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Synergy between LPS and immobilized anti-human CD3ϵ mAb for activation of cord blood CD3+ T cells

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Abstract

Despite an attenuated proliferative response in human cord blood mononuclear cells (CBMCs) to activation of its TCR *in vitro*, the neonate is capable of mounting a mature T_h1-type response to BCG vaccination. We hypothesized that in the context of other innate triggers, activation of the TCR can be restored. In order to test this hypothesis, we analyzed CBMC response to LPS, with LPS serving as a surrogate activator of the innate system. We performed proliferative assays on 34 maternal–neonatal pairs of PBMCs and CBMCs, respectively. In all, 30/34 (88%) of CBMCs proliferated in response to LPS (10 $\mu g\ ml^{-1}$, $P < 2.7 \times 10^{-7}$), in contrast to only 10/32 (31%) of their respective maternally derived PBMCs, despite having a comparatively greater response to PHA than did their CBMC counterparts ($P < 0.0002$). LPS synergized with immobilized anti-human CD3ϵ mAb (1.25–10 $\mu g\ ml^{-1}$) to augment the proliferative response in CBMCs but failed to do so in maternally derived PBMCs. LPS responsiveness and its synergy with activation of the TCR in CBMCs were independent of accessory cells. These results are the first evidence that LPS and anti-CD3 mAb are synergistic, demonstrating a critical link between the innate and adaptive immune systems.

Introduction

Important differences exist between the adult and neonatal human immune systems despite the apparent presence in the neonate of a full repertoire of T_h1, T_h2, cytotoxic T cells, B cells and dendritic cells (1). These differences are noted in many of the cellular components of the immune system with wide-ranging clinical implications. For example, dendritic cell immaturity during infancy may be one cause restricting the capacity to express vaccine-specific T cell memory (2). On the T cell level, responses to environmental allergens *in utero* are skewed towards a T_h2 type (3). Finally, the inability to mount a T cell-independent B cell response to polysaccharide antigens until 2 years of age leads to susceptibility to infection by encapsulated bacteria (4). *In vitro*, as well, differences between the neonate and the adult are noted. This includes a neonatal hyporesponsiveness for cytokine secretion after LPS stimulation (5), a decrease in IFN-γ secretion after PHA stimulation in the neonate compared with the adult (6) and a decreased response to activation of the TCR by an activating anti-CD3ϵ mAb in neonates (7).

The attenuated neonatal response to TCR activation *in vitro* presents somewhat of an enigma, since, for example, human neonates develop a mature T_h1 cell response in response to BCG vaccination (8). Apparently, there must be additional critical mechanisms endowing the neonate to respond to antigens at this stage. One potential possibility is that while an individual pathway may be insufficient for the neonate to mount a mature response, synergistic combinations may allow for an adequate response. One appealing candidate pathway is through the activation of the innate immune system. As an example of such a mechanism, despite a reported T_h2 bias in the neonate, Toll-like receptor (TLR)-4-dependent signals provided by the intestinal commensal flora inhibit the development of T_h2-mediated allergic responses to food antigens (9). In addition, recent epidemiological evidence indicates that early exposure to LPS can minimize the incidence of asthma in populations at risk (10).

In previous studies, the attenuated cord blood mononuclear cell (CBMC) proliferative response to TCR activation *in vitro* was linked to inefficient activation of phospholipase C and decreased Lck expression, thus impairing its phosphorylation cascades for activation (11). Also required for TCR activation by anti-CD3ϵ mAb is nuclear factor-kappa B-inducing kinase (NIK). T cells from alymphoplasia (aly) mice that contain mutant NIK showed impaired proliferation and

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IL-2 production in response to anti-CD3ε stimulation (12). These effects were the result of impaired nuclear factor (NF)-kappa B activity in the T cells. The aim of this paper was to examine the proliferative response of CBMCs and maternal PBMCs to LPS. In addition, since LPS is a strong inducer of NF-kappa B activity (13), we tested in CBMCs whether it would restore activation of the TCR by anti-CD3ε mAb.

