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Effect of retinoic acid and vitamin D on the expression of interleukin-1β, tumour necrosis factor-α and interleukin-6 in the human monocytic cell line U937

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SUMMARY

We have previously described a synergism between the two physiological hormones, retinoic acid (RA) and 1α,25-dihydroxyvitamin D_3 (VD) in the induction of U937 cell differentiation towards a more mature state. Herein, we investigated the regulation of cytokine production during RA and/or VD treatment of U937 cells. Cell differentiation was followed by measurement of their capacity to give oxidative responses, and interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and IL-6 gene and protein expression were determined in RA/VD-treated cells, activated or not with lipopolysaccharide (LPS). The undifferentiated and RA-treated U937 cells were unable to produce monokines even when they were stimulated by LPS. VD induced the monokine mRNA expression in U937 cells but failed to induce protein release. However, unlike RA, it primed the cells to secrete monokines upon endotoxin stimulation. A large enhancement of the production of the monokines both at mRNA and protein levels was observed in the U937 cells exposed to the combination of RA + VD. Nevertheless, protein release required a further step of activation of the RA + VD-primed cells. The co-inducer effect of RA and VD was not observed in HL-60 or THP-1 cells and seems to be restricted to U937 cells. These results on cytokine expression support our previous finding that a combination of RA and VD brings the U937 cells to a high stage of myeloid differentiation with major characteristics of monocytes/macrophages.

INTRODUCTION

Cells of the monocyte/macrophage lineage are an important source of cytokines, including interleukin-1 (IL-1), tumour necrosis factor (TNF) and IL-6, which play a pivotal role in immune and inflammatory reactions.[1] At the functional level, macrophages are heterogeneous and differ in their ability to produce these monokines.[2] It has become increasingly clear that monocyte subpopulations may exist and that different phenotypes might be generated through distinct developmental pathways.[3] In addition to this intrinsic determinism, environmental factors may control the progression towards terminal differentiation.[4]

The physiological derivatives of vitamin A and D, all-*trans* retinoic acid (RA) and 1α,25-dihydroxyvitamin D_3 (VD), are among the agents likely to modulate macrophage differentiation. RA has a profound effect on cell differentiation and pattern formation during development[5] and has been described as a regulator of macrophage functions and cytokine production.[6] The immunomodulatory role of VD is well recognized.[7,8] It interacts with cells of the monocyte/macrophage lineage[9-11] and activated macrophages can themselves convert the relatively inactive form of vitamin D, 25-dihydroxyvitamin D_3 to the most biologically active form, 1α,25-dihydroxyvitamin D_3 (calcitriol).[7]

Human myelomonocytic cell lines provide an experimental model to study the effects of RA and VD on monocytopoiesis.[12,13] Results from our laboratory have shown that both agents act synergistically to induce macrophage-like properties in U937 cells.[14] The RA/VD-treated cells become more phagocytic and show a dramatic enhancement of the oxidative burst. In order to characterize this co-operative effect further, we examined the capacities of the cells treated by RA and VD alone or in combination, to express cytokine genes and to release biological activity upon stimulation by bacterial endotoxin (LPS). We found that RA and VD co-operatively participate to induce cytokine expression in U937 cells. This effect seems to be specific to this myelomonocytic cell line.

MATERIALS AND METHODS

Chemicals and reagents

All-*trans* RA, formyl-methionyl-leucyl-phenylalanine (FMLP), lipopolysaccharide (LPS) and Zymosan A were purchased from

Abbreviations: RA, retinoic acid, VD, 1α,25-dihydroxyvitamin D_3.

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Sigma (St Louis, MO). VD was a generous gift from Hoffman-LaRoche (Basel, Switzerland). Luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) was from Boehringer Mannheim (Grenoble, France). RA and VD were dissolved in absolute ethanol and stored at −70° at an initial stock concentration of 10^{-3} M. Dilutions were performed in RPMI-1640 medium. The final concentration of ethanol had no effect on cell growth and differentiation. Human recombinant IL-6 (rhIL-6), rhIL-2, rhIL-1β and rhTNF-α were a generous gift from Dr P. Casellas (SANOFI Recherche, Montpellier, France). [^{3}H]methylthymidine ([^{3}H]TdR) was purchased from Dositeck (Paris, France).

