Research article

Phorbol esters and CAMP differentially regulate the expression of CD4 and CD8 in human thymocytes

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Abstract

Background: Intrathymic development and selection of the T lymphocyte repertoire is restricted by the interactions of the T cell antigen receptor and CD4 or CD8 co-receptors with self major histocompatibility complex molecules. Positive or negative selection depends on a tight regulatory control of CD4 and CD8 expression. Determining the intracellular signals that differentially regulate the expression of CD4 and CD8 is important to understand the mechanisms that are implicated in selection of single positive CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$.

Results: The present study shows that stimulation of human thymocytes by phorbol esters or cAMP result in a differential regulation of CD4 and CD8 expression, both at the mRNA and cell surface glycoprotein level.

Conclusions: The differential regulation of CD4 and CD8 gene expression suggests that the selective activation of protein kinase C (PKC) and cAMP-dependent protein kinases (PKA) may be required for the selection of single positive CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells during Intrathymic differentiation

Background

T-lymphocytes expressing the α:β T-cell antigen receptor (TCR) consist of two major subsets: T cells expressing the CD4 surface glycoprotein, which are restricted by the major hystocompatibility complex (MHC) class II proteins, and CD8$^+$ T cells, which interact with antigen presented by MHC class I proteins [1]. CD4 and CD8 molecules bind to monomorphic regions on class II and class I MHC proteins respectively, thereby increasing the avidity in the interaction of the TCR with antigen presenting cells [2]. An intriguing question concerns to the mechanism by which selection and tolerance to self-components is mediated in developing lymphocytes. This is particularly relevant to T Lymphocytes because of their ability to recognize antigen only if this is associated with the class I or the class II MHC molecules [3], and also because of the unique selection process which operates within the thymus during T cell development. Early during T-cell development in the thymic cortex, thymocytes bear both of the T cell surface glycoproteins CD4 and CD8 and express the α and β heterodimer of the TCR [4]. Compelling data have demonstrated that CD4 and CD8 molecules participate in the selection of MHC class I or class II restricted T cells in the thymus. It is known that during clonal selection in the

thymus, immature CD4, CD8 double positive cells develop into single positive CD4 or CD8 cells according to the relative affinity of their TCR to class II or class I molecules respectively [5,6]. These immature T-cells are selected in the thymic epithelium on the basis of their TCR interaction with either class I or class II MHC molecules [7,8], thus generating two mutually exclusive subpopulations that are characterized by the expression of either CD4 or CD8 glycoproteins [9]. Earlier studies in the murine system have demonstrated that PMA and cAMP can modulate the expression CD4 and CD8 on developing thymocytes [10–13]. In keeping with these findings, ex vivo experiments with human thymocytes previously reported by our group, revealed that while phorbol esters can coordinately regulate the transcription of α and β TCR during intrathymic T cell differentiation [14], cAMP selectively affected the transcription of the α and δ-TCR [15,16]. Together the murine data and our own findings support the hypothesis that in humans, TCR expression and CD4, CD8 commitment and selection may be regulated by the same signal-transduction pathways. Since (a) it is generally accepted that CD4 or CD8 are physically associated to the TCR in the course of T cell activation [17,18] and (b) it is well known that mitogen activated protein (MAP) kinase signaling is involved in the selection of the T cell repertoire [19,20], the present study further proposes that the PKC and PKA-mediated signaling, which regulates TCR expression, can coordinately modulate the selection of CD4+CD8- and CD4-CD8+ T cells during human intrathymic differentiation.

Results

The effect of the phorbol ester 12-tetradecanoylphorbol-13 acetate (PMA) and exogenous cAMP on the levels of mRNA encoding the CD4 and CD8 glycoproteins was examined in human thymocytes. Total post-Ficoll un-fractionated thymocytes were incubated in the presence or the absence of 10 nM PMA and/or 0.1 mM cAMP + 1 mM of the phosphodiesterase inhibitor isobutylemthylxnthine (IBMX). The fundamental reasons for the design of our *ex vivo* experiments are three-fold: (a) The need to evaluate intrathymic differentiation as close as it could be physically possible to an *in vivo* model; (b) We sought to perform the experiments on "untouched" cells with the least of manipulations. In purifying thymocyte subsets, there is a risk that the antibodies used for the selection of the DP subset can stimulate cells through a TCR-like signaling, thus masking the read-out of the experimental stimuli and; (c) At least 80% of thymocytes are double positive (DP) cells. Therefore the contribution of single positive (SP) cannot be a concern. After 3.0 h incubation RNA was extracted and analyzed for CD4 and CD8 expression by northern blot hybridizations. As shown in Fig. 1, PMA caused a marked decrease in the levels of both CD4 and CD8 mRNAs, whose constitutive expression is high in de-
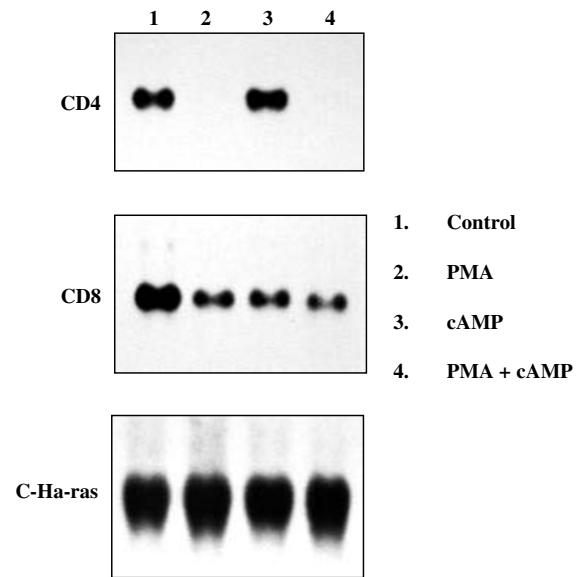

Figure 1
Northern blot analysis of the effect of PMA and cAMP on CD4 and CD8 mRNA levels in human thymocytes. Cells were incubated for 4 h at 37°C in culture media as described in the methods, in the absence (lane 1) or in the presence of 10 nM PMA (lane 2) or 1 mM dibutyryl cAMP plus 1 mM IBMX (lane 3), and the combination of both (lane 4). RNA was subsequently extracted and analyzed. The expression of the proto-oncogene c-Ha-ras served as an internal control.

veloping thymocytes. In contrast, cAMP selectively decreased CD8 mRNA levels. On the other hand, apoptosis was ruled out as a mechanism to explain the differential down-regulation of CD4 and CD8 by PMA and cAMP, on the grounds of two independent observations: (a) After a maximum of 4.0 hr incubation with either PMA or cAMP+ IBMX the cell viability was the same as the untreated controls (usually between 90–95%); (b) Treatment with dexamethasone, which is a known pro-apoptotic molecule did not have any effects on the expression of either CD4 or CD8 (data not shown) and; (c) As demonstrated earlier in the murine system, TCR engagement of CD4+CD8+ induces positive selection but not apoptosis [12]

To further investigate whether the effect of PMA on CD4 and CD8 expression is consistent with the activation of protein kinase C (PKC), we examined structurally distinct phorbol esters that either have or lack the capacity to activate PKC, for their effectiveness to modulate the levels of CD4 and CD8 mRNAs. As shown in Fig. 2, the α-configuration of the phorbol ester that lacks the ability to activate PKC [21] had no effect on the expression of CD4 and CD8. In contrast, the β-configuration that has the capacity to activate PKC [21] decreased the levels of CD4 and CD8
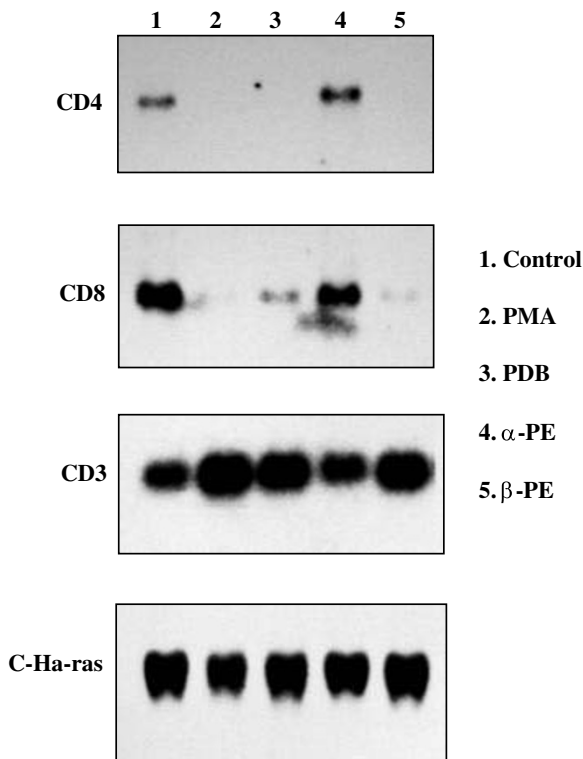
Figure 2
Northern blot to determine the specificity of the Phorbol ester effect on the expression of CD3, CD4, CD8, and C-Ha-ras by human thymocytes. Experimental conditions are number-coded as follows: I = control; 2 = PMA-treated; PDB-treated; 4 = α-Phorbol Ester-treated; and 5 = β-Phorbol Ester-treated.

mRNAs. The water-soluble phorbol 12,13 dibutyrate (PDB) had similar effects to those of PMA. The specificity of the signals driven by PMA was further stressed by the up-regulation of CD3 expression and by the unaltered mRNA levels of the proto-oncogene c-Ha-ras.