Methods

Materials

Ficoll-Paque PLUS and tritiated thymidine ([^{3}H]TdR) were bought from Amersham Biosciences (Sweden). BSA was obtained from USB (Cleveland, OH, USA). RPMI, FCS, L-glutamine, HBSS and Dulbecco's PBS were purchased from Biological Industries Ltd (Beit Haemek, Israel) and Quicksafe A from Zinsser Analytic (UK). PHA and LPS from *Escherichia coli* serotype 0111:B4 were purchased from Sigma (St Louis, MO, USA). Anti-human CD3ε antibody mouse IgG1, clone UCHT1, was purchased from R&D Systems (USA). U-bottom 96-well culture plates were bought from Costar Co. (Corning, NY, USA).

Isolation of blood mononuclear cells via density gradient centrifugation

The Institutional Review Board at the Medical Center approved the study protocol according to the Helsinki Declaration and written consent was obtained from the donors. Thirty-four maternal–infant pairs were analyzed. All infants were full term (>38 weeks). Only routine uncomplicated pregnancies were included in our analysis. Diabetics on insulin, patients with pre-eclampsia or patients with fever suggesting of chorioamnionitis and any other high-risk pregnancies were excluded from our analysis. Nineteen neonates were born through elective cesarean section while 15 were born through a vaginal delivery. In cases of cesarean sections, the mothers were not in labor and all were elective. Blood was drawn from the mother upon placement of the intravenous line. Cord blood was obtained from catheterization of the umbilical vein with an 18-gauge syringe and placed into vacutainers containing EDTA. CBMCs and PBMCs were isolated after a Ficoll-Paque centrifugation and washed twice with RPMI. Neonatal CBMCs were treated with 5 ml water for 10 s to lyse the RBCs and then washed twice with RPMI containing 5% FCS.

Purification of CD3+ cells

CD3+ cells were purified by magnetic cell sorting using CD3 microbeads and MACS MS separation columns (Miltenyi Biotec, Germany). A maximum of 25×10^6 CBMCs were magnetically labeled with CD3 microbeads and loaded on the column as per the manufacturer's instructions. Preliminary experiments demonstrated by FACS analysis that >95% of the cells collected from the column were CD3+.

Proliferation assay

For the determination of the proliferative response to LPS, 2×10^5 cells of isolated PBMCs and CBMCs were seeded in a U-bottom 96-well plate in 100 μl volume of RPMI containing 5% FCS. PHA (5 μg ml^{-1}) or LPS (1 ng ml^{-1} or 10 μg ml^{-1}) were then added in 5-μl volume. LPS stock was kept frozen at 1 mg ml^{-1} and sonicated for 10 min in a water bath before each dilution. After 48 h in culture, cells were pulsed for 16 h with [^{3}H]TdR (1 μCi per well) and then harvested. Filters were placed in Quicksafe A and counted in an LKB liquid scintillation counter. Each point was performed in triplicate. Background counts determined from wells without cells were subtracted from the data. Maternal–neonatal pairs with cells unresponsive to PHA stimulation (proliferative response <3-fold) were excluded from our analysis. In experiments in which the synergistic response to LPS and anti-human CD3ε were evaluated, the cells were initially seeded at 1×10^5 cells per well onto wells pre-coated with anti-human CD3ε mAb. Pre-coated plates were prepared as per the manufacturer's instructions. Briefly, varying doses of anti-human CD3ε mAb (1.25–10 μg ml^{-1}) were plated in U-bottom 96-well plates at 25°C. After 24 h, excess antibody was washed away with PBS and the plates were air-dried prior to use.

Statistics

The results of thymidine incorporation from 34 CBMCs and maternally derived PBMC pairs were evaluated for the determination of the average proliferative response to LPS. A paired *t*-test was utilized for comparison of maternal and neonatal responses to LPS and PHA relative to their respective controls. To compare between the proliferative responses of PBMCs versus CBMCs responding to LPS, the proliferative index was first obtained for each respective mother and infant. The response to LPS between each mother–infant pair was then compared using a paired *t*-test.