Cell culture and treatment

The human myeloid cell lines U937, HL-60 and THP-1 from the American Type Culture Collection (ATCC; Rockville, MD) were cultured at 37°, 5% CO_2, in complete RPMI-1640 medium (Gibco BRL, Sarl, France) with 10% (v/v) foetal calf serum (FCS) (Sigma) and gentamycin (50 μg/ml). The cells were regularly screened for mycoplasma contamination by 4,6-diamino-2-phenylindole (DAPI) fluorescence and found to be negative. Cell viability was assessed by trypan blue exclusion. Cells in the logarithmic phase were incubated at 2×10^5 cells/ml for up to 4 days in the presence of 100 nM of VD and/or 100 nM RA. The cells were then washed and incubated in fresh medium, alone or supplemented with 25 μg/ml LPS. For RNA extraction, the cells were recovered after 5 hr incubation. In parallel experiments, cell supernatants were recovered after 24 hr for cytokine bioassays.

Respiratory burst activity

The oxidative bursts elicited by opsonized zymosan or FMLP were assayed by luminol-dependent chemiluminescence as previously described.[14] Briefly, 5×10^5 cells in 500 μl RPMI were transferred to a counting tube filled with 500 μl luminol solution at 6 μM in phosphate-buffered saline (PBS). The suspension was prewarmed at 37°, before stimulation with 50 μl of 1 mg/ml opsonized zymozan or with 10^{-5} M FMLP. Light emission was measured by an automatic luminescence analyser Lumicon (Hamilton, Switzerland).

IL-6 bioassay

IL-6 was measured in a bioassay based on the proliferation of IL-6-dependent hybridoma B9 cells, kindly provided by Dr L. Aarden (University of Amsterdam, Amsterdam, The Netherlands).[15] Briefly, after being extensively washed and kept 4 hr in IL-6-free medium, 5×10^3 B9 cells were cultured in 100 μl RPMI-1640 medium supplemented with 1 mM pyruvate, 50 μM mercaptoethanol, 10% FCS, containing serial dilutions of the supernatants to be assayed. Dilutions of rhIL-6 were included as standard. After 72 hr, B9 cell growth was measured by adding 0·75 μCi [^{3}H]TdR 4 hr before cell harvesting. In control experiments, the biological activity was blocked with anti-human IL-6 neutralizing antibodies.

IL-1 bioassay

IL-1 activity was measured as previously described,[16] using the mouse thymoma EL4-NOB1 cell line (which can be stimulated by IL-1 to produce IL-2) and the IL-2-dependent cytotoxic T-lymphocytic line (CTLL). EL4-NOB1 cells were cultured at 5×10^5/ml in RPMI, 5% FCS in the presence of 5×10^4 CTLL cells/ml for 24 hr at 37° with rhIL-1β or appropriate dilutions of cell culture supernatants to be assayed. The CTLL cell proliferation was measured by incorporation of [^{3}H]TdR added 4 hr before cell harvesting. The biological activity was expressed in U/ml.

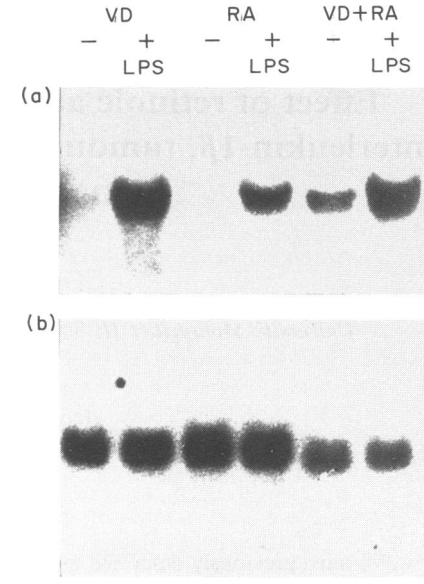

Figure 1. (a) Northern blot analysis of IL-1β mRNA of U937 cells treated for 4 days with 100 nM RA, VD or RA + VD. The induced cells were cultured for 5 hr with medium alone (−), or in the presence of 25 μg/ml of LPS (+). Fifteen micrograms of total RNA per lane was run and hybridized with the IL-1β probe. (b) The same filter was subsequently hybridized with the GAPDH probe to control the amounts of RNA loaded in each line.

