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All-*trans*-Retinoic Acid and Erk1/2 Signaling Synergistically Regulate the Expression of CD300B in Human Monocytic Cells

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Abstract

The regulation of the cell-surface receptors that constitute the gene cluster, CD300, also known as the Myeloid Activating/Inhibitory Receptor (MAIR) family, is poorly understood. In the present study, we tested the hypothesis that all-*trans*-RA (RA), a bioactive form of vitamin A long recognized for its role in regulation of immune cell activities, may be a potent regulator of the expression of human CD300B. In monocytic THP-1 cells, RA (20 nM) alone significantly increased CD300B mRNA within 2 h and up to 20-fold after 24 h; however, CD300B protein determined by flow cytometry and confocal microscopy showed little change. A search for coactivating molecules revealed that phorbol myristyl acetate (PMA), a mimetic of diacylglycerol, alone increased CD300B mRNA by less than 5-fold; however, the combination of at-RA and PMA increased CD300B mRNA nearly 60-fold. Moreover, CD300B protein was increased. CD300B molecules were mainly located on the plasma membrane and in the endosomal compartment, sharing a distribution/recycling pattern similar to transferrin receptor CD71. The induction of CD300B mRNA by PMA required signaling through the MEK/ERK branch of the MAP kinase pathway, as PD98059, a MEK1/2 inhibitor, abrogated this response, while SB203580, an inhibitor of the p38 pathway, had no effect. Our data suggest a model in which RA alone induces a CD300B mRNA response in which transcripts accumulate but remain untranslated and therefore “sterile,” whereas RA combined with signals from the ERK1/2 pathway results in both increased CD300B transcription and protein expression on the cell surface and in endocytic vesicles.

Keywords

phorbol ester; MAPK; gene expression; CD71; endosome; THP-1 cells

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1. Introduction

CD300 molecules are a family of membrane-spanning glycoproteins predicted to be located on the leukocyte surface [1]. Six human CD300 family members (CD300A, CD300B, CD300C, CD300D, CD300E, and CD300F) were identified by using the CMRF-35 monoclonal antibody [2, 3], and were mapped to a locus on chromosome 17q22-25 [4, 5]. Structurally, CD300 molecules are type I transmembrane proteins all of which have a single IgV-like extracellular domain [6]. CD300A and CD300F are differentiated from other family members by containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) [7, 8], whereas the other CD300 molecules have a charged amino acid positioned in their transmembrane domains, which enables association with adaptor molecules such as DAP12 [9, 10], acting as an immunoreceptor tyrosine-based activation motif (ITAM) that mediates signal transduction. The molecular structure indicates that CD300 proteins trigger or inhibit immune responses [1, 7]. The amino acid sequences of human CD300 family members are 40% to 80% similar and can be divided into three pairs [6], where CD300A is similar to CD300C, CD300B is most similar to CD300F, and CD300D is closest to CD300E [1]. Orthologues for different CD300 genes have been reported in mammals, birds and fish, indicating that this family has been conserved during evolution [11]. Most of the CD300 molecules are expressed, with different patterns, on peripheral blood leukocytes [5, 9]. Previous studies have shown that the cross-linking of various CD300 molecules on different leukocyte populations broadly modulates phagocytosis, migration, gene transcription, cytokine production, and survival [12, 13], supporting the view that the CD300 family contributes to innate immunity by tuning, directing, or terminating active immune responses. However, very little is yet known of the specific signals these receptors may transduce, or of the signals that may alter their expression.

CD300B, like its paired CD300 family member CD300F, is distributed mainly on myeloid cells, and appears to be a nonclassical activating receptor. When epitope-tagged CD300B protein was expressed and cross-linked, CD300B not only delivered signals through the associated membrane adaptor DAP-12, but also transmitted messages by recruiting Grb2 to its cytoplasmic tail [9]. It is commonly accepted that the binding of CD300B to adaptor proteins such as DAP-12 and Grb2 relies on the presence of a positively charged lysine residue in the CD300B transmembrane domain. In addition, CD300B, a relatively newly-identified receptor protein, was reported to trigger the immune response through its cell membrane adaptor protein in human cell lines [9]. However, factors that regulate the CD300 system are poorly understood, and we know of no previous research regarding factors or mechanisms by which the level of CD300B is regulated.

