture Collection, Manassas, VA); T112 (CD2, IgG2a; American Type Culture Collection, Manassas, VA).

Abbreviations used in this paper: CBTC, cord blood T cells; mlg, mouse Ig; WN, wortmannin; PI-PLC, phosphatidylinositol-specific phospholipase C; CBMC, cord blood mononuclear cells; PI3-K, phosphatidylinositol 3-kinase; pTy, phosphotyrosine; GPI, glycosyl phosphatidylinositol.
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).

The cells were then washed twice with PBS, and 200 l 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 l of PBS. Residual binding sites were saturated by 1-h incubation at room temperature with 200 l of complete medium.

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 using 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-mlgGs (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 MLC.

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% CO_2 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 CD37 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 FACSscan (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 1E9 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. 1E9 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-reactivity between CD38 and CD37 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 mAb by washing in cold IMDM, incubation with a Fab’ fragment 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 Na_3VO_4, 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 preclariﬁed by incubation with recombinant protein A-Sepharose beads (Repligen, Cambridge, MA) for 1 h and then supplemented with anti-mlgG (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 (Hoeffer, San Francisco, CA) in Tris-glycine buffer containing 20% methanol. Filters were blocked in 1% nonfat dry milk and reprobed with 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-mlgG 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 flat-bottom microtiter plate (Costar) in triplicate wells in complete medium at 37°C in a humidified atmosphere of 5% CO_2 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; Amer sham, 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% CO_2 in air. Cell proliferation was evaluated by pulsing 200 μl of cells with 1 μCi of [3H]TdR (47 Ci/mmol; Amer sham, 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%). CD3^+ and CD56^+ cells accounted for >80% of CD38^+ cells (CD3^+ cells, 54 ± 21%; CD56^+ cells, 27 ± 11%), whereas CD19^+ and CD14^+ cells made up only 12 ± 7 and 5 ± 2%, respectively. The large majority of CD3^+ and CD56^+ cells were CD38^+ (CD3^+ cells, 85 ± 10%; CD56^+ cells, 92 ± 5%), whereas only a minority were CD73^+ (CD3^+ cells, 8 ± 10%; CD56^+ cells, 5 ± 3%). We have previously reported that CD38 stimulation with appropriate mAbs induces CD73 expression in cord blood T cells and NK cells (18). As expected, a marked increase in the percentage of CD73^+ 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, alternatively, 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 1E9, 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 CD73 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 1E9 and FITC-conjugated goat anti-mlgG were used as the standard reagents for cytofluorometric analyses.

CD73 was detectable on the cell surface after 20 min and remained uniformly up-regulated for 6 h as previously reported in...
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.

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). CBMC were incubated for 2 h with 10 μg/ml soluble T16 mAb in the presence of WN, a potent inhibitor of PI3-K (32). WN completely blocked the induction of CD73 expression at concentrations of 2–20 nM (Fig. 1). Cell viability was similar in WN-treated cultures and controls, as shown by the trypan blue dye test and FACS analysis of cell size and granularity (data not shown). Kinetic analysis showed that WN 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). 1,10-Phenanthroline was added to CBMC cells after 4 h of incubation, i.e., after CD73 was fully induced on the surface. CD73 disappearance was prevented, indicating that CD73 is released by enzymatic hydrolysis of the GPI anchor (Fig. 1). In selected experiments, WN, brefeldin A, an inhibitor of intracellular protein transport (35), and monensin, an inhibitor of degradation of endocytosed molecules (36), were added to CBMC after 4 h of incubation. None of these inhibitors blocked CD73 release (Fig. 1).

One mechanism for activation of PI3-K is tyrosine phosphorylation of the p85 regulatory subunit (37). To directly evaluate the role of PI3-K in the signaling cascade elicited by CD38 stimulation, highly purified CBTC were stimulated with soluble T16 mAb and immunoprecipitated with an mAb reacting with the p85 subunit, and the extent of tyrosine phosphorylation was evaluated by Western blotting (Fig. 2). A marked increase in phosphorylation was observed in several distinct substrates coprecipitated by anti-p85 mAb. WN did not influence the phosphorylation pattern induced by CD38 stimulation, as previously reported for progenitor B cells (31). Indeed, WN inhibits the protein serine/lipid kinase activity of the p110 catalytic subunit of PI3-K, i.e., downstream activation of the p85 subunit (37). Changes in the extent of phosphorylation were not as evident in total cell lysates immunoblotted with anti-pTyr mAb (Fig. 2A). Stripping and reprobing of the same filters with anti-p85 mAb confirmed that the tyrosine-phosphorylated 85-kDa protein indicated by the arrow was indeed the p85 subunit of PI3-K (Fig. 2B). The specificity of the highlighted band was confirmed by positive immunoblotting of control lysates (Fig. 2B). Together, these data showed that both the p85 regulatory subunit and the p110 catalytic subunit of PI3-K are involved in the signaling cascade leading from CD38 stimulation to CD73 expression.

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. 3A). 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).
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 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 × 10^5) 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]ThdR/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 × 10^5) 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]ThdR/well and were harvested 4 h later. Results are expressed as the mean counts per minute ± SD from three experiments, each performed in triplicate.
CD73 mAbs, they proliferated vigorously in the absence of any other activatory signal (Fig. 3B). These data indicate that the CD73 induced on the cell surface upon CD38 stimulation acts as a potent agonistic transducer and lowers the activation threshold of CBTC by the CD3/TCR complex via a monocyte-independent mechanism. These data are consistent with our previous observations in adult CD8+ CD45RA+ T cells. These cells, however, are constitutively CD73+ (22, 23).

