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All-trans Retinoic Acid Stimulates IL-2-Mediated Proliferation of Human T Lymphocytes: Early Induction of Cyclin D3

Nikolai Engedal,* Tone Gjevik,* Rune Blomhoff,† and Heidi Kiil Blomhoff2,*

Vitamin A is established as an important immune regulator, but the mechanisms whereby vitamin A regulates T cell biology are poorly defined. In this study, we show that an active metabolite of vitamin A, all-trans retinoic acid (RA), potently stimulates T cell proliferation by modulating IL-2-mediated signaling downstream of IL-2R and independent of the induction of IL-2. Thus, at concentrations as low as 0.1 nM, RA enhanced the division of normal human T lymphocytes that were simultaneously stimulated with anti-CD3 mAbs and saturating concentrations of IL-2. At the optimal concentration of RA (50 nM), a 3-fold increase in T cell proliferation was observed. The induced proliferation was preceded by increased phosphorylation of the retinoblastoma protein and enhanced G1-to-S-phase progression. Interestingly, the promitogenic effect of RA was found to be particularly directed toward increased expression of cyclin D3 at both the mRNA and protein level. Furthermore, the stimulatory effect of RA on cyclin D3 expression as well as on cell proliferation was completely abolished in the presence of the JAK inhibitor AG-490 or blocking IL-2Rα mAbs, and RA also enhanced cyclin D3 expression and T cell proliferation in the presence of IL-2 alone. Finally, we showed that the proliferative effect of RA was mimicked by agonists of the retinoic acid receptor (RAR) and completely inhibited by a RAR-selective antagonist. In conclusion, our results indicate that RA, via RAR, stimulates IL-2-induced signaling in a JAK-dependent manner to enhance cyclin D3 expression and thereby promote T cell proliferation. The Journal of Immunology, 2006, 177: 2851–2861.

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1 Abbreviations used in this paper: RA, all-trans retinoic acid; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; PI, propidium iodide; PKB, protein kinase B; pRB, retinoblastoma protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; TTNPB, 4-(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-1-2-naphthalenyl)-1-propenylbenzoic acid.

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G₁ to S-phase progression and proliferation by enhancing the production of IL-2 (6, 7). However, in our previous studies, we had observed that RA also had a significant, albeit less potent effect on the proliferation of T cells under IL-2-R-saturating conditions (6). Moreover, the amount of exogenous IL-2 needed to mimic the effect of RA was higher than that of IL-2 that was actually produced by RA (6). These observations led us to hypothesize that in addition to modulating TCR/CD3-initiated signals and thereby increasing IL-2 production, RA may also stimulate IL-2-induced mitogenic signaling per se. In the present study, we tested this hypothesis by assessing the effect of RA on highly purified human peripheral blood T lymphocytes that were simultaneously stimulated with anti-CD3 mAbs and high concentrations of IL-2. We found that T cell proliferation was potently enhanced by physiologic concentrations of RA, and that this event was closely linked to an early induction of cyclin D3 at both the RNA and protein level. Furthermore, we found that the effect of RA was mediated by the retinoic acid receptor (RAR) and that it was completely dependent on IL-2-induced JAK activity.

Materials and Methods

Reagents

RA, 4-(E)-2-(5,6,7,8-tetrahydro-5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTPNB), Am580, phosphodiode (PI), and BrdU were from Sigma-Aldrich. Ro 41-5253 was provided by M. Klaus (Hoffmann-LaRoche, Basel, Switzerland). Retinoids were dissolved in ethanol or DMSO, flushed with argon, and stored in lightproof containers at −20°C. Experiments with retinoids were performed in subdued light. RA stability was checked weekly on the basis of UV spectra. Human rll-2, anti-IL-2Rα mAb (MAB223), and isotype-matched control mAb (MAB002) were from R&D Systems. AG-490 was provided by Ciba-Geigy. OKT-3 (anti-CD3 mAb) was purified from OKT-3-producing hybridoma supernatant. Anti-phospho-Ab (G3-254) was from BD Pharmingen. Abs against p27KIP1 (C-19), p21WAF1 (C19), STAT5 (C-17), C-Myc (9E10), cyclin D2 (C-17), E (HE12), and A (C-19) were from Santa Cruz Biotechnology. Anti-cyclin D3 mAb (DSC-22) was from MBL. Abs against phospho-STAT3 (Tyr705, 9135), phospho-STAT3 (Ser727, 9134), STAT3 (9132), phospho-STAT5 (Tyr904, 9351), phospho-Akt (Ser473, 9271), phospho-p44/42-MAK (9101), and p44/42-MAK (9102) were from Cell Signalling Technology.

T lymphocyte isolation, culture, and treatment

PBLs were isolated from healthy human blood donors (Ullevaal Hospital Blood Bank), as previously described (6). Next, CD4¹ T lymphocytes were positively selected by use of magnetic CD4 MicroBeads (Miltenyi Biotec), as described by the manufacturer. More than 98% of the isolated cells were positive for CD3 and CD4, as measured by flow cytometry using FITC- or PE-conjugated Abs against CD3 (SK7) and CD4 (SK3) (BD Biosciences). T cells were cultured in standard 96- or 24-well flat-bottom microtiter plates (BD Biosciences; Falcon 3072 and 3047) at 1×10⁶ cells/ml in RPMI 1640 supplemented with 2 mM glutamine, 125 U/ml penicillin, and 125 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. In some experiments, the culture medium was in addition supplemented with heat-inactivated FBS to final concentrations of 2.5 or 10% (v/v). OKT-3, IL-2, and RA were added simultaneously at the start of the cultures. Unless otherwise stated, the final concentrations of OKT-3, IL-2, and RA were 2.5 μg/ml, 4 ng/ml (~250 pM), and 50 nM, respectively.

Determination of retinol concentration in FBS

The retinol concentration in heat-inactivated FBS was determined by HPLC analysis, as previously described (28). The FBS did not contain detectable amounts of RA.

Cell proliferation assays

To determine cell divisions, freshly isolated T lymphocytes were stained with CFSE (Molecular Probes), as previously described (7), except that the final concentration of CFSE was 0.15 μM. Cell cycle distribution was analyzed by incubating cells with BrdU (10 μM) for 1 h, followed by staining with a FITC-conjugated anti-BrdU Ab (556028; BD Pharmingen) and PI, as previously described (29). For determination of DNA synthesis, T cells were cultured in triplicates in 96-well plates, pulsed with 1.25 μCi of [3H]thymidine (TRA120; Amersham Biosciences) for 18 h, transferred to Unifilter-96 GF/C filters (PerkinElmer) with a Packard FilterMate cell harvester, and counted on a Topcount liquid scintillation counter (Packard Instrument) using 25 μl of MicroScint mixture (Packard Instrument) per well.

Analysis of CD69, CD25, CD122, and CD132 cell surface expression

For Western blot analyses, T cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, as previously described (29). Protein concentrations were determined by Bradford assay (Bio-Rad) of duplicate samples and equalized with RIPA buffer. Equal amounts of protein were loaded onto SDS-polyacrylamide gels, and Western blot analyses were performed, as previously described (30). Northern blot analyses were performed, as previously described (30). RNA was isolated from 1×10⁶ T cells using the RNeasy Mini Kit (Qiagen). The probes were as follows: for cyclin D3, a 1.4-kb EcoRI/BamHI fragment from pEFl-Hist-cyclin D3 (see Ref. 45), and for c-myc, a 1.4-kb ClaI/EcoRI fragment from pMC41-3RC (31).

Statistical analyses

SPSS12.0.1 for Windows was used to perform paired samples t test analyses.

Results

RA potentiates T cell proliferation induced by OKT-3 and IL-2

We wished to determine whether RA is able to modulate IL-2-induced mitogenic signaling in T lymphocytes. Previously, we had shown that RA stimulates IL-2 production in isolated human T cells (6, 7). To avoid the contribution to T cell proliferation caused by RA-induced IL-2 production in the present study, we added IL-2-R-saturating concentrations of human rll-2 (4 ng/ml) (32) together with anti-CD3 mAbs (OKT-3) at the beginning of the cultures. In initial experiments performed in the presence of 10% FBS, we found that RA treatment resulted in a slight increase in T cell proliferation (data not shown and Fig. 1A). FBS is known to contain the parent vitamin A compound retinol (33, 34), which may be intracellularly metabolized to RA and thereby mask the effect of exogenously added RA. The retinol content in a given batch of FBS will vary depending on, for example, conditions of storage and handling. Because the heat-inactivated FBS used in the present study was found to contain ~0.6 μM retinol (data not shown), we decided to compare the effect of RA in the absence or presence of FBS. Cell divisions were tracked by CFSE staining after 4 days of culture, as previously described (7). Only a slight effect of RA was observed in the presence of 2.5 or 10% FBS (Fig. 1A). In the absence of FBS, however, RA potently enhanced T cell proliferation induced by OKT-3/IL-2. Thus, under serum-free conditions, a >3-fold increase in the percentage of divided cells was observed in the presence of RA (Fig. 1A). Serum-free conditions were therefore used in the subsequent experiments to study the effect of RA on T cell proliferation.
RA potentiates T cell proliferation in a dose- and time-dependent manner

Using the CFSE-staining technique, we tested the dose dependency of the effect of RA on OKT-3/IL-2-induced T cell proliferation. RA enhanced T cell proliferation at doses as low as 0.1 nM, whereas optimal effects were seen at 10–100 nM (Fig. 1B). At 1 μM, the effect of RA was essentially lost (Fig. 1B), probably due to the increased cell death observed at this concentration of RA (data not shown). T cell division was not stimulated by RA alone (Fig. 1B). The dose-dependent effect of RA was confirmed by measurement of DNA synthesis; as shown in Fig. 1C, optimal uptake of [3H]thymidine was obtained at 100 nM RA, and again effects of RA were noted at very low concentrations. In fact, in these experiments, effects of RA were observed already at 10 pmol (Fig. 1C).

Next, we examined the kinetics of the RA effect. CFSE staining was used to track cell divisions at various time points after T cell stimulation. As shown in the dot blots from one representative experiment (Fig. 1D), T cells cultured in medium alone did not undergo cell division. No cell division was observed in stimulated T cells at day 1 or 2 (data not shown), but at day 3 RA had induced one round of cell division in a proportion of T cells (Fig. 1D). At day 4, a larger proportion of T cells had divided once and a comparable proportion of the cells had undergone two cell divisions in the presence of RA. Thus, 42% of RA-treated T cells had divided.
compared with only 10% in the absence of RA. After 5 days, 52% of RA-treated cells had divided, among which a substantial proportion of cells had undergone three cell divisions. In comparison, only 21% of the cells had divided in the absence of RA (Fig. 1D).

FIGURE 2. RA stimulates the cell cycle machinery and S-phase entry in OKT-3/IL-2-treated T cells. A, T cells were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA. After 24, 48, or 72 h, whole cell extracts were prepared, and Western blot analyses, using Abs against the indicated proteins, were performed, as described in Materials and Methods. Hypo- and hyperphosphorylated pRB proteins are indicated as pRB and ppRB, respectively. One representative experiment of three is shown. B, T cells were cultured in medium alone, or stimulated with OKT-3, IL-2, and RA, as indicated. At days 1 or 2, cells were pulsed for 1 h with BrdU and analyzed for BrdU incorporation and DNA content, as described in Materials and Methods. Dead cells were excluded from the dot plots by gating for living cells on forward/side scatter plots (data not shown). Percentages of cells in S phase are indicated. One representative experiment of three is shown. C, The same CFSE-stained T cells that had been analyzed for percentages of divided cells in Fig. 1E were now analyzed for number of discernible cell divisions from dot plots, such as the example shown in Fig. 1D. Average numbers of cell divisions ± SEMs at days 3, 4, and 5 for T cells from the eight different blood donors are shown.

FIGURE 3. AG-490 inhibits T cell proliferation, cyclin D3 expression, and late IL-2Rα, β, and γ expression, but not the early induction of IL-2Rα. A, T cells were treated with various concentrations of AG-490 as indicated and either cultured in medium alone, or stimulated with OKT-3 and IL-2 in the absence or presence of RA. After 48 h, cells were pulsed with [3H]thymidine for 18 h, as described in Materials and Methods. A representative experiment of three is shown. B, T cells were either cultured in medium alone, OKT3/IL-2, or OKT-3/IL-2/RA, respectively. After immunopробing with cyclin D3- or phospho-STAT3-specific Abs, each membrane was stripped and reprobed with Abs recognizing total ERK (tot-ERK) or total STAT3 (tot-STAT3) proteins, respectively, to control for equal loading. One reproducible experiment of two is shown. C and D, T cells were treated with OKT-3, IL-2, RA, and AG-490 (20 μM), as indicated, and analyzed for cell surface expression of CD25 (C) after 24 or 72 h (bold lines), or CD122 and CD132 (D) after 72 h (bold lines). In each case, FITC- or PE-conjugated isotype-matched control Abs were included as negative controls (dotted lines). CD25 positively stained cells and induced expression of CD122 or CD132 relative to untreated cells are indicated (%). The values in parentheses indicate mean fluorescence intensities of the gated cells. One representative experiment of three is shown.
Next, we assessed the effect of the enhanced cell division observed in the presence of RA could be a result of decreased cell death. As shown in Fig. 1F, RA did not affect T cell viability at day 3 and caused only a minor reduction in cell death at days 4 and 5. Thus, the effect of RA on T cell division could not be explained by inhibition of cell death.

RA stimulates the cell cycle machinery and S-phase entry in T cells

Multiple phosphorylation of the pRB protein is a major requirement for G₁ to S-phase transition (14). Hyperphosphorylated forms of pRB can be detected by their slower migration in SDS-polyacrylamide gels. As shown in Fig. 2A, pRB phosphorylation was strongly enhanced by RA; the effect of RA was first notable at day 2, and at day 3 pRB was predominantly observed in its hyperphosphorylated forms in the presence of RA.

Phosphorylation of pRB is mediated by CDKs, whose activities are positively and negatively regulated by cyclins and CKIs, respectively. Each cyclin family comprises several members. T cells express cyclin D2, D3, E1, E2, and A2 (8, 9). As shown in Fig. 2A, the protein levels of all these cyclins were clearly enhanced upon OKT-3/IL-2 treatment. Whereas cyclin D2 expression was unaffected by RA, cyclin D3 protein levels were increased by RA early at day 1, and at days 2 and 3 a strong up-regulation was observed (Fig. 2A). Cyclin E levels were unaffected by RA, whereas cyclin A expression was considerably up-regulated by RA at days 2 and 3. p27Kip1 levels were markedly, and in a time-dependent manner reduced by OKT3/IL-2 treatment (Fig. 2A). RA augmented this effect, with a minor effect noted at day 2, and a more evident, but still relatively modest effect observed at day 3 (Fig. 2A). The expression of p21Cip1 was unaffected by RA (data not shown). In conclusion, the earliest and most striking effect of RA on the cell cycle machinery was enhanced expression of cyclin D3, followed by increased pRB phosphorylation.