Vitamin A is an essential micronutrient for all vertebrates [14]. Metabolites of vitamin A are necessary for vision, differentiation of epithelial tissues, normal reproduction, and for the optimal functioning of both the innate and adaptive immune systems [15–19]. Retinoic acid, the active oxidized metabolite of vitamin A, has long been recognized to play a role in immune regulation. The functions of all-*trans*-RA are now becoming better defined, with evidence for roles in the control of B and T cell proliferation and differentiation [15, 20–22], differentiation and activation of dendritic cells (DC) [23, 24], regulation of integrins and chemokines receptors involved in T-cell and B-cell homing [25, 26], and differentiation of lymphocytes subsets, most notably in the differentiation of FoxP3⁺ regulatory T cells [27, 28]. RA acts as a regulator of gene transcription, either directly through the activation of nuclear retinoic acid receptors (RAR α , β , and γ) by binding to specific DNA sequences termed RA response elements, or in an indirect manner [29–31]. In the present study, which was based initially on results from a microarray study, we hypothesized that CD300B mRNA is significantly regulated by RA. The results of our studies have supported this initial hypothesis but, in addition, have revealed a much more complex relationship in which two

signals, one from RA and another from the extracellular signal-regulated kinase (ERK)1/2 pathway, converge to synergistically increase the expression of CD300B molecules both on the cell surface and in endocytic vesicles.

2. Materials and Methods

2.1. Cell and Culture Conditions

THP-1, a human monocytic leukemia cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Rockville, MD) at 37°C in an atmosphere of 95% air and 5% CO₂. In order to keep the cells in an optimized condition, cells were passed for no more than 3 months before renewal from frozen stocks [32]. For each experiment, the cells were collected and resuspended in fresh medium supplemented with 10% FBS and aliquots of cell suspension (0.5×10^6 cells/ml) were transferred to a 6-well or 12-well culture plate and incubated for various times with indicated reagents as described for each experiment.

2.2. Reagents

All-*trans*-RA (prepared as a concentrated stock in ethanol), calcium ionophore A23187, lipopolysaccharide (LPS from *E. coli*), 12-O-tetradecanoylphorbol-13-acetate (PMA), dibutyryl-cAMP, and cycloheximide were from Sigma (St. Louis, MO). 9-*cis*-RA was from Sigma, retinyl trimethyl benzyl ether and Ro40-6055 (Am580) were gifts from Hoffman-La Roche. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β were from R&D Systems (Minneapolis, MN). Interferon (IFN)-γ was purchased from PreproTech Inc. (Rocky Hill, NJ). Poly-I:C stabilized with poly-L-lysine and carboxymethylcellulose was used as previously described [33]. Mitogen-activated protein kinase (MAPK) pathway inhibitors, SB203580 and PD98059, were from Calbiochem Inc. (San Diego, CA).

2.3. RNA isolation, reverse transcription and real-time quantitative PCR

THP-1 cells [34] were cultured in 6-well or 12-well plates for various times and treatments. Upon harvest, the total cellular RNA was isolated by using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Total RNA isolated from HL-60 and U937 cell preparations were used as prepared previously [35]. One microgram of total RNA was subjected to reverse transcription (Promega, Madison, WI). One-tenth of the reaction mixture was used for real-time quantitative PCR (qPCR) tests with SYBR Green labeling (BioRad, Hercules, CA). cDNA was denatured at 94°C for 3 min followed by 35 repeated cycles of 30 s at 94°C, 45 s at 62°C, and 30 s at 72°C. Primer sets used were as follows: human CD300B, sense, 5'-GCGGTACCGGGAAGGCAGAGCCATGT-3', antisense, 5'-GCCTCGAGTGCAGATCCATCTCTCTAAG-3'; human CD71, sense, 5'-ATCAGGATTGCCTAATATACCTGTC-3', antisense, 5'-GTTCAACTCAGGAATCCTCTCAATC-3'; human GADPH, sense 5'-TGAAGGTCCGAGTCAACGGATTGGT-3', antisense 5'-CATGTGGGCCATGAGGTCCACCAC-3'. GADPH was chosen as the internal control gene, and the mRNA expression level of each sample was corrected by its relative ratio to GADPH mRNA. Data were normalized to the average value for the control group, set at 1.00, prior to statistical analysis.

2.4. Flow cytometry

THP-1 cells were washed twice with PBS at the time of harvesting and prepared for the following staining. For intracellular protein detection, cells were permeabilized by using BD

Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) and then incubated with appropriately diluted anti-CD300B-goat IgG (R&D System Inc., Minneapolis, MN) and anti-CD71-rabbit IgG antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 1 h, and then incubated with secondary antibodies to CD300B and CD71, Alexa 488-anti-goat-IgG (Invitrogen, Carlsbad, CA) and PE-anti-rabbit-IgG (Invitrogen, Carlsbad, CA), respectively, at 4°C for 1 h. For cell surface protein detection, living cells were incubated at 4°C with the same antibodies as for permeabilized cells. Instead of CD71, PE-conjugated-anti-CD11b antibody (BD PharMingen, San Diego, CA) was used in cell surface staining at 4°C for 1 h [36]. Cells stained with secondary antibody only were considered as the non-specific binding control and used to set up the gates for flow cytometry analysis. After incubation, the cells were washed and fixed with 1% paraformaldehyde in PBS, and positive-stained cells were measured by flow cytometry. Data were analyzed by FLOWJO software (Tree Star, Ashland, OR).

2.5. Confocal microscopy

THP-1 cells after various treatments were harvested and incubated with antibodies. The incubation procedures and antibodies were the same as for the flow cytometry tests. After the fixation of the cells (by the same procedure as for flow cytometry), samples were visualized using an Olympus Fluoview 1000 Confocal Laser Scanning Microscope (Olympus America Inc.; Melville, NY). Images were analyzed by the Fluoview software (Olympus).

2.6. Statistical analysis

Statistical analysis was performed by using Prism software (Graphpad Software Inc., La Jolla, CA) for one-way analysis of variance (ANOVA), or two-way ANOVA. Differences among groups were determined using Tukey's Multiple Comparison Test. Data are presented as the mean $\pm$ SEM for experiments with four repeats. For tests having only duplicates, the data are shown as the mean $\pm$ range. A value of $P < 0.05$ was considered statistically significant.

On-line supplementary material—Methods relevant to supplementary Figs. S1, S2 and S3 have been described above.

3. Results

3.1. Retinoic acid alone increases CD300B gene mRNA expression

Human THP-1 cells were treated with a physiological concentration (20 nM) of all-*trans*-RA for 24 h and the level of CD300B mRNA was measured by qPCR (Fig. 1A). Under these conditions RA alone increased the CD300B gene expression by 20-fold, supporting the results of the microarray study that first identified this gene.

To further confirm these results and determine if other human monocytic cell lines respond similar to all-*trans*-RA, experiments were conducted with U-937 and HL-60 cells (Table 1). Generally, these cell lines represent somewhat less mature monocytes as compared to THP-1 cells [37]. In both U-937 and HL-60 cells, CD300b mRNA increased ~ 4-times in all-*trans*-RA-treated cells, about one-third that in THP-1 cells. To further examine the specificity for all-*trans*-RA, THP-1 cells were also incubated with other retinoids in parallel with all-*trans*-RA. Neither a retinyl ether, which is not a ligand for RARs, nor oleic acid, used as a lipid control, had any effect on CD300b expression. 9-*cis*-RA was effective, with a 19-fold increase, and Ro40-6056 (Am580), a ligand that selectively binds to RAR- α [38], also increased expression but less than for either isomer of RA. Although 9-*cis*-RA was most

effective, we chose to continue studies using all-*trans*-RA due to its clear status as a natural metabolite of vitamin A [39].

3.2. PMA combined with RA increased the expression of CD300B protein

To address whether the protein expression is also induced, we measured surface and intracellular CD300B protein in human THP-1 cells after various cultured conditions using an anti-human CD300B antibody followed by flow cytometry analysis. For comparison, we also detected the cellular distribution of the transferrin receptor, CD71, because its location on plasma membrane and endocytic vesicles has been well defined [40, 41]. In addition, the expression of cell surface protein CD11b was measured as an indicator of cell differentiation, since RA has previously been shown to induce the expression of CD11b concomitant with macrophage-like differentiation in THP-1 cells[36] and other monocytes [42]. There was, however, no increase of CD300B protein when THP-1 cells were treated only with RA (Fig. 1B). A similar result was found for CD71. However, CD11b showed a marked shift to brighter, more heterogeneous cells, indicating RA did induce THP-1 cell differentiation. Because CD300B is considered as an immune-related gene and THP-1 cells can perform immune-related functions, we next considered whether CD300B protein expression might be induced by certain cytokines, or chemokines, or other immune-related stimuli. Thus we added cytokines, such as TNF- α , IFN- γ , IL-1 β , and bacterial antigens, such as LPS, into the cell culture. Although cell differentiation was driven by RA and the various stimuli, as shown by the increased expression of CD11b on the cell surface (Fig. 1B), CD300B protein showed no change under the same culture conditions, for both cell surface and intracellular protein staining (Fig. 1B).

Previous reports regarding the stimulating of various activities in THP-1 cells with PMA, poly-I:C, and cAMP, led us to include them in our 'super mix'. Indeed, the 'super mix' and RA increased the expression of CD300B protein, as early as 4 h after addition, with a peak at 24 h (Supplementary Fig. S1). Increased cell differentiation was also observed under the same conditions, as indicated by the elevated expression of CD11b on the cell surface.

To investigate which of these additional factors increased the expression of CD300B protein expression, we treated THP-1 cells with the combination of RA and PMA, RA and poly-I:C, and RA and cAMP, respectively, for 24 h. The protein expression of CD300B was significantly increased by the combination treatment of RA and PMA (Fig. 1C), but not by RA and poly-I:C, nor RA and cAMP (Supplementary Fig. S2). Notably, although RA and cAMP had no effect on CD300B protein level, cell differentiation was induced to a similar extent as for RA and PMA treatment, as indicated by the increased expression of CD11b. Thus, cell differentiation *per se* was not necessarily associated with the increase of CD300B mRNA or protein expression.

3.3. RA and PMA synergistically increase CD300B mRNA expression

Because the results above showed that the combination of RA and PMA could induce the protein expression of CD300B, we then tested the effect of PMA on CD300B mRNA in THP-1 cells. THP-1 cells were treated with RA, PMA, and the combination for 24 h. PMA alone increased the CD300B mRNA by 5 fold, while the combination of RA and PMA further increased the gene expression by as high as 60 fold, thus indicating that both together act in a synergistic manner (Fig. 1D), and suggesting the interaction of two regulatory pathways. The effect of the combined treatment with RA+PMA on CD300B mRNA expression was rapid, with a significant increase by 2 h, increasing to about 12 fold higher by 8 h (Fig. 1E), and further increasing by 24 h (shown in Fig. 1D).

These results also led us to question whether new protein synthesis is involved in CD300B mRNA expression. To address this, we pretreated THP-1 cells with cycloheximide to inhibit new protein synthesis for half an hour and then cultured the cells with RA, PMA, and the combination for another 6 h. The result showed no difference between the cycloheximide-pretreated cells and control cells (Supplementary Fig. S3), indicating that the induction of CD300B mRNA expression is likely to be by a direct mechanism that utilizes preexisting cellular proteins.

3.4. CD300B is distributed similarly to CD71 in RA, PMA and RA plus PMA-treated cells

The distribution of CD300B protein on THP-1 cells was investigated by confocal microscopy (Fig. 2A,B). A specific monoclonal antibody was used to tag CD300B, followed by a fluorescence-labeled secondary antibody. CD71, which is known to be located to the plasma membrane and endosomal membrane, was used as an indicator for comparison to the localization of CD300B protein. The CD300B staining (green) showed the similar cellular distribution to CD71 (red), with slightly more CD300B towards the cell surface, indicated by the overlapping color ('Merged column', Fig. 2A,B). No staining was observed in the second antibody control (top), and only weak staining was observed in the control cells and RA-treated cells. Treatment with PMA increased the intensity of CD300B protein on the edges of the cells, while the combination of RA and PMA induced a stronger expression of CD300B protein within the cell (Fig. 2A). On the other hand, the surface expression of CD300B protein was detected even without treatment, although dimly, indicating a preexisting basal level of CD300B protein, and this staining was increased by PMA or the combination treatment (Fig. 2B). Furthermore, treatment with PMA altered the shape of THP-1 cells (Fig. 2B, DIC column), resulting in apparently larger and more adherent cells, which was correlated to the cell differentiation detected by CD11b expression, as indicated in Fig. 1C.

3.5. PMA induced CD300B mRNA expression through the MEK/ERK pathway

PMA, as a mimetic of diacylglycerol, may activate THP-1 cells by activating protein kinase C (PKC) and initiating a downstream signaling cascade [43, 44]. Two possible signaling pathways of PMA induction could be involved: one is the MEK 1/2, ERK 1/2 pathway, the other is the MKK 3/6, p38 MAPK pathway. To investigate which pathway may be essential to the regulation of CD300B expression, we applied specific inhibitors to MEK 1/2 and p38 MAPK to THP-1 cells treated RA, PMA, or the combination. The data for all treatment conditions were plotted on the same scale, with the control without inhibitors was set to 1.0 (Fig. 3A). In the absence of stimulation, the inhibitors, alone or together, did not affect the level of CD300B mRNA. SB203580, a p38 inhibitor, had no effect on the CD300B mRNA expression in any of the treatments, either with RA alone (Fig. 3B), PMA alone (Fig. 3C), or the combination (Fig. 3D). However, PD98059, a MEK1/2 inhibitor, significantly reduced the induction of CD300B mRNA in the PMA-treated and the RA+PMA-treated cells (Fig. 3C and D). We thus concluded that PMA induces CD300B mRNA transcription through the MEK/ERK pathway but not the p38 MAPK pathway.

To further explore the effects of these inhibitors on CD300B protein, we again performed flow cytometry and confocal microscopy. Surface CD300B expression was determined by flow cytometry. PMA increased CD300B fluorescence intensity as compared to cells treated with medium alone (Fig. 4A). When the inhibitors were added with PMA, there was a marked reduction in fluorescence intensity with both inhibitors (Fig. 4B), suggesting that while ERK1/2 signaling was important for CD300B mRNA expression, both branches of the downstream pathway from PKC affected CD300B protein levels. The result was confirmed by confocal microscopy, with a similar distribution of staining but less bright staining in cells that were treated with PMA plus either PD98059 or SB203580 (Fig. 4D).