TNF bioassay

Biological activity of TNF was evaluated by a cytotoxic assay performed on the L929 murine fibroblast cell line using the method previously described.[17] Briefly, 100 μl of L929 cells at 5×10^5/ml were seeded in 96-microwells for 24 hr. Appropriate dilutions of cell culture supernatants, or rhTNF-α were then added in quadruplicate to culture wells together with actinomycin D (at a final concentration of 1 μg/ml). The TNF activity was assessed by measuring the cell viability with crystal violet. The results were expressed in pg/ml.

Northern and slot blot analysis

Probes. The monokine probes (TNF-α, IL-1β and IL-6) used were a 1·3 kilo base (kb) human TNF-α, a 0·6 kb human IL-1β and a 1·1 kb human IL-6 cDNA fragment (ATCC). The control probe was a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe made of a 1·3 kb hamster cDNA fragment (kindly provided by Dr P. Fort, CNRS, Montpellier, France). The probes were labelled with ^{32}P-dCTP by random priming.

RNA extraction and Northern blot. Total RNA was extracted using the guanidine/thiocyanate method as described by Chomczynski *et al.*[18] and electrophoresed in a 1·2% agarose formaldehyde gel. Fifteen micrograms of total RNA were used for each lane. After transfer onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), RNA blots were hybridized with a ^{32}P-dCTP labelled probe. The blots were then washed under high stringency conditions and exposed at −80° to a Kodak X-Omat AR film for 1–5 days. Northern blot

Table 1. Determination of the oxidative burst and monokine secretion in U937, HL-60 or THP-1 cells. Cells were cultured for 4 days in the presence of the medium (none), 100 nM RA, 100 nM VD, or 100 nM of both agents. They were harvested and the chemiluminescence of 10^5 cells induced by opsonised zymosan or FMLP was determined. In parallel, the amounts of IL-6, IL-1β and TNF-α secreted in the supernatants upon 24 hr LPS stimulation were determined as described in Materials and Methods

		Differentiation inducers			
	Cell line	None	RA 10^{-7} M	VD 10^{-7} M	RA+VD 10^{-7} M
Zymosan+induced luminescence (c.p.m.)	U937	1556	8720	18,574	260,000
	HL-60	2000	11,250	25,200	42,500
	THP-1	3060	61,801	14,144	83,304
FMLP-induced luminescence (c.p.m.)	U937	26	25	38	3242
	HL-60	30	35	58	71
	THP-1	92	70	80	152
LPS-induced IL-6 (U/ml)	U937	700	400	4200	45,000
	HL-60	1000	250	11,500	9600
	THP-1	36,880	17,720	42,000	51,130
LPS-induced IL-1β (U/ml)	U937	70	60	410	940
	HL-60	ND	ND	32	23
	THP-1	452	300	1100	987
LPS-induced TNF-α (pg/ml)	U937	8	15	63	270
	HL-60	ND	ND	ND	ND
	THP-1	100	50	520	370

ND, not detectable.

analyses enabled control of the size of the cytokine mRNA as shown in Fig. 1 for IL-1β mRNA. The RNA appeared undegraded in all samples.

Quantification by slot blot analysis. Serial dilutions of RNA between 0·3 and 10 μg of RNA were spotted on nitrocellulose membrane using a BRL slot blot apparatus. Filters were made in triplicate, hybridized as described above with the three cytokine probes and further hybridized with the GAPDH probe. Densitometric analysis was used to quantify the signals on the autoradiogram with a Bioimage System (Millipore, Molsheim, France). The values of GAPDH mRNA signal were used to normalize the values obtained from the cytokine mRNA signals.