To confirm that the observed effect of RA on the cell cycle machinery was due to RA stimulating S-phase entry rather than enhancing the rate of cell division among dividing cells, we directly determined percentages of cells entering S phase after T cell stimulation by use of BrdU incorporation and PI staining (29). As shown in Fig. 2B, the first T cells entered S phase at day 2 after stimulation with OKT-3/IL-2. In the presence of RA, the percentage of cells in S phase was clearly increased (from 4.8 to 15.5%; Fig. 2B), demonstrating that RA indeed stimulated S-phase entry. That RA stimulated S-phase entry rather than enhancing the rate of cell division among dividing cells was further confirmed by examining the number of cell divisions that had occurred over time. When the same cells as those described in Fig. 1E were analyzed for number of cell divisions, we found that the rate of division was not significantly enhanced by RA after day 3, i.e., after the first cell division had taken place (Fig. 2C). Thus, we concluded that RA exerts its effects on T cell proliferation primarily by stimulating the cell cycle machinery and S-phase entry during the first cell division.

RA-mediated enhancement of T cell proliferation and cyclin D3 expression is dependent on IL-2-induced signaling

By using IL-2R-saturating concentrations of IL-2, we had ruled out the possibility that RA stimulated the cell cycle machinery and T cell proliferation by increasing IL-2 production. Still, we could not exclude the possibility that RA mediated its effect by modulating TCR/CD3-, and not IL-2-induced signaling. To elucidate which of these two signaling pathways was affected by RA, we used a specific inhibitor of JAK, AG-490 (35). By inhibiting the activity of JAK-3, AG-490 efficiently blocks mitogenic signaling from IL-2R (35). First, we assessed the effects of various concentrations of AG-490 on OKT-3/IL-2-induced T cell proliferation. AG-490 inhibited T cell proliferation in a dose-dependent manner, both in the absence and presence of RA (Fig. 3A), without affecting cell viability (data not shown). Moreover, the potency of RA to enhance T cell proliferation was gradually diminished with increasing concentrations of AG-490. Thus, whereas RA induced a 4-fold increase in [³H]thymidine uptake in the absence of the JAK inhibitor, only 2.9-, 1.8-, and 1.2-fold increases were observed in the presence of 2.5, 10, or 25 µM AG-490, respectively (Fig. 3A).

Next, we assessed the effect of JAK inhibition on the expression of cyclin D3. Strikingly, as shown in Fig. 3B, OKT-3/IL-2-induced cyclin D3 expression was almost totally blocked by AG-490, both in the absence and presence of RA. To verify that AG-490 inhibited JAK activity, we assessed the effect of the inhibitor on STAT3 tyrosine phosphorylation, which is known to be mediated by JAKs (36). As shown in Fig. 3B, OKT3/IL-2-induced tyrosine phosphorylation of STAT3 was indeed markedly reduced by AG-490.

TCR/CD3-induced signaling leads to a rapid up-regulation of IL-2Rα on the T cell surface (22). Thus, if AG-490 only blocks signaling from IL-2R and not from the TCR/CD3 complex, the early expression of IL-2Rα should not be inhibited by AG-490. Indeed, AG-490 did not affect the cell surface expression of IL-2Rα at day 1 (Fig. 3C). Moreover, IL-2Rα expression was unaffected by RA. We also examined expression of the activation marker CD69, whose early expression is mediated by TCR/CD3-induced signaling (37). Like IL-2Rα, early CD69 expression was not inhibited by AG-490 and was also unaffected by RA (data not
shown). Once IL-2Rα is expressed, its levels can be further up-regulated by IL-2-induced signals (10). Thus, at day 3, it is expected that the expression of IL-2Rα is at least partly regulated by IL-2-induced signaling. Indeed, we found that the cell surface expression level of IL-2Rα was partly inhibited by AG-490 at day 3 (Fig. 3C). Moreover, although RA did not increase the percentage of IL-2Rα-positive cells, the average intensity of the staining was doubled, and this effect was totally blocked by AG-490 (Fig. 3C). Cell surface expression levels of IL-2Rβ and γ are enhanced upon T cell activation and may be regulated by IL-2-induced signaling (20–22). Thus, we examined whether RA affected β- and γ-chain expression in our system. Strikingly, the expression of both IL-2Rβ and γ was substantially increased by RA at day 3, and in both cases this effect was completely abolished by AG-490 (Fig. 3D). These results indicate that RA stimulates T cell proliferation and enhances the expression of cyclin D3 and IL-2Rα, β, and γ, by modulating IL-2-induced signaling.