4. Discussion

Recently, there has been significant progress in elucidating the roles of RA in the regulation of immune cell activities [21–23, 45–48]. In the present study, we investigated the role of RA in the expression regulation of CD300B, a novel member of the leukocytes surface molecule family, and we also investigated the putative signaling pathways involved in the regulation of CD300B expression. Among CD300 family members, CD300B is one of the least studied, perhaps due to the limited availability of specific antibodies. In the human, the CD300 molecules are present in many tissues, including lung (CD300D and CD300F), spleen and thymus (CD300C), and heart and placenta (CD300G), but are likely to be monocyte-associated within these tissues [13]. For instance, CD300E is restricted to mature CD14⁺ monocytes, whereas CD300A and CD300C are expressed by all CD14⁺ monocytes and nearly DC [49]. Cell surface CD300F is found on a limited subpopulation of CD14⁺ monocytes and is restricted to a small portion of DC [8]. The distribution of CD300B and CD300D remains uncertain, but it is believed that they also have limited expression on monocytes and DC [50, 51]. Together, these profiles suggest that the CD300 molecules are expressed on resident or migratory tissue leukocytes, specially monocytes, macrophages, or DC, and they might play an important role in myeloid cell function and innate immunity.

We chose human monocytic leukemia THP-1 cells as our research model because this cell line resembles human monocytes, with various similar criteria such as secretory products, oncogene and membrane antigens expression, and its potential to differentiate into macrophages or DCs [34]. Several aspects of CD300B regulation were elucidated by this approach. At the beginning of this study, we observed the increase of the CD300B mRNA after we treated THP-1 cells with a physiological concentration (20 nM) of RA. This increase was time dependent and robust, with a 10-fold increase in 24 h. Moreover, U937 and HL-60 cells exhibited a similar although lesser increase in CD300b mRNA after treatment with all-*trans*-RA, while THP-1 cells did not respond to either a retinoid without retinoid receptor binding activity, or to a general lipid control. THP-1 cells did, however, exhibit increased CD300b mRNA in response to treatment with 9-*cis*-RA as well as a synthetic retinoid with RAR- α binding selectivity [38]. We chose to use all-*trans*-RA in our further studies since this isomer is well recognized as a naturally-occurring, functionally important product of retinol metabolism [39]. However, analysis of CD300B protein by flow cytometry detected no more than the basal level of CD300B protein after RA treatment. It is possible that RA, as an immune-response inducer, can sensitize cells for the expression of responsive genes, such as CD300B and thereby increase its mRNA accumulation; however, these transcripts appeared to be nonfunctional, it may need a secondary signal to proceed to the active translation. Hence, we then looked for potential stimulatory molecules, especially those related to innate immunity, which might increase the protein expression of CD300B. Although TNF- α , IFN- γ , and IL-1 β have been shown to induce the differentiation of THP-1 cells and increase the production of other immune-related proteins [52], none of these factors alone or in combination had a regulatory effect on the expression of CD300B protein. The bacterial antigen LPS, a ligand for TLR4, also had no effect on the level of CD300B protein. Additionally, zymosan, a yeast cell wall extract used to induce experimental inflammation in macrophages [53], was also added into our treatment (data not shown), but did not affect CD300B expression. Among these stimulatory candidates, PMA was identified as the only mediator that increased the level of CD300B protein both on cell surface and intracellularly. A concentration titration study (not shown) indicated that PMA at quite low concentration, 5 ng/mL, was sufficient to increase CD300B protein as detected by flow cytometry and confocal microscopy. In addition, although certain combinations such as RA and cAMP had no effect on the level of CD300B protein, they induced THP-1 cell differentiation as determined by CD11b expression to a similar extent as after RA and PMA treatment, indicating that the increase of CD300B protein is not required for cell

differentiation. Our results suggest that PMA plays a main role in the induction of CD300B protein expression on the cell surface and also in the cell compartment (Fig. 2).

In our confocal microscopy studies we included the detection of transferrin receptor CD71 because CD71 could potentially be useful as an indicator of the cellular localization of the CD300B protein. According to the merged images, CD300B protein is expressed on the cell surface, presumably on the plasma membrane (Fig. 2B). CD300B was also visualized below the plasma membrane in the region where endosomal compartment membranes exist. CD300B shared the same distribution pattern as CD71 for which plasma membrane-to-endocytic vesicle cycling is well described [40, 41]. Even though CD300B is considered a cell surface protein based on its structure, it was also observed inside the cell, even around the nucleus. It is likely that the newly synthesized CD300B protein was on its way to being relocated to the surface and was labeled by the detecting antibody that diffused into the permeabilized cells. It is also possible that the membrane-bound CD300B protein was internalized along with the phagocytic vesicles into the cell. Thus, the localization pattern provides new information on endogenous CD300B expression and suggests additional questions for future studies. It is noteworthy that several pattern recognition receptors of the TLR family, like TLR3, TLR7 and TLR9, reside in endosomal vesicles [54–56], and thus the combined surface plus intracellular location of CD300B suggests it could possibly sense signals in more than one compartment, or it may serve to internalize a bound ligand from the cell surface into intracellular vesicles, similar to the role of CD71. At present, a major gap exists in current knowledge about all of the CD300 protein family members as the putative ligands and/or the external signals they might sense and respond to have not been identified. Research on the CD300 family of proteins is quite new, and further studies are needed.