RESULTS

Assessment of U937 cell differentiation

The cells were seeded at an initial density of 2×10^5/ml and cultured in the presence of 100 nM RA and VD either alone or in combination. With each agent alone, cell proliferation was reduced but not completely abolished. When both agents were used in combination, cell growth arrest occurred after 3 days incubation and cell viability was not affected upon further incubation for at least 3 more days.[14] Measurements of the oxidative burst triggered by opsonized zymosan or FMLP demonstrated that: (1) undifferentiated U937 cells did not respond to these stimuli; (2) RA- and VD-treated cells became capable of producing chemiluminescence in response to zymosan; (3) both agents synergized to enhance this response; and (4) U937 cells produced an oxidative burst in response to FMLP only when exposed simultaneously to RA and VD. These criteria were used throughout this work to monitor ongoing cell differentiation and are shown in Table 1.

Production of monokines by U937 cells following RA and VD treatment

Undifferentiated U937 cells did not produce significant amounts of monokines; mRNA levels were very low; a small increase of mRNA was detected following LPS stimulation and the cells produced only trace amounts of biologically active monokines (Figs 2–4).

IL-1β and TNF-α mRNA levels were increased in RA-treated cells exposed to LPS. However, these cells did not release IL-1 and produced low amounts of TNF-α. The effects of RA treatment on IL-6 production seemed negative both at the mRNA and at the protein level.

On the other hand, VD had clearly positive effects on the production of monokines. It did not induce protein expression by itself, but increased mRNA level. Upon LPS stimulation, mRNA synthesis was still enhanced and was accompanied by the release of significant amounts of monokines in the culture medium.

The major finding was that RA, which by itself did not render U937 cells able to release monokines, potentiated the action of VD. Without LPS stimulation, the cells treated by both agents displayed mRNA levels comparable to those of LPS-stimulated VD-treated cells. Exposure to the endotoxin

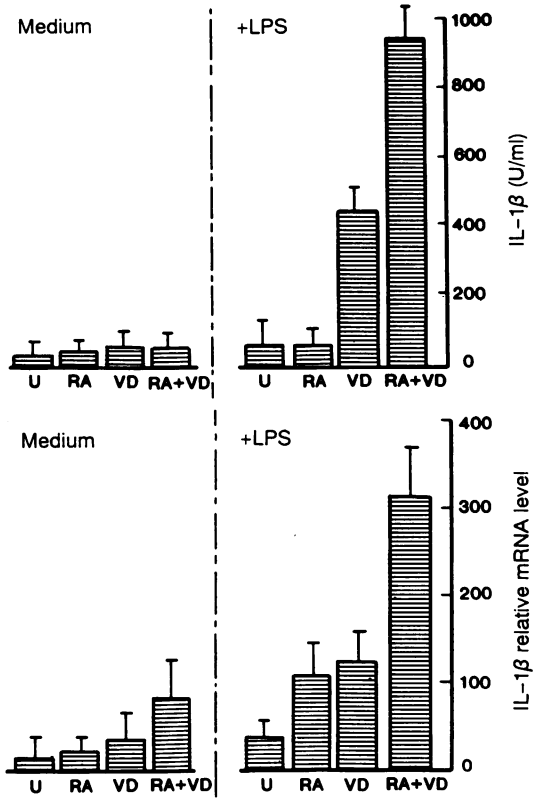

Figure 2. Expression of IL-1 bioactivity (top) and IL-1β mRNA (bottom) in U937 cells previously exposed for 4 days to different treatments (medium alone U, 100 nM RA, 100 nM VD, or 100 nM RA + VD). The induced cells were incubated with medium alone (left), or with LPS (right) for 5 hr for RNA recovering, or 24 hr for IL-1 assay. Dot blot of total RNA was prepared and hybridized with IL-1β probe, the autoradiographs were quantified densitometrically, and the signals were integrated and normalized to GAPDH mRNA signals. Cell supernatants were collected and assayed for IL-1 activity as described in Materials and Methods. Each bar represents the arithmetic mean $\pm$ SD of three independent experiments.