Results

Responsiveness of CBMCs and PBMCs to LPS

In preliminary experiments (data not shown), an increased LPS-proliferative effect was noted in CBMCs when compared with regular adult volunteer-derived blood. To control for hormonal changes during the birthing process, maternal blood was next compared with umbilical-derived cord blood cells. Proliferative assays on 34 maternal–neonatal pairs of human PBMCs and CBMCs were performed. CBMCs and PBMCs were isolated and stimulated with LPS for 72 h. Similar results were obtained whether the neonate was born through a vaginal delivery or a cesarean section (data not shown). PHA was used as a positive control for T cell polyclonal activation. CBMCs proliferated on average 1.64- to 1.93-fold compared with its control in response to LPS concentrations of 1 ng ml^{-1} ($P < 1.7 \times 10^{-6}$) to 10 μg ml^{-1} ($P < 2.7 \times 10^{-7}$), respectively. In contrast, there was no response to LPS on average noted in maternal PBMCs.

It should be noted, however, that the average proliferative response masks some true responders in the adult. Figure 1 charts individual responses of PBMCs and CBMCs to PHA and LPS. The proliferative index was obtained by comparing the PBMC and CBMC responses to their respective controls. In all, 27/33 (82%) and 30/34 (88%) of CBMCs proliferated in response to LPS 1 ng ml^{-1} and 10 μg ml^{-1}, respectively. In contrast, only 7/33 (21%) and 10/32 (31%) of maternally

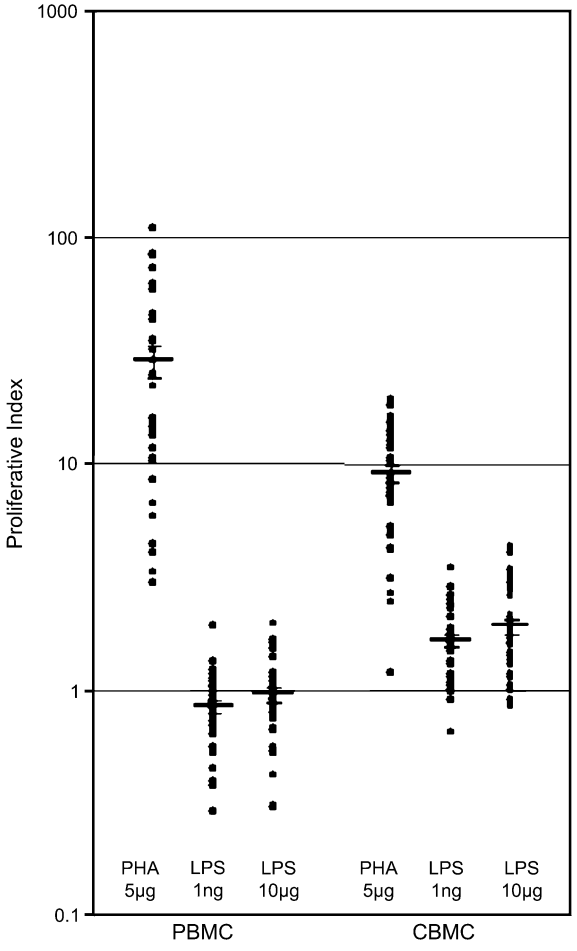

Fig. 1. Proliferative responses of PBMCs and CBMCs to LPS. The proliferative index was obtained by dividing the PBMC and CBMC responses to a given stimulant by their respective controls. Each dot represents the average of an individual's response performed in triplicate. The average for the group is represented by a bar in the figure ±SE. Statistically significant differences were noted in the response of CBMC to LPS relative to its control (1 ng ml $^{-1}$, $P < 1.7 \times 10^{-6}$ and 10 μg ml $^{-1}$, $P < 2.7 \times 10^{-7}$), as well as relative to the response of its respective mother (1 ng ml $^{-1}$, $P < 2.7 \times 10^{-5}$ and 10 μg ml $^{-1}$, $P < 7.0 \times 10^{-5}$).

derived PBMCs responded to 1 ng ml^{-1} and 10 μg ml^{-1}, respectively. No correlation was noted between the degree of LPS response in CBMCs and the corresponding maternally derived PBMCs. The difference in the LPS response of CBMCs compared with its respective maternally derived PBMCs was statistically significant (LPS 1 ng ml^{-1}, $P < 2.7 \times 10^{-5}$, and LPS 10 μg ml^{-1}, $P < 7.0 \times 10^{-5}$). Maternal cells, however, had a comparatively greater response to PHA than did their CBMC counterparts ($P < 0.0002$), demonstrating the specificity of the LPS-mediated proliferative effect.

Synergy between LPS and anti-CD3 mAb for activation of CBMCs

We next examined whether LPS can reconstitute the attenuated neonatal proliferative response to immobilized anti-human CD3ε mAb, which has been previously noted in the literature (14, 15). A dose–response curve to immobilized anti-CD3ε mAb in the presence and absence of LPS (1 ng ml^{-1}) was generated (Fig. 2A). As one increases the concentration of the anti-human CD3ε mAb, a proliferative response is noted in PBMCs (closed diamond in Fig. 2A) but not in CBMCs (open triangle in Fig. 2A). The proliferative response in CBMCs to the anti-human CD3ε mAb can be reconstituted to half the maximum adult level in the presence of LPS (1 ng ml^{-1}) ('cross symbol' in Fig. 2A). Similar findings were noted at LPS (10 μg ml^{-1}) and are charted in Fig. 2(B) according to the counts per minute (c.p.m.) incorporated to better illustrate this synergy. Figure 2(B) depicts the c.p.m. for CBMCs and PBMCs incubated in medium (control), LPS (10 μg ml^{-1}), immobilized anti-CD3 mAb (coated at 10 μg ml^{-1}) and in LPS and anti-CD3 mAb together. LPS synergized with anti-CD3 mAb to augment the proliferative response to the activation of the TCR in CBMCs but failed to do so in maternally derived PBMCs. The hypothetical sum of the effects, if they were just additive, is demonstrated as the 'dotted bar' in the figure. These results suggest that LPS and anti-CD3 mAb activate CBMCs through complementary pathways.

LPS-mediated proliferation in CBMCs is independent of accessory cells

In order to gain insight into the cellular requirements for LPS-mediated proliferation in CBMCs, the response of CBMC-derived CD3+ cells to LPS was next evaluated. CBMCs were isolated by Ficoll-Paque centrifugation and CD3+ cells were then MACS column purified (Miltenyi Biotec). CBMCs as compared with their respectively derived CD3+ cells were evaluated for their response to LPS or the combination of LPS and immobilized anti-human CD3ε mAb (Fig. 3). CD3+ purified cells on average responded greater than total CBMC to LPS and to the synergistic combination treatments. A greater response in CD3+ cells would be expected since the responding population is now pure. These results clearly demonstrate that the proliferative response to LPS in CBMCs is independent of accessory cells.

Discussion

This paper provides evidence for the first time of a synergistic proliferative response in CBMCs to TCR activation by anti-human CD3ε mAb and LPS. LPS and anti-human CD3ε mAb partially reconstituted the CBMC response to an adult level, which, if extrapolated to an antigen-specific T cell response, may help explain the mechanism by which the neonate responds to foreign antigens. Supporting this hypothesis, in a previous report, low concentrations of LPS (100 pg ml^{-1}) synergized with peptides to augment adult human T cell proliferation (16). In addition, in a whole blood model, no synergy was noted between LPS and immobilized anti-CD3 mAb in the adult, similar to our findings here (17). However, adult CD4+ cells are capable of mounting a synergistic response to immobilized anti-CD3 and flagellin (a TLR5 ligand) or R-848 (a TLR7/8 ligand) (18), highlighting the somewhat specific developmental differences in the innate immune system between the neonate and the adult.