Thirdly, RA and PMA synergistically increased the expression of human CD300B at the mRNA level. RA alone increased CD300B mRNA by 20-fold, while PMA alone increased it by less than 5-fold. Interestingly, if we treated the THP-1 cells with RA and PMA at the same time, the combination increased CD300B mRNA by 60-fold, in comparison to the control. In addition, the RA+PMA-induced increase of CD300B mRNA was a rapid and cumulative effect. According to a previous report, PMA has long been recognized as a cell activation inducer, and induction of cell differentiation has been observed in HL-60 and NB4 cells [57], among others. Phorbol 12-myristate 13-acetate and similar phorbol esters, which share structural similarity to diacylglycerol, can induce monocytic cells such as THP-1 cells to differentiate into macrophage-like cells [58], by a mechanism dependent on the activation of PKC [59]. It is possible that PMA, through activation of MAPK or other pathways, could enhance the expression of a related protein, to form a positive feedback loop, therefore resulting in the observed synergistic effect. However, our protein synthesis inhibition experiment showed that the synergistic induction of CD300B mRNA by RA and PMA was independent of newly synthesized protein.

Finally, our studies provide evidence that PMA induces CD300B mRNA expression through the MEK/ERK pathway. Phorbol esters can activate PKC and trigger the related signaling pathways (reviewed in [60]). In general, it is believed that the upstream kinase MAPK kinase kinase (MAPKKK) is phosphorylated by activated PKC through the phosphorylation of Raf, which can initiate the activation of one of multiple other kinases of the three major downstream signaling pathways: the Jun kinases, the p38 kinases, and the extracellular signal-regulated kinases (ERKs) [43]. The activation of protein kinase(s) is an essential step in PMA-induced differentiation of monocytic cells and is tightly connected to the Raf/MAP kinase-signaling pathway [44]. Our results showed that the induction of CD300B expression by PMA was through the MEK/ERK signaling pathway, supported by the evidence that the inhibitor of MEK1/2 abolished the increase of CD300B mRNA by PMA. In contrast, inhibiting the p38 MAPK kinase pathway had no effect on the increased expression of

CD300B mRNA. However, it is noteworthy that both inhibitors reduced surface CD300B expression (Fig. 4), suggesting there may be a complex regulation of CD300B trafficking in monocytes. In other studies in which COS-7 cells transfected with CD300B were studied, ligation of CD300B enhanced signaling of the Ras/mitogen-activated protein kinase (MAPK) pathway through the association with the adaptor protein Grb2 [9]. There may, however, be additional regulation within this pathway, as IL-1 β , which did not affect CD300B, can also activate ERK signaling. Further studies on the precise signaling pathways involved will be necessary. Additionally, definitive studies with CD300 family molecules will require confirmation using physiological CD300 ligands and endogenously expressed CD300 protein. Only very recently, Yamanishi et al. [61] identified a possible endogenous ligand for CD300B/LMIR5, as T cell Ig mucin 1 (TIM-1). Work with other members of the CD300 family showed that cross-linking of CD300A on eosinophils altered cell mobility, whereas cross-linking of CD300F on monocytes enhanced migration to CCR7 and CXCR4 ligands [1]. In cell survival testing in mice, cross-linking of CD300A and CD300F promoted cell proliferation, possibly through protein kinase B/AKT activation, but the same test in a human cell line indicated the opposite effect [7, 8]. However, all these results and any findings based on use of current and future antibodies will eventually require confirmation once physiological ligands of the CD300 proteins are established.

Our study also revealed that the some treatments that we designed specifically to modulate CD300B expression also affected the expression of the transferrin receptor CD71. The transferrin receptor, as an essential player in iron uptake, has been reported to be vital for the proliferation of all cells including those of the immune system [40, 41]. Structurally, CD71 is a disulfide-bonded homodimeric type II transmembrane molecule, with a long extracellular domain (671 amino acids) which binds transferrin, a transmembrane domain (28 amino acids), and a short cytoplasmic tail (61 amino acids) which mediates the rapid endocytosis and recycling [62]. As a cell surface protein, CD71 is mainly found on the plasma membrane. However, the uptake of iron-carrying transferrin involves the internalization of the complex of transferrin and its receptor CD71. Therefore, CD71 is also found in endosomes and other cellular compartment while recycling to the cell surface [63]. In the present study, both CD71 mRNA and protein increased after the combination treatment of THP-1 cells with RA and PMA, in a manner like that of CD300B (Fig. 1B,C and Fig. 2A,B), suggesting they could share a similar regulatory mechanism.