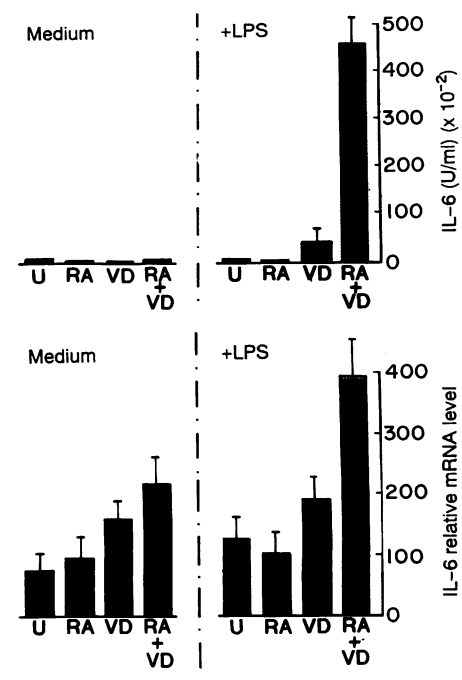

Figure 3. Expression of IL-6 bioactivity (top) and IL-6 mRNA (bottom) in U937 cells previously exposed for 4 days to different treatments (medium alone, 100 nM RA, 100 nM VD, or 100 nM RA + VD). The induced cells were subsequently incubated with medium alone (left), or with 25 μg/ml LPS (right). Dot blot of total RNA was prepared and hybridized with IL-6 probe, the autoradiographs were quantified densitometrically, and the signals were integrated and normalized to GAPDH mRNA signals. Cell supernatants were collected and assayed for IL-6 activity using the B-9 cell line. Each bar represents the arithmetic mean $\pm$ SD of three independent experiments.

increased mRNA synthesis and caused a release of large amounts of monokines. Compared to the protein levels measured with VD alone, a twofold increase was observed for IL-1β, an approximate fourfold increase for TNF-α and more than an 11-fold increase for IL-6 were observed in RA/VD-treated cells.

Comparison between U937, HL-60 and THP-1 cells

Our previous studies (Table 1) suggested that the synergy between RA and VD may be a specific feature of U937 cells. Experiments performed with HL-60 and THP-1 cells showed that RA or VD alone can prime these cells to the zymosan-induced oxidative burst. However, although this zymosan-induced oxidative burst was enhanced when RA and VD were used in combination, the effect of both vitamins was no more than additive. Moreover, these cells did not display FMLP-induced chemiluminescence.

The enhancement of monokine production seen with U937 cells was not observed with the two other cell lines. LPS-treated HL-60 cells released IL-6 only when preincubated with VD. Preincubation with RA reduced the very low production detected with LPS-stimulated undifferentiated HL-60 cells. A combination of RA and VD did not increase the production of IL-6. Without any differentiation treatment, THP-1 cells were able to release IL-6 upon LPS stimulation. Incubation with RA alone reduced this production, while treatment with VD alone increased it. A combination of both agents resulted in a small increase over the level produced by VD-treated cells. The amounts of IL-6 released by THP-1 were of the same order of magnitude as that observed in the RA/VD-differentiated U937 cells. Similarly, the addition of RA during the VD treatment of THP-1 or HL-60 cells did not increase their ability to produce TNF-α or IL-1β upon LPS activation. Moreover, RA which promoted a negative effect when it was used alone, slightly decreased the capacity of the VD-induced THP-1 cells to release TNF-α or IL-1β in response to the endotoxin.