Although the JAK inhibitor AG-490 efficiently repressed IL-2-induced signaling, while leaving TCR-induced signaling unaffected, we could not formally exclude the possibility that AG-490 was repressing the activity of other bioactive molecules than IL-2, which were mediating the effects of RA. Therefore, to directly test the dependency of RA on IL-2-induced signaling, we used a specific mAb against IL-2Rα, which blocks signaling downstream of the high affinity IL-2R. As shown in Fig. 4A, the blocking IL-2Rα mAb completely inhibited the effect of RA on OKT-3/IL-2-induced DNA synthesis. Moreover, OKT-3/IL-2-induced DNA synthesis was by itself almost fully repressed, indicating that T cell proliferation under our conditions was almost entirely dependent on signaling from IL-2R. Next, we tested the effect of the IL-2Rα-blocking mAb on cyclin D3 expression. As shown in Fig. 4B, the Ab against IL-2Rα totally repressed OKT-3/IL-2-induced cyclin D3 expression, both in the absence and presence of RA. Thus, we concluded that the effect of RA on cyclin D3 expression and T cell proliferation was indeed strictly dependent on IL-2-induced signaling.

RA stimulates signaling induced by IL-2 alone

Resting T cells express IL-2Rs with intermediate IL-2 affinity (consisting of IL-2Rβγ heterodimers) (18, 19). Therefore, high concentrations of exogenously added IL-2 can induce some proliferative IL-2R-mediated signaling in the absence of T cell-activating stimuli (18, 38). Thus, we tested whether RA could stimulate the proliferation of T cells that were stimulated with IL-2 alone. Indeed, as assessed by CFSE-staining experiments, RA enhanced T cell division in the presence of IL-2 in a dose-dependent manner (Fig. 5A). Furthermore, RA clearly potentiated the ability of IL-2 to induce DNA synthesis (Fig. 5B), S-phase entry (Fig. 5C), and cyclin D3 expression (Fig. 5D), as well as the cell surface expression of IL-2Rγ (Fig. 5E). These results demonstrate that RA is able to modulate IL-2-induced signaling also in the absence of RA.
TCR/CD3-initiated signals, and importantly that RA can enhance cyclin D3 expression and S-phase entry when acting in concert with IL-2-mediated signals alone.

RA-mediated enhancement of IL-2R expression is a relatively late event

Having established that the effects of RA on OKT-3/IL-2-induced cyclin D3 expression and T cell proliferation most likely were mediated via regulation of IL-2-induced signaling, we next studied the potential mechanism(s) involved. Because we had observed that RA increased the expression of all three subunits of the IL-2R at day 3 (Fig. 3), we first examined the kinetics of this RA effect. As shown in Fig. 6, none of the receptor subunits were up-regulated by RA at day 1, and only the expression of IL-2Rβ was increased at day 2. At day 3, the cell surface expression levels of both IL-2Rβ and γ were strongly increased (Fig. 6, B and C). The percentage of cells staining positive for IL-2Rα was not enhanced by RA (Fig. 6A). However, the average intensity of positively stained cells was substantially increased at day 3, but not at day 1 or 2 (data not shown and Fig. 3C). These results indicate that RA-mediated enhancement of IL-2R expression is a relatively late event, making it unlikely that increased IL-2R expression can explain the early and strong effects of RA on cyclin D3 expression.

RA does not affect OKT-3/IL-2-induced phosphorylation of STAT5 or STAT3

JAK-mediated tyrosine phosphorylation of STAT5 is a proximal IL-2-induced signaling event that is involved in IL-2-mediated T cell proliferation (23, 26, 27). We therefore examined whether RA-induced enhancement of cyclin D3 expression and T cell proliferation could be mediated by increased STAT5 phosphorylation.

As shown in Fig. 7A, STAT5 was strongly phosphorylated after 1 day of OKT-3/IL-2 treatment, and the level of phospho-STAT5 proteins remained high at days 2 and 3. However, at neither of these time points was STAT5 phosphorylation enhanced by RA. STAT3 requires both tyrosine and serine phosphorylation for full activation (39). As shown in Fig. 7A, both tyrosine and serine phosphorylation of STAT3 was strongly induced by OKT-3/IL-2. However, RA did not affect either phosphorylation events at any of the time points examined.

We also examined STAT5 phosphorylation at earlier time points. Elevated levels of phospho-STAT5 proteins were observed already after 15 min of OKT-3/IL-2 treatment and peaked between 2 and 24 h (Fig. 7B). However, RA did not increase phospho-STAT5 levels at any of the time points examined (Fig. 7B). In separate experiments, and in accordance with a previous study (26), the early phosphorylation of STAT5 was found to be induced by IL-2 and not by OKT-3 (data not shown).

RA does not affect OKT-3/IL-2-induced phosphorylation of PKB/Akt or MAPK/ERK, but enhances the expression of c-Myc

IL-2R ligation activates the PI3K-PKB/Akt and Raf-MAPK/ERK pathways, both of which have been reported to up-regulate the

![Figure 6](image-url) Effect of RA on the cell surface expression levels of IL-2Rα, β, and γ. T cells were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA. After 24, 48, or 72 h, whole cell extracts were prepared, and Western blot analyses, using Abs against the indicated proteins, were performed. After immunoprecipitation with phospho-STAT-specific Abs (pSTAT), each membrane was stripped and reprobed with Abs recognizing total STAT5 (tot-STAT5) or total STAT3 (tot-STAT3) proteins. B, T cells were either harvested immediately after isolation (C), or stimulated with OKT3 and IL-2 in the absence (OI) or presence (OIR) of RA. At the indicated time points, whole cell extracts were prepared, and Western blot analyses, using Abs against the indicated proteins, were performed. One representative experiment of four is shown. C, T cells were either harvested immediately after isolation (C), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA. After 24, 48, or 72 h, total cellular RNA was isolated and subjected to Northern blot analysis of c-myc mRNA. The ethidium bromide-stained 28S and 18S rRNA bands are shown as loading control. One representative experiment of three is shown.