Although relatively little is yet known concerning the CD300 family proteins, various aspects are beginning to come into focus. It has been reported that CD300B protein interacts with other members of the CD300 family, and with itself, and that these interactions may modulate cell signaling [64]. It has also been reported that CD300A and CD300C molecules may function as NK cell activating and inhibitory proteins, although CD300A was not inhibitory in all NK cell clones tested [65]. Much more research is needed to better understand this gene locus and the functions of its proteins.

In summary, RA and PMA acted synergistically to increase the expression of CD300B at both the gene expression and protein levels in human THP-1 cells. A model of the regulation of CD300B expression suggested by these data is illustrated in Fig. 5. RA can diffuse through the cell membrane and then enter the nucleus where it binds to the nuclear receptor complex of RAR and RXR, increasing the gene transcription of CD300B up to 20 fold, as shown in Fig. 1. On the other hand, PMA can also induce the mRNA expression of CD300B, although less potently (~5 fold, Fig. 1D). Interestingly, the combination of RA and PMA synergistically increases the CD300B mRNA expression by 40–60 fold (Fig. 1D and 3D). The action of PMA on the CD300B gene transcription requires signaling via the MEK/ERK1/2 signaling pathway (Fig. 3). Moreover, the gene transcription process is independent of new protein synthesis (Supplementary Fig S3) and is time dependent (Fig. 1E). The

CD300B protein level apparently can be increased by PMA, but not RA alone. This suggests that the CD300B transcripts induced by RA are “sterile” in that they accumulate in the cell but are not efficiently translated unless or until a second signal, which in these experiments was delivered by PMA, results in CD300B protein expression. The true upstream factor which would provide the natural signal leading to diacylglycerol formation is not yet known. Our data suggest that the newly synthesized CD300B protein can locate to the plasma membrane (Fig. 2B). The positively charged lysine in the transmembrane domain of CD300B could provide a means for its interaction with another, putative coupled protein. It has been shown that upon stimulation by an unknown ligand, which has not yet been determined but can be mimicked artificially by cross-linking CD300B cross-linking, CD300B can be phosphorylated, as shown in studies of expressed CD300B protein [9], and can pass the signal to downstream proteins, such as DAP12, to initiate an immune response. Our working model thus integrates the results of the present investigation, showing that all-*trans*-RA and PMA act cooperatively to regulate CD300B expression, and it provides a basis for additional studies to elucidate how CD300B may potentially function in the regulation of innate immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

DC	dendritic cells
ERK	extracellular signal-regulated kinase(s)
ITAM	immunoreceptor tyrosine-based activation motif
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
PKC	protein kinase C
PMA	phorbol myristyl acetate
RA	retinoic acid
RAR	retinoic acid receptor
TNF-α	tumor necrosis factor α

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Fig. 1. Retinoic acid and PMA induces the expression of CD300B mRNA and protein in human THP-1 cells. In each experiment, THP-1 cells were treated with a physiological concentration (20 nM) of RA for the times indicated. The expression level of CD300B mRNA was measured by real-time qPCR and normalized to GAPDH mRNA expression, and the CD300 protein was determined by flow cytometry staining. (A) CD300B mRNA was significantly increased by RA alone (24 h). Bars show the mean $\pm$ SEM for $n=4$ experimental repeats. The CD300B mRNA value of the control group was set at 1.0 (*: $P<0.001$). (B) Protein expression of CD300B, CD71, and CD11b in cells treated with RA for 24 h are shown as black curves. Gray curves are positive staining in untreated control cells. Gates were set according to non-specific antibody staining (negative control).

Numbers on the bars indicate the percentage of cells expressed the target protein. (C) Protein expression of CD300B, CD71, and CD11b in cells treated with RA+PMA (5 ng/ml) for 24 h, shown as black curves, compared to control cells (gray curves). Results from duplicate cultures were analyzed for cell surface protein as in panel B. (D) Synergistic increase in CD300B mRNA in cells treated for 24 h with medium, RA, PMA (5 ng/ml), and both in combination (R+P). Data were analyzed as in panel (a). ANOVA $P < 0.05$; $a > b > c$. (E) THP-1 cells were incubated with RA and PMA for 2 h, 4 h, and 8 h. The induction of CD300B mRNA transcription by RA and PMA was apparent as early as 2 h and continued to increase at 4 h and 8 h (and further to 24 h, see panel d). Bars show the mean $\pm$ SEM; ANOVA $P < 0.05$; $a > b > c$.

Fig. 2.

RA combined with PMA increases the expression of CD300B protein. THP-1 cells were incubated with RA (20 nM) and/or PMA (5 ng/ml). For CD300B staining, cells were permeabilized (A, intracellular staining) or not permeabilized (B, surface staining) and incubated with monoclonal anti-CD300B antibody and followed with an Alexa488-conjugated secondary antibody (green color). For CD71 staining, cells were incubated with monoclonal anti-CD71 antibody and followed with an Alexa594-conjugated secondary antibody (red color). The sensitivity and brightness of fluorescence detection was set according to the control using the secondary antibody for non-specific staining (2nd Ab control). Bright field views (DIC) of the same cells are shown in the left column. Overlaid pictures (Merged, right column) of Alexa488 and Alexa594 stains show the co-localization of CD300B protein and CD71 protein. The photomicrographs shown are representative of at least three independent experiments.