On the other hand, it has been previously demonstrated that direct activation of adenylate cyclase by Forskolin or cholera toxin, inhibition of cAMP-specific phosphodiesterase, and treatment with a cell permeable cAMP analog, induces a rise in intracellular cAMP [22–26]. Since cAMP is known to modulate the expression of CD8 in mice [27], we sought to determine whether cAMP-dependent protein kinase (PKA)-mediated signaling plays a role in modulating the mRNA levels of CD3, CD4, and CD8 in ex vivo examined human thymocytes. Fig. 3 shows that the signal, which regulates the expression of human CD8, is cAMP-mediated, since direct activation of adenylate cyclase by cholera toxin produced the same effects as the di-butiryl

cAMP analog. The requirement of phosphodiesterase inhibition by IBMX further supports this statement. As it can also be observed, cAMP coordinately regulated the expression of both the α and β chains of the CD8 molecule. CD4 remained unchanged and CD3 was minimally affected, thus stressing the selectivity of the cAMP-dependent down-regulation of CD8 expression (Fig. 3). To further investigate whether PMA and cAMP regulate CD4 and CD8 expression at the protein level, surface detection of the CD3, CD4, and CD8 was also assessed. As observed in Fig. 4a PMA dropped the proportion of CD4+ cells from 60% to zero, produced a slight decrease (from 71% to 64%) in CD8+ cells, and did not affect the expression of CD3. cAMP on the other hand had little effect on the surface expression of the glycoproteins. These findings are in contrast with the drastic down-regulation of CD8 transcript levels exerted by cAMP. However, regulation gene expression is complex and engages numerous checkpoints that include transcription, RNA processing, nuclear/cytosol transport, translation, and RNA and Protein stability. Since the half-lives of mRNA and protein can be dramatically different, we hypothesize that an increased CD8 protein stability may result in a delay of CD8 surface disappearance, thus favoring its transient selection. As CD8 gene transcription has been turned-off by cAMP-mediated signaling, surface CD8 subsequently fades with the concomitant emergence of CD4+ cells. In keeping with this rationale, the kinetics of the PMA-mediated down-regulation of CD4 (Fig. 4b) reveals a sharp and rapid decrease on CD4 surface expression as early as 15 min. of incubation with PMA, with a complete disappearance by 2.0 hr. The relatively short kinetics of surface CD4 down-regulation and the delayed CD8 surface disappearance can be interpreted as a PKC-mediated selection signaling in favor of CD8+CD4-/α:β-TCR cells that may operate during intrathymic T cell differentiation. Binding of specific ligands to CD4 (including phorbol esters) results in internalization of the molecule by endocytosis, which in turn is phosphorylated [28] and possibly immobilized. It is conceivable that a similar mechanism might be used by extracellular signals in the thymic epithelium during intrathymic differentiation.

Discussion
An accepted scheme of the T cell development program [29,30] has demonstrated that as immature cells enter the thymus, cycling CD4-CD8- precursors begin to rearrange δ, and γ genes, which generates CD4-CD8-, and δ+γ+ cells. CD4+CD8+ on the other hand, rearrange and express the α:β-TCR heterodimer. A few of these cells are selected through the specificity of their antigen receptor and mature into either CD4+CD8-, or CD4-CD8+ T cells which exit from the thymus.

1. **Control**
2. **IBMX**
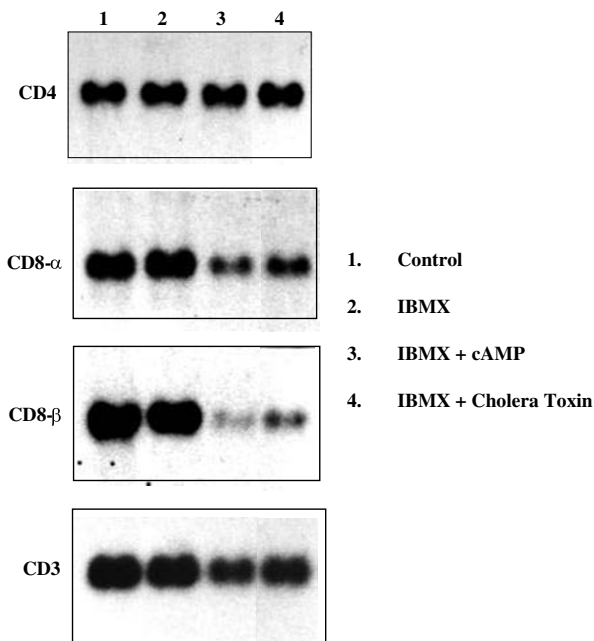3. **IBMX + cAMP**
4. **IBMX + Cholera Toxin**