DISCUSSION

Proliferating U937 cells had low (or no) levels of cytokine expression, and did not spontaneously respond to LPS stimulation. These data are consistent with the immature character of the cells.[19]

A 4-day VD treatment of U937 cells promoted a significant increase of monokine mRNA in these cells. These results are in accordance with our previous data[20] and those of others,[21,22] demonstrating that VD induces U937 cells towards monocyte/macrophage lineage.[21,22] However, since VD alone failed to trigger protein expression, it is probable that an event or events

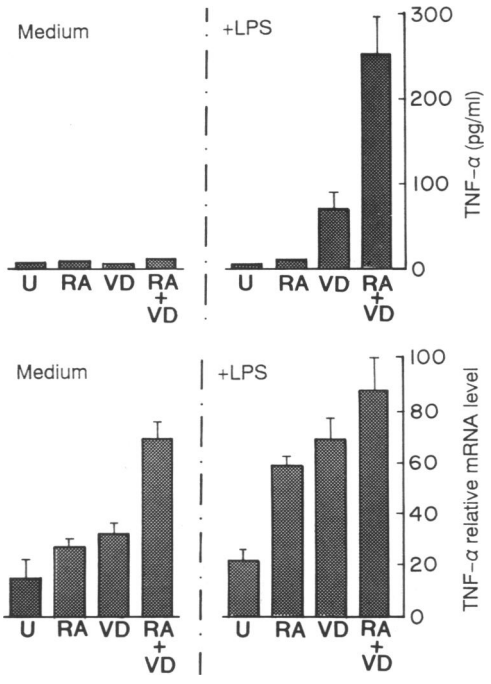

Figure 4. Expression of TNF bioactivity (top) and TNF-α mRNA (bottom) in U937 cells previously exposed for 4 days to different treatments (medium alone U, 100 nM RA, 100 nM VD, or 100 nM RA + VD), in the absence (left) or the presence (right) of 25 μg/ml LPS. The cells at 10^6 cells/ml which had been pretreated with the different agents, were incubated with LPS for 5 hr for RNA recovering, or 24 hr for TNF bioactivity assay. Dot blot of total RNA was prepared and hybridized with a TNF-α probe, the autoradiographs were quantified densitometrically, and the signals were integrated and normalized to GADPH mRNA signal. Cell supernatants were tested for TNF activity in the L929 lytic assay as described in Materials and Methods. Each bar represents the arithmetic mean ± SD of three independent experiments.

other than those induced by VD are necessary to allow the post-transcriptional cascade to result in monokine secretion.[23] VD-treated U937 were primed to produce the post-transcriptional event(s): they released biologically active monokines in response to LPS. This demonstrates that VD-triggered U937 cells reached a transient macrophage intermediate state where they became LPS sensitive, the monokine processing occurring during the terminal step of maturation induced by LPS. It has been shown elsewhere that VD has no effect on IL-1β and TNF-α gene expression although it causes a synergistic increase in PMA-treated U937 cells.[24] The shorter period of VD treatment (18 hr) used in this study certainly explains the discrepancy between these results and ours. On the other hand, our findings are in accordance with a positive effect on IL-6 production observed when normal monocytes were cultured in the presence of VD.[11]

RA alone is claimed to have little effect on a differentiation inducer.[25] In RA-treated cells, the expression of cytokine genes was unchanged, but LPS increased IL-1β and TNF-α mRNA expression in these cells. However, the cells were not primed to the secretion of monokines in response to endotoxin. Moreover, the low amount of IL-6 produced by LPS-stimulated U937 cells was reduced by RA pretreatment, and a similar decrease was also detected in HL-60 cells and THP-1 cells (Table 1). IL-1β and TNF-α secretion were also inhibited in RA-pretreated THP-1 cells. Recently, Sidell *et al.* have also shown that RA treatment of U937 cells decreases IL-6 receptor expression of these cells.[26] As a direct negative effect of RA on the post-transcriptional events is unlikely, it seems that RA differentiate the U937 cells into a cell stage able to give an oxidative response to opsonized zymosan but unable to produce monokines upon LPS action. Our data support a role of RA in U937 cell differentiation, and suggest that RA alone is able to give a signalling pathway leading to an ill-defined intermediate stage of differentiation.