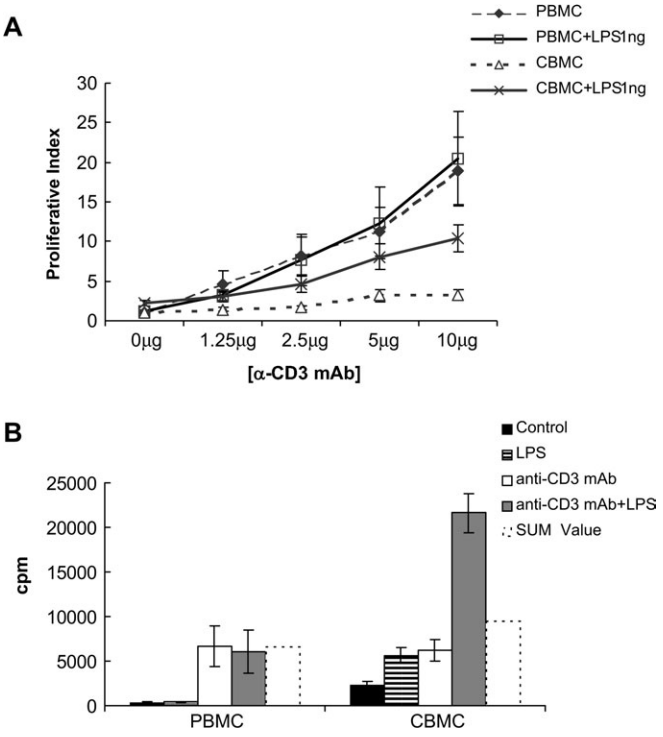

Fig. 2. The effects of LPS on anti-human CD3ϵ mAb-mediated proliferation in PBMCs and CBMCs. (A) A dose–response curve to immobilized anti-human CD3ϵ mAb in the presence and absence of LPS (1 ng ml $^{-1}$) is illustrated. The data are the average, ±SE, derived from five independent PBMCs and eight independent CBMCs. Each sample was performed in triplicate. (B) CBMCs were incubated in medium (control), LPS (10 μg ml $^{-1}$), immobilized anti-human CD3ϵ mAb (coated at 10 μg ml $^{-1}$) and in LPS and anti-human CD3ϵ mAb together (10 μg ml $^{-1}$ each). The SUM value (dotted bar) illustrated in the figure is the theoretically expected additive effect (calculated from the sum of the c.p.m. obtained from incubating with LPS and anti-CD3 mAb, respectively, minus the c.p.m. of the control). A synergistic proliferative response to LPS and immobilized anti-CD3 mAb was noted in CBMCs but not in PBMCs.

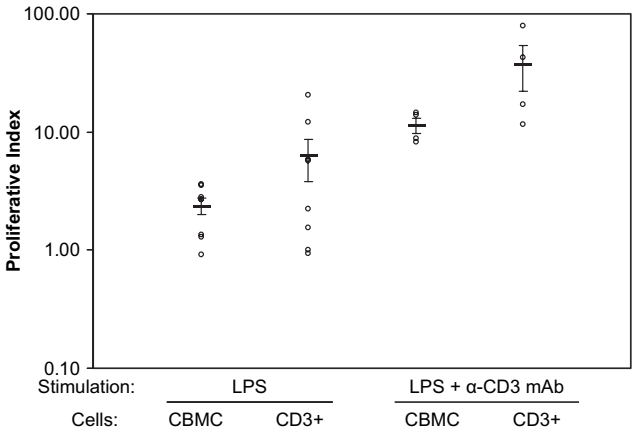

Fig. 3. Response of CBMCs and CBMC-derived CD3+ cells to LPS. CBMCs were isolated by Ficoll-Paque centrifugation and CD3+ cells were then MACS column purified (Miltenyi Biotec) as described in Methods. A comparison of the proliferative responses of CBMCs and purified CD3+ cells to LPS (10 μg ml $^{-1}$, $n = 8$) and LPS (10 μg ml $^{-1}$) plus anti-human CD3ϵ mAb (immobilized at 10 μg ml^{-1}, $n = 4$) is illustrated.