Fig 3.

PMA-induced CD300B mRNA transcription requires MEK/ERK pathway signaling but is not reduced by inhibition of the p38 MAPK pathway. THP-1 cells were incubated with PD98059 (MEK1/2 kinase inhibitor) and/or SB203580 (p38 kinase inhibitor) under four treatment conditions: (A) control, (B) RA (20 nM), (C) PMA (5ng/ml) or (D) both RA and PMA, for 24 h. The expression of CD300B mRNA was analyzed by real-time qPCR. The expression level of CD300B mRNA was normalized to GAPDH mRNA expression. The CD300B mRNA value of the control group with no inhibitors in panel A was set at 1.0 and data for all other conditions and all panels were expressed relative to this value in order to illustrate both treatment effects and inhibitor effects for each treatment. Note that the residual CD300 mRNA level in cells treated with RA+PMA and PD98059 (~13 fold, bottom right) is similar to the increase by RA alone without inhibitor, (10 fold, upper left), indicating that the effect of RA is still apparent in PD98059 and PD98059+SB302580 (PD+SB)-treated cells. Bars show the mean $\pm$ SE for four experimental repeats. Different letters above the bars indicate significant differences between groups (ANOVA $P < 0.05$; $a > b$).

Fig. 4. MEK/ERK and p38 inhibitors alter the distribution of cell-surface and intracellular CD300B. (A) THP-1 cells were incubated PMA (5 ng/ml) and treated with either PD98059 (PD, MEK1/2 kinase inhibitor) or SB203580 (SB, p38 kinase inhibitor). Surface CD300B was analyzed after 24 h. Left, PMA-treated cells compared with negative staining control, control cells (Medium) with CD300B staining. Right, PMA-treated cells compared to PMA-treated cells with either PD or SB, showing reduced CD300B expression on the cell surface. (B) Confocal microscopy of cells treated with PMA alone compared to cells treated with PMA and either PD or SB, showing fluorescent staining and the same cells as bright field (DIC) images.

Fig. 5.

Model of the regulation for CD300B expression. Retinoic acid can diffuse through the cell membrane, and then enter the nucleus where it binds to the complex of RAR and RXR, increasing the gene transcription of CD300B up to 20 fold (Fig. 1). On the other hand, PMA can also induce the mRNA expression of CD300B (~5 fold). Interestingly, the combination of RA and PMA synergistically increases the CD300B mRNA expression by 60 fold (Fig. 1D). Indeed, PMA induces the CD300B transcription through MEK/ERK signaling pathway (Fig. 3). Moreover, the gene transcription process is independent of new protein synthesis and is time dependent. The CD300B protein amount can be increased by PMA or RA+PMA, but not by RA alone (Fig. 2). The newly synthesized CD300B protein can relocate to the plasma membrane. Based on these data and the structure of CD300B [9], we hypothesize that CD300B can interact with a putative coupled protein by the positively charged lysine in the transmembrane domain of CD300B, and, upon ligand stimulation, CD300B can be phosphorylated and pass the signal to the downstream proteins, such as DAP12, to initiate a proper innate immune response, which currently is unknown for CD300B.

Table 1

CD300b response of U937, HL-60 and THP-1 human myeloid cell lines to retinoid treatment.

Monocyte cell line	Treatment	Relative CD300B mRNA
		mean $\pm$ SD
U937	Vehicle control	1.00 $\pm$ 0.35 ^a
	all- <i>trans</i> -RA	4.15 $\pm$ 2.17 ^b
HL-60	Vehicle control	1.00 $\pm$ 0.35 ^a
	all- <i>trans</i> -RA	3.90 $\pm$ 1.94 ^b
THP-1	Vehicle control	1.00 $\pm$ 0.20 ^a
	Retinyl trimethoxybenzyl ether	0.77 $\pm$ 0.30 ^a
	all- <i>trans</i> -RA	12.9 $\pm$ 2.76 ^c
	9- <i>cis</i> -RA	19.4 $\pm$ 5.15 ^d
	RAR- α agonist, Ro40-6055	5.82 $\pm$ 1.91 ^b
	Oleic acid	0.74 $\pm$ 0.13 ^a

Cells were treated, n=3 wells/treatment condition, with ethanol vehicle or retinoid or oleic acid at a concentration of 20 nM for 24 hours. Real-time PCR results for CD300b were calculated relative to GAPDH for the same sample, and the value for control group for each cell type was set to 1.00. Comparisons within cell type having different letters differed significantly, $P < 0.05$, a<b<c<d.