![Figure 7](image-url) RA enhances cyclin D3 mRNA levels and c-Myc protein and mRNA levels, but does not affect OKT3/IL-2-induced phosphorylation of STAT5, STAT3, PKB/Akt, or MAPK/ERK. A, T cells were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA. After 24, 48, or 72 h, whole cell extracts were prepared, and Western blot analyses, using Abs against the indicated proteins, were performed. After immunoprecipitation with phospho-STAT-specific Abs (pSTAT), each membrane was stripped and reprobed with Abs recognizing total STAT5 (tot-STAT5) or total STAT3 (tot-STAT3) proteins. B, T cells were either harvested immediately after isolation (C), or stimulated with OKT3 and IL-2 in the absence (OI) or presence (OIR) of RA. At the indicated time points, whole cell extracts were prepared, and Western blot analyses, using Abs against the indicated proteins, were performed. One representative experiment of four is shown. C, T cells were either harvested immediately after isolation (C), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA. After 5 h, total cellular RNA was isolated and subjected to Northern blot analysis of c-myc mRNA. The ethidium bromide-stained 28S and 18S rRNA bands are shown as loading control. One representative experiment of three is shown.
expression of D-type cyclins (40, 41). We therefore asked whether RA under our experimental conditions may enhance cyclin D3 expression by stimulating the activities of Akt and/or ERK. Phospho-Akt protein levels were enhanced within 2 h of OKT-3/IL-2 treatment and remained up-regulated for at least 22 h (Fig. 7B). However, at neither of the time points examined was Akt phosphorylation significantly affected by RA (Fig. 7B). OKT-3/IL-2 induced a strong and sustained phosphorylation of ERK; elevated levels of phospho-ERK proteins were observed already after 15 min of stimulation and peaked between 2 and 24 h (Fig. 7B). However, RA did not significantly alter phospho-ERK levels at any of the time points examined (Fig. 7B). Together, these results suggest that RA-mediated induction of cyclin D3 neither involves the PI3K-PKB/Akt nor the Raf-MAPK/ERK pathway. In support of this conclusion, we found that neither wortmannin (a PI3K inhibitor) nor U0126 (a MEK inhibitor) could diminish the effect of RA on cyclin D3 expression (data not shown). Furthermore, we found that these inhibitors did not affect the ability of RA to stimulate OKT3/IL-2-induced DNA synthesis (data not shown), suggesting that the potentiating effect of RA on IL-2-induced T cell proliferation is independent of both Akt and ERK.

e-Myc is a transcription factor that is often implicated in the promotion of cell proliferation. The expression of e-Myc is up-regulated by IL-2 through a Jak3-dependent pathway (10). It was recently reported that e-Myc can enhance the expression of cyclin D3 (42). We therefore examined whether RA could enhance e-Myc expression. Protein levels of e-Myc were consistently increased within 2 h of OKT3/IL-2 treatment and remained up-regulated for at least 22 h (Fig. 7B and data not shown). In the presence of RA, e-Myc expression was even further enhanced, the effect of RA being most prominent at 5 h (Fig. 7B). RA also increased the mRNA levels of e-myc at 5 h, as assessed by Northern blot analysis (Fig. 7C). These results indicate that an early induction of e-Myc could at least in part mediate the effect of RA on cyclin D3 expression.

RA up-regulates cyclin D3 mRNA levels

It is evident that cyclin D3 protein expression can be regulated at multiple levels. These include regulation of gene transcription (43), mRNA stability (44), mRNA translation (30), and protein stability (45). To elucidate how RA enhances cyclin D3 protein levels under our experimental conditions, we determined cyclin D3 mRNA levels by Northern blot analysis. As shown in Fig. 7D, OKT3/IL-2 treatment potently induced cyclin D3 mRNA expression, and in the presence of RA the mRNA levels were markedly further increased.

The effect of RA on OKT3/IL-2-induced T cell proliferation is mediated via RAR

Most of the biological effects of RA are believed to be exerted via binding to RAR, which, when heterodimerized with the retinoid X receptor (RXR) functions as a ligand-dependent transcription factor capable of regulating several hundred genes (46). In some cases, however, RA may also act via RAR-independent mechanisms (47, 48). We assessed whether RA was involved in RAR-mediated potentiation of OKT3/IL-2-induced T cell proliferation by the use of two different RAR-selective agonists, TTNPB (49) and Am580 (50), and the RAR-selective antagonist Ro 41-5253 (51). TTNPB and Am580 almost completely mimicked the effect of RA on OKT3/IL-2-induced thymidine incorporation (Fig. 8A). Furthermore, the effect of RA was completely abolished in the presence of Ro 41-5253 (Fig. 8B). Essentially, the same results were obtained when cell proliferation was determined by the use of CFSE staining (data not shown). We therefore concluded that the effect of RA on OKT3/IL-2-induced T cell proliferation was mediated via RAR.