Interestingly, utilizing a mouse model (6–8 weeks old), in the presence of anti-CD3 mAb, CD4+ CD25+ T regulatory (Treg) cells were activated and induced to proliferate in the presence of LPS (10 μg ml^{-1}) (19). While it remains to be seen whether the same is true of Treg cells in the human, Treg cells are present in cord blood (20) and therefore further phenotypic characterization to identify the responding cells in this report is under progress.

The cellular requirements and kinetics of the proliferative response to LPS reported here differ from previously published reports (21, 22), which demonstrated a requirement for direct cell-to-cell contact with accessory monocytes. Interestingly, this latter requirement for monocytes was MHC unrestricted, but dependent on B7 interactions (21). We noted, however, that purified CD3+ cells were able to respond directly to LPS. A possible explanation is related to the kinetics of the experimental observations. In our assay, an increase in proliferation was seen by 72 h in culture. In contrast, an accessory cell requirement was noted in the reports above after 7 days. In addition, perhaps accessory monocytic cells and CD3+ cells differ in the TLRs utilized or in their affinities for LPS. For instance, LPS was shown to be sufficient to stimulate the maturation of dendritic cells using dosing in the ng ml^{-1} range (23). We therefore chose concentrations at both 1 ng ml^{-1} and 10 μg ml^{-1} to uncover potential LPS responses on different cell types. One more reported requirement for LPS responsiveness, which is perhaps most intriguing, is the finding that exposure to CD34+ hematopoietic stem cells may account for the differences between LPS responsiveness of adult PBMCs and CBMCs (24). We therefore cannot rule out the possibility that in our experiments CBMCs were primed *in vivo* by CD34+ hematopoietic stem cells and thus were able to proliferate in response to LPS.

It will be of great interest to define at what age the LPS-mediated proliferative effects seen here revert to the adult phenotype. From the evidence presented here and from the work of others, the majority of adult PBMCs do not respond to LPS (25). The responsiveness to LPS appears to persist into at least early childhood since in a study of explants from the nasal mucosa from atopic children (mean age 3.2 years), LPS caused a 5-fold increase of T cell proliferation as measured by 5-bromo-2-deoxyuridine incorporation compared with mucosa stimulated by allergen alone. This response was not noted in the atopic adults (26). Additional evidence that LPS may influence the T_h1–T_h2 balance in the neonate is our observation that CBMCs generate significantly greater amounts of IFN-γ in response to LPS compared with matched maternal PBMCs (M. R. Goldberg, unpublished observations). Further experimentation is in progress to determine whether the cytokine-producing cells are also the same cells that are proliferating.

It is also of importance to note that the greater proliferative response to LPS in CBMCs is in contrast to the decreased *in vitro* proliferative response of umbilical cord blood to PHA (Fig. 1), pokeweed mitogen, and allo-antigen stimulation (mixed lymphocyte reaction) (27) and TCR activation (Fig. 2A and B) compared with their respective mothers'

peripheral blood response (14, 15). Interestingly, we were able to identify the response of CBMCs to LPS despite its higher background proliferation. This background proliferation is likely due to a stimulant in the FCS to which the neonate responds (28) and may also be dependent on cell-to-cell contact (29). Thus, the specificity of the LPS-mediated effect suggests that identifying the time course of responsiveness for the proliferative effect noted here could set the stage for identifying a therapeutic window for modulation of the immune system.

In summary, a unique synergistic response between LPS and the activation of the TCR by immobilized anti-CD3 mAb was detected in CBMCs. This synergistic combination, when translated into physiologic terms, defines a potentially potent innate immunological response to antigenic exposure during this critical period.

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Abbreviations

CBMC	cord blood mononuclear cell
c.p.m.	counts per minute
[^{3}H]TdR	tritiated thymidine
NF	nuclear factor
NIK	nuclear factor-kappa B-inducing kinase
TLR	Toll-like receptor
Treg	T regulatory

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