The RA-induced effect contrasted with the strong production of cytokines observed in U937 cells preincubated with the combination of RA + VD and then stimulated by LPS. The co-treated U937 cells had increased levels of cytokine mRNA compared to those treated by each agent alone, but above all they acquired a marked responsiveness to LPS. RA, which did not prime the cells to release the cytokines in response to endotoxin, synergized with VD to exert such an effect. This synergistic action of the two vitamins is supported by the oxidative burst response of the RA/VD-treated cells to FMLP, each agent alone being unable to induce response to chemotactic peptide (Table 1). Our previous reports[14,20] have shown that whatever the differentiation marker assayed (oxidative response, expression of the CD11b/CD18 or CD14 antigens, proliferation, morphological or functional changes) RA was found to enhance the VD-evoked effect on U937 cells. In fact, it seems that RA potentiated the VD-induced maturation of U937 cells with, as a result, monocyte/macrophage characteristics.[20] The present data on cytokine expression greatly support this possibility, the ability to produce cytokines being one feature of the monocyte/macrophage cell lineage. It has been reported that upon phorbol 12-myristate 13-acetate (PMA) differentiation U937 cells produced IL-1β and TNF-α but not IL-6.[19] Nevertheless, we observed that after LPS activation, PMA-differentiated cells produced as much IL-6 as those differentiated by RA + VD (M. Taimi *et al.*, submitted for publication). This establishes that the cells differentiated by both pathways are in fact primed to secrete IL-6 upon a secretory activation signal.

Leukaemogenesis may occur at distinct stages of development and the differences between U937 and other cell lines are probably linked to their respective levels of myeloid differentiation.[13] HL-60 resembles non-leukaemic human promyelocytes. The lack of synergy between RA and VD in this cell line is in agreement with numerous data establishing that these agents drive the cells along different pathways, inducing respectively granulocyte-like and monocyte-like phenotypes.[12] In THP-1 cells, which are more mature and display monoblastic features,[27] RA in association with VD caused zymosan-induced oxidative burst, a small increase in IL-6 production and a decrease in TNF-α or IL-1β secretion. Therefore, it appears that RA and VD which taken separately are capable of changing the phenotype of the three cell lines, act synergistically only in U937 cells (Table 1). Since leukaemic cells retain numerous properties of their non-leukaemic counterparts, the results presented here may indicate that the synergy occurs at a specific stage of normal cell development.

In U937 cells, RA is capable of exerting two kinds of events, one which brought the cells into an early ill-defined state of differentiation, a second which synergistically potentiated the VD-induced effect. Recent data on the molecular mechanism of action of RA and VD support the notion that these two agents

may display co-operative effects.[28] Both molecules bind to specific nuclear receptors (RAR or RXR and VDR) which are expressed in myeloid cells[29,30] and act as transcription regulators. These receptors belong to the steroid and thyroid hormone (T3) superfamily of transcription factors.[31] The DNA-binding sites of all these receptors are themselves structurally related[32] and some members of this family have been shown to form heterodimers, thereby increasing their activity on gene transcription.[33] For example, the RXR receptor, the ligand of which is the 9-*cis* RA resulting from the endogeneous isomerization of the all-*trans* retinoic acid,[33] forms a heterodimer RXR–VDR, and may play a central role in modulating the response conferred through VDR activation.[34] Furthermore, a co-operative effect between T3 and RA has recently been described in erythroid differentiation.[35] It is therefore possible that our data provide another example of this type of mechanism, through which RA, poorly potent by itself, may co-operate with VD to modulate monocytopoiesis and control the acquisition of LPS responsiveness in monocytes/macrophages.

In conclusion, our results on myelomonocytic cell maturation[14,20] and cytokine expression underscore the fact that studies on the immunomodulatory effects of RA and VD must take into account the possibility of combinatorial effects of these compounds. Such combinatorial effects could have therapeutic implications in the treatment of some kinds of myelomonocytic leukaemia.

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