Discussion

In the present study, we demonstrate for the first time that vitamin A can stimulate IL-2-mediated proliferation of peripheral blood T cells. Physiological concentrations of RA potently augmented DNA synthesis and promoted the division of freshly isolated human T lymphocytes that were simultaneously stimulated with anti-CD3 mAbs and saturating concentrations of IL-2.

Of interest is that the effect of RA on T cell proliferation was observed in the absence of serum. In fact, the addition of FBS largely abrogated the RA effect. One explanation for this could be that FBS-contained retinol is metabolized by the T cells, producing enough RA to stimulate T cell proliferation under our experimental conditions. Indeed, we found that OKT-3/IL-2-induced T cell proliferation was very sensitive to RA, responding to RA concentrations as low as 10 pmol. Moreover, the FBS that was used in the present study contained ~0.6 μM retinol (data not shown), a concentration that is comparable to that found by others (33, 34). Accordingly, when studying the effects of RA or retinol on T cells, serum has frequently been omitted from the culture medium (52–54). An alternative explanation as to why the RA effect was diminished in the presence of FBS could be that serum contains factors (other than retinol) that replace and/or inhibit the effect of RA on IL-2-mediated signaling. Indeed, serum has been reported to contain factors that can either stimulate (55) or inhibit (56) IL-2-induced T cell proliferation. Irrespective of how serum affects IL-2-mediated signaling, the fact that we in the present study found RA to enhance IL-2-mediated proliferation in the absence of serum suggests that RA stimulates IL-2-induced signaling directly.
and not by modulating the effects of factors that are present in serum. It should also be noted, however, that the effect of RA was not exclusively observed in the absence of serum. Indeed, we have found that RA significantly enhances OKT-3/IL-2-induced T cell proliferation in the presence of 0.5 or 1% FBS (data not shown).

We argue that the increased T cell proliferation observed with RA in the present study is due to RA stimulating IL-2-, rather than TCR/CD3-initiated signaling. First, because we used IL-2-saturating concentrations of IL-2, a possible contribution from RA-mediated enhancement of IL-2 production was excluded. Second, in the presence of the JAK inhibitor AG-490, which blocked IL-2-induced signaling (as demonstrated by inhibition of STAT3 tyrosine phosphorylation), but not TCR/CD3-induced signaling (as demonstrated by the intact induction of early IL-2-α expression), RA-mediated enhancement of cyclin D3 expression and T cell proliferation was abolished. Third, the effect of RA on cyclin D3 expression and DNA synthesis was also abolished in the presence of blocking anti-IL-2Rα mAbs. Fourth, we found that RA increased cyclin D3 expression and T cell proliferation in the presence of IL-2 alone. Finally, our proposal that RA stimulates IL-2-mediated proliferation of peripheral blood T cells is supported by previous studies showing that RA can potentiate IL-2-induced DNA synthesis in resting and PHA-pretreated human thymocytes (57) as well as in Con A-pretreated murine splenocytes and in the murine IL-2-dependent T cell line HT-2 (58). Interestingly, RA has also been reported to enhance IL-2-induced cytolytic activity of lymphokine-activated killer cells (59).

We found that RA substantially up-regulated the cell surface expression of all three subunits of the high affinity IL-2R. In a previous study, it was proposed that RA stimulates the proliferation of murine T splenocytes through an early and transient potentiation of mitogen-induced up-regulation of IL-2Rα expression (5), and another study suggested that RA enhances the proliferation of IL-2-maintained human thymocyte lymphoblasts by augmenting the expression of both IL-2Rα and β (60). However, the data obtained in the current study indicate that the effects of RA on cyclin D3 expression and T cell proliferation are not mediated by an up-regulation of IL-2Rs. First, RA-mediated enhancement of IL-2R expression was a relatively late event; RA did not increase cell surface expression of the α- and γ-chains before day 3, and RA-induced enhancement of IL-2β expression was first noted at day 2. In comparison, RA-mediated up-regulation of cyclin D3 protein levels could be observed already at day 1. Second, if the effect of RA on T cell proliferation was mediated by induction of IL-2Rs, RA would be expected to enhance proximal IL-2 signaling, e.g., the phosphorylation of STAT proteins. However, the phosphorylation statuses of both STAT5 and STAT3 were unaffected by RA, even at day 3. This suggests that proximal IL-2 signaling was already saturated in the absence of RA. Finally, we found that RA-mediated induction of IL-2Rα, β, and γ was totally abolished when IL-2 signaling was blocked by AG-490. We therefore believe that RA-induced up-regulation of IL-2Rs is a result, rather than the cause of RA-mediated stimulation of IL-2-induced signals.