Figure 3
Northern blot to examine the effect of cyclic AMP analogs and cholera toxin on CD4, CD8, and CD3 mRNA levels in human thymocytes. Experimental conditions are number-coded and respectively identified

Figure 4
Effect of PMA and cAMP on the surface expression of CD3, CD4, and CD8 by human thymocytes. (**a**) Immunofluorescent staining and (**b**) CD4 surface expression kinetics. Experimental conditions and detailed time course is respectively indicated.

Much progress has been accomplished in determining the molecular steps that lead to a better understanding of murine T cell progenitor commitment and selection of the functional repertoire, which has gained important insights into mechanisms of CD4/CD8 lineage commitment (reviewed in 31). For instance, changes in expression of CD5, TCR, Bcl-2, RAG and TdT genes can be induced by in vitro TCR engagement of DP thymocytes, thus indicating that at least some aspects in the T cell maturation program are directly coupled to TCR signaling. It is therefore accepted that positive selection are directly coupled to TCR signaling pathways in DP thymocytes. However, the signals that promote selective down regulation of CD4 and CD8 remain have not been that conclusive, at least in humans. Earlier (again murine) studies (reviewed in 31) have suggested that a transitional phenotype of CD4$^+$ CD8^{low} is not a signature of commitment towards the CD4 lineage. The confirmation of such conclusion in the humans and the determination of the signals that lead to the phenotype remain to be assessed.

We have previously shown that PMA strongly induces the transcription of α and β TCR in human thymocytes, whereas cAMP exclusively affected the transcription of α-TCR [15,16]. The data reported herein, suggest that following rearrangement, protein kinase C activation mim-
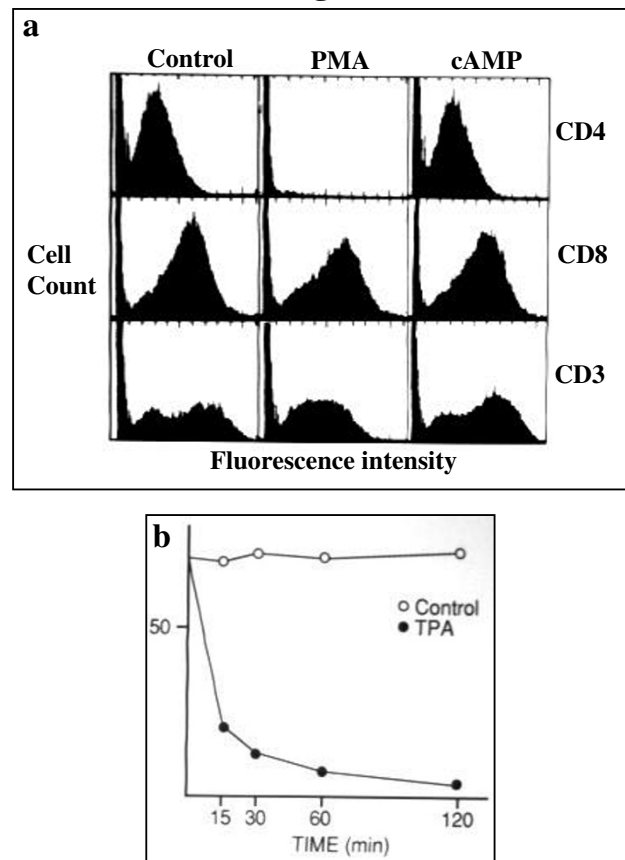
icked by the phorbol esters induces the expression of the α and β-TCR on CD4+CD8+ precursor T cells. Since only a proportion of developing thymocytes succeed in expressing the α:β TCR heterodimer, PKC activation also provides the signal to turning off the CD4 and CD8 genes on cells that failed to express the TCR, which are non-functional and potentially harmful. On the other hand, cAMP is known to participate in the growth and differentiation of varied cell types (including thymocytes), through the transcriptional regulation of specific genes [32–34]. The unique cAMP signaling which specifically targets the expression of the CD8 mRNAs (α and β), suggests that PKA activation may result in the transduction of signals that determine the selection of T lymphocytes in favor of the CD4+CD8- lineage, by down-regulating and possibly turning off the expression of the CD8 transcripts. In keeping with this notion, regulatory elements, within the CD4 and CD8 promoters have been identified an characterized