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 CD73 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 OKT3 mAb (data not shown). The second strategy exploited the GPI anchor used by CD73 to bind the cell membrane. T16-treated CBMC were exposed to PI-PLC to remove CD73 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 CD73 expression induced by CD38 ligation. CBMC were mixed with allogeneic PBMC after 12-h incubation with T16 mAb, i.e., after CD73 had completely disappeared from the surface. No alloreactivity was triggered. The third strategy was to incubate T16-treated CBMC, fully expressing CD73 on the cell surface, with different mAbs to allogeneic PBMC 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.

Enhancement of alloreactivity was mediated by CD73

Three lines of experiments were pursued to determine the role of CD73 in the CBMC alloreactivity triggered by T16 treatment (Fig. 6). The first strategy exploited the GPI anchor used by CD73 to block the cell membrane. T16-treated CBMC were exposed to PI-PLC to remove CD73 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 CD73 expression induced by CD38 ligation. CBMC were mixed with allogeneic irradiated PBMC after 12-h incubation with T16 mAb, i.e., after CD73 had completely disappeared from the surface. No alloreactivity was triggered. The third strategy was to incubate T16-treated CBMC, fully expressing CD73 on the cell surface, with different mAbs to CD73 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 (CD73) 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).

FIGURE 5. The enhanced alloreactivity observed in T16-treated CBTC is associated with cell aggregate formation. Aliquots of CBTC were incubated for 2 h in complete medium or in the presence of 10 μg/ml soluble T16 mAb (CD38). After washing, MLC were established with irradiated allogeneic PBMC (3000 rad) at a responder to stimulator cell ratio of 5:1. On day 3 cultures were microphotographed at ×40 with an inverted microscope. MLC conditions were CBTC and allogeneic PBMC (A), and T16-treated CBTC and allogeneic PBMC (B).
Triplicate wells were pulsed with 1 mT16-treated CBMC incubated with 10^6 after CD73 had disappeared from the cell surface (T16 (after 24 h)), and activate the same transduction pathway and share the same early 219 (38). Thus, it is not surprising that different agonistic mAbs but also on a sequence(s) starting from the amino acid residue of located not only on the carboxyl-terminal sequence of 273–285, and IB4 mAbs are not identical; they are discontinuous and are effective (38). In addition, the epitopes recognized by T16, HB7, and IB4 mAbs, the latter being much less tyrosine phosphorylation have been reported in THP-1 cells stim-
ulated with HB7, T16, and IB4 mAbs. The agonistic activity of CD73, HB7, T16, and IB4 mAbs not inducing CD73 expression (these cells have surface- extended CD38 signaling to other signal transducers, including CD73 expression. IB4 mAb may not be as efficient as the other CD3 signaling pathway, is resistant to PI3-K inhibition (45). A series of findings ruled out the possibility that the prolifera-
tion of T16-treated CBMC was merely due to nonspecific cross-
linking of CD38 and/or CD73 mAbs. First, isotype-specific mouse Lgs (FLOPC-21) did not induce the proliferation of CD38 (even though they have surface-bound, CD37-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 CD37 mAbs). The agonistic activity of CD73 was also evaluated in puri-
fied CBTC after extensive removal of monocytes and other FcR responsive to plastic-immobilized CD3 and CD73 mAbs, whereas their reac-
tivity to PHA, a mitogenic combination of soluble CD2 mAbs, and soluble or plastic-immobilized CD3 mAb was not modified. Thus, reactivity to CD37 stimulation was specifically related to CD37 expression and not to a general state of hyper-reactivity induced by T16 mAb. As expected, WN, by blunting CD37 induction, abrogated the ability of T16-treated CBMC to proliferate in response to plastic-immobilized CD37 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).

The next issue concerned the functional competence of CD73 induced by T16 treatment. CD73 delivers potent agonistic signals to mature adult peripheral blood T cells (22, 23, 44). T16-treated CBMC responded very vigorously to CD37 stimulation with three independent plastic-immobilized CD37 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 CD37 induction, abrogated the ability of T16-treated CBMC to proliferate in response to plastic-immobilized CD37 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).

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A series of findings ruled out the possibility that the prolifera-
tion of T16-treated CBMC was merely due to nonspecific cross-
linking of CD38 and/or CD73 mAbs. First, isotype-specific mouse Lgs (FLOPC-21) did not induce the proliferation of CD38 (even though they have surface-bound, CD37-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 CD37 mAbs). The agonistic activity of CD73 was also evaluated in puri-
fied CBTC after extensive removal of monocytes and other FcR responsive to 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 com-
plex. This is the first time that such an agonistic signaling is doc-
umented as a rapid and transient event related to the de novo sur-
facture-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 non-
specific FcR-mediated cross-linking, since it was not observed in
MHC class I and II molecules are essential for alloreactivity, but their presence alone is not sufficient for the generation of IL-2-responsive cells. Therefore, it is necessary to induce the expression of MHC class I and II molecules in the alloantigen-presenting cell. This can be achieved through the expression of CD73 molecules, which play a critical role in alloreactivity. CD73 molecules are expressed on the surface of both CD8+ and CD4+ T cells, and they are involved in the regulation of T cell activation. CD73 molecules are induced by the expression of CD38 molecules, which are also involved in T cell activation. CD73 molecules are involved in the regulation of T cell activation through the expression of IL-2 and other cytokines. CD73 molecules are also involved in the regulation of T cell proliferation and differentiation through the expression of CD80 and CD86 molecules. Therefore, the expression of CD73 molecules is essential for the generation of IL-2-responsive cells and the induction of alloreactivity.