IL-2 initiates T cell proliferation by promoting G1- to S-phase transition (10). Thus, we examined potential effects of RA on essential components of the cell cycle machinery that regulate G1- to S-phase transition in T cells. Consistent with the potent enhancing effect of RA on DNA synthesis noted at day 3, the phosphorylation of pRB was strongly enhanced by RA at days 2 and 3. The earliest and most striking effect of RA was the induction of cyclin D3, which was observed already at day 1, and was very strong at days 2 and 3. Cyclin D3 may contribute to cell cycle progression in at least two ways: 1) direct activation of CDK4/6, and 2) indirect activation of CDK2 through cyclin D3-CDK4/6 complex binding and thereby sequestration of p21Cip1 and p27Kip1 (16). Down-regulation of p27Kip1 expression has been reported to be the critical IL-2-mediated event for CDK2 activation and thereby G1- to S-phase transition in murine T cells (8, 17), and interestingly, we did observe that RA modestly reduced p27Kip1 protein levels. However, the effect of RA on p27Kip1 was observed at days 2 and 3 only, and could thus well be a result, rather than the cause of G1- to S-phase transition. RA did not alter p21Cip1 protein levels, and the expression of cyclin D2 and E was also unaffected by RA. Therefore, it is tempting to speculate that RA allows CDK2 activation and pRB hyperphosphorylation predominantly by cyclin D3-CDK4/6 complex-mediated sequestration of p21Cip1 and p27Kip1. RA also increased the expression of cyclin A. However, because cyclin A gene transcription has been shown to be a downstream regulatory target of both cyclin D (61) and pRB (62), the induced expression of cyclin A could well be a consequence of RA-mediated up-regulation of cyclin D3 and enhancement of pRB phosphorylation. Interestingly, previous studies indicate that whereas the expression of cyclin D2 (9, 63, 64), E (8, 9, 17), and A (8) is strongly induced by TCR/CD3-initiated signals and only modestly induced by IL-2-initiated signals, cyclin D3 expression seems to be primarily induced by IL-2 signaling (9, 63, 64). In line with this, we found that cyclin D3 expression was almost completely abolished, both in the absence and presence of RA, when IL-2 signaling was repressed by AG-490 or blocking anti-IL-2Rα mAbs. Moreover, RA augmented cyclin D3 expression also in the presence of IL-2 alone, indicating that IL-2-induced signaling may be sufficient as well as required for the effect of RA on cyclin D3 expression.

We believe that the present study provides some important clues with respect to the molecular mechanism(s) involved in RA-mediated stimulation of cyclin D3 protein expression and T cell proliferation. First, because RAR agonists mimicked and a RAR antagonist abolished the effect of RA on T cell proliferation, we believe that RA acts primarily through RAR-dependent transcriptional regulation. Second, considering the strong induction of cyclin D3 mRNA by RA, it seems likely that RA predominantly increases cyclin D3 protein expression by enhancing its mRNA levels. Because, to our knowledge, there does not exist any RAR-responsive elements in the cyclin D3 promoter, we believe that the effect of RA on cyclin D3 mRNA expression is indirect. RAR/RXR-dependent, de novo synthesized proteins could act to modulate a single, upstream IL-2-induced signaling event or alternatively cooperate with IL-2 via multiple mechanisms. The data obtained with AG-490 indicate that the effect of RA is dependent on JAK activity. However, because the levels of phospho-STAT5 and phospho-STAT3 proteins were not increased by RA, we concluded that RA mediates its effects downstream or independently of proximal JAK-STAT signaling. Furthermore, we could exclude the involvement of two other major IL-2-induced signaling pathways, namely the PI3K-PKB/Akt and the Raf-MAPK/ERK pathways. Thus, at present, we do not have any indications that RA regulates a single, upstream IL-2-induced signaling event. Rather, it is possible that RA via RAR/RXR acts further downstream, e.g., at the level of gene transcription, to modulate the expression of IL-2-responsive genes. Interestingly, we found that the expression of c-myc, a candidate RAR/RXR target gene (46) and a known target of IL-2-induced signaling (10), was up-regulated by RA at both the RNA and protein level within 5 h after stimulation with OKT-3/IL-2. Because c-Myc has been shown to have the capacity to induce the expression of cyclin D3 (42), RA-mediated up-regulation of c-Myc could provide a mechanistic explanation for the observed induction of cyclin D3 by RA. We have put strong effort
into assessing this possibility by the use of siRNA against c-myc RNA. However, it has proven to be very difficult to obtain specific knockdown of c-myc under serum-free conditions in primary T cells, and thus we have not been able to verify our hypothesis.

In conclusion, we have demonstrated that RA potently stimulates IL-2- induced proliferation of freshly isolated T lymphocytes, and have linked the effect of RA to an early induction of cyclin D3. The elucidation of the molecular mechanism(s) involved in RA-mediated potentiation of IL-2-induced T cell proliferation may be particularly important from a physiological point of view, considering the recently described role of Ag-presenting dendritic cells in providing T cells with both IL-2 (65, 66) and RA (67) during dendritic cell-T cell interaction. IL-2 is a promising therapeutic agent for the treatment of HIV/AIDS (68) and several types of cancers (69). The results obtained in the present study imply that RA may be exploitable as an enhancer of IL-2-mediated immunotherapy.

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Disclosures

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References