including the presence of cAMP regulatory elements binding (CREB) sites [35]. However it is also likely that the control of CD4 and CD8 expression and thus the selection of CD4 or CD8 lineage-committed cells may also be controlled by the differences in mRNA and protein stability. Lastly, some of the TCR signaling pathways involved in positive selection have been identified in the mouse and there is some evidence that these may be different from those involved in negative selection. It remains to be determined whether same interpretation applies to human T cell selection.

Conclusion

In conclusion, the results of the ex vivo experiments of human thymocytes reported in the present study are in complete agreement with the earlier studies in the murine system and strongly support the conclusions. We further propose that the transduction of intracellular signals initiated by the activation of PKC and PKA by phorbol esters and cAMP respectively, are not only critical for the differential regulation of CD4 and CD8 gene expression but more importantly in the commitment and selection of the functional T cell repertoire.

Materials and Methods
Cells

Human thymocytes were purified from fresh residual thymic tissue that was obtained as an incidental specimen from immunologially normal pediatric patients undergoing cardiac surgery and according with institutional guidelines. Purification was carried out by a single step Ficoll/Hypaque gradient centrifugation. Purified cells were cultured at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum at a density of 5×10^6 cells/ml and in the presence or absence of 10 nM PMA phorbol ester and or 1 mM dibutyryl cAMP + 1 mM phosphodiesterase inhibitor isobutylemthylxnthine (IMBX).

Reagents

The phorbol esters 12-tetradecanoylphorbol-13 acetate (PMA), 4x-phorbol, 12,13 didecanoate (αPD2), 4B-phorbol 12,13 didecanoate (βPD2), the phorbol 12,13 dibutyrate (PDB), dibutyryl cyclic AMP, and the IBMX were obtained from Sigma Chemical Co. (St. Louis, MO.). Deionized formamide Distilled Phenol and guanidinium isothiocyanate were purchased from Fluka (Milwaukee, Wl). Nylon Hybond membranes (0.45 mm), and ^{32}p-dCTP were obtained from Amersham (Arlington Heights, IL). The CD3 plasmid was commercially obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the respective cDNA insert was generated by a Pst I digestion. CD4 [36] and CD8 [37] were kind gifts from Dr. Dan Littman and the respective cDNA probes were obtained by digestion with the EcoR 1 restriction endonuclease. The cHa-ras was also from ATCC and the respective

insert probe was excised by a Barn H1 endonuclease digestion. The monoclonal antibodies to CD3, CD4, and CD8 were commercially obtained (Pharmigen, San Diego, CA).

RNA Extraction

Total unfractionated RNA was extracted in guanidiniun isothiocyanate as previously described [38].

Northern Blots

Total RNA (10 μg) was electrophoresed in 1.0% agarose gel and transferred onto nylon membranes as described earlier [39,40]. Blots were then prehybridized overnight at 42°C in 50% formamide, 4X SSC, 5X Denhart's solution, 25 mM sodium phosphate, pH 6.5, 1.0% sodium deodecyi sulphate (SDS), and 200 μg/ml salmon sperm DNA. Hybridization was carried out for 16 hr. at 42°C, simply by adding [a-^{32}P]dCTP-labeled probes. After hybridization, northern blots were stringently washed for 2.0 hr. and autoradiographed. Equal amounts of RNA loading of the different experimental samples, was confirmed by ethidium bromide staining and ultraviolet transilluminator visualization. The constitutively expressed proto-oncogene c-Ha-ras was used as internal control.

Immunofluorescent Staining

Freshly isolated thymocytes were incubated in the presence or the absence of either PMA or cAMP+IBMX. At the indicated times cells were harvested, washed twice with phosphate-buffered saline (containing 0.1% serum bovine albumin and 0.1% sodium azide), and incubated for 30 min at 4°C with specific monoclonal antibodies to either CD3, CD4 or CD8. Cells were then washed twice with PBS and incubated for 30 min at 4°C with the appropriate FITC-conjugated second antibody. After the second incubation the cells were washed twice with PBS and analyzed on a fluorescence-activated Cell Sorter (FACS) II.

List of Abbreviations
IBMX: isobutylemthylxnthine/PMA: 12-tetradecanoylphorboMS acetate/PKA: cAMP-dependent Protein Kinase/PKC: Protein Kinase C

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