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Retinoic acids exert direct effects on T cells to suppress T_h1 development and enhance T_h2 development via retinoic acid receptors

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Abstract

The vitamin A metabolite, retinoic acid (RA), affects T_h1 and T_h2 development. The effect is partly exerted through the modulation of antigen-presenting cell functions, but it remains unclear whether RA directly exerts its effect on T cells to influence T_h1/T_h2 development. To clarify this problem, we used two experimental systems with isolated T cells *in vitro*. In one system, isolated $CD4^+CD8^+$ thymocytes differentiated into T_h1 and T_h2 by two transient stimulations with defined combinations of ionomycin and phorbol myristate acetate followed by treatment with IL-2 and IL-4 and/or IL-12. In the second system, functional differentiation was induced in purified naive CD4 T cells from DO-11.10 TCR-transgenic and RAG-2-deficient mice with cytokines and antibodies to CD3 and CD28. In both systems, all-*trans*-RA at $\geqslant$1 nM concentrations suppressed T_h1 development, but enhanced T_h2 development. 9-*cis*-RA elicited similar effects. The optimal enhancement of T_h2 development in the second system, however, was achieved with a delayed addition of RA. The presence of RA during the initial stimulation period often suppressed T_h2 development. The RA receptor (RAR) antagonists, LE540 and LE135, but not the retinoic X receptor (RXR) antagonist, PA452, inhibited the effect of RA on T_h1/T_h2 development. Accordingly, the RAR agonists, Am80 and Tp80, but not the RXR agonists, HX600 and TZ335, mimicked the effect of RA. The RXR agonists enhanced the effect of the RAR agonists only slightly, if at all. These results indicate that, via RAR, RA directly suppresses T_h1 development and directly enhances T_h2 development with its timely addition.

Introduction

Vitamin A deficiency causes immune dysfunction with IFN-γ overproduction and impaired antibody responses. There is a T_h cell imbalance with excess T_h1 and insufficient T_h2 function (1). On the other hand, high-level dietary vitamin A enhances T_h2 cytokine production and IgA responses (2,3), and is likely to decrease T_h1 cytokine production (2). The vitamin A retinol contributes to signal transduction in immune cells by serving as a cofactor to augment the activation of c-Raf and protein kinase C (4), and the vitamin A metabolite retinoic acid (RA) influences various cellular functions including proliferation, differentiation and apoptosis in a variety of cell types, including immune cells (5–7). RA bind to two families of receptors, the RA receptor (RAR) isotypes (α, β and γ) and the retinoid X receptor (RXR) isotypes (α, β and γ). 9-*cis*-RA binds to both RAR and RXR, whereas the major physiologic RA, all-*trans*-RA, binds to RAR and may bind to RXR at high concentrations (8,9). These receptors function as ligand-inducible transcription factors, mainly as RXR/RAR heterodimers (9).

Both all-*trans*-RA and 9-*cis*-RA inhibit IL-12 production in activated macrophages, and RA pretreatment of macrophages reduces IFN-γ production and increases IL-4 production in antigen-primed $CD4^+$ T cells (10). Since the antigen-presenting cell (APC)-derived cytokine IL-12 is critical for T_h1 development (11), RA may indirectly affect T_h cell differentiation. Accordingly, Hoag *et al.* (12) have recently shown that all-*trans*-RA enhanced T_h2 development through an effect on APC function in the presence of exogenous IL-4. However, Stephensen *et al.* (13) have suggested that 9-*cis*-RA and the RXR agonist, AGN194204, but neither all-*trans*-RA nor a RAR

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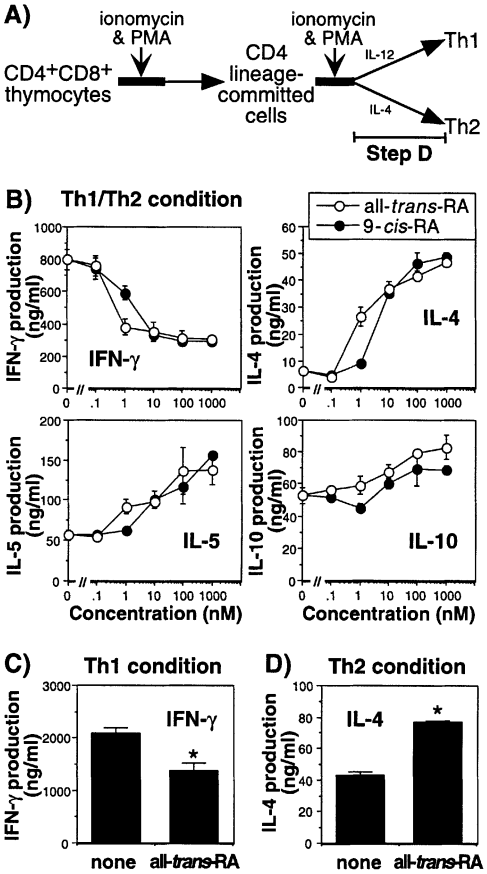

Fig. 1. Effects of all-*trans*-RA and 9-*cis*-RA on the functional differentiation of cultured thymocytes into T_h1 and T_h2. (A) CD4 lineage commitment and functional differentiation were induced in DKO mouse thymocytes *in vitro* as described in Methods. (B) Graded concentrations of all-*trans*-RA (open circles) or 9-*cis*-RA (closed circles) were added to the Step D culture in the presence of IL-4, IL-12 and IL-2 (T_h1/T_h2 condition). (C and D) All-*trans*-RA (10 nM) was added to the Step D culture either (C) in the presence of IL-12, IL-2 and anti-IL-4 mAb (T_h1 condition) or (D) in the presence of IL-4, IL-2, anti-IFN-γ mAb and anti-IL-12 mAb (T_h2 condition). After 4 days of culture, the cells were collected, washed, and re-stimulated with plate-bound antibodies to CD3 and CD28. Culture supernatants were collected after 48 h of culture, and were assessed for concentrations of IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Vertical bars represent SD in triplicate cultures. Statistical analysis was performed with Student's *t*-test and asterisks indicate that the inhibition or enhancement by RA is significant ($P < 0.001$).

agonist, enhanced T_h2 development in DO-11.10 TCR-transgenic (Tg) mouse-derived T cells bearing CD4 and the clonotypic TCR upon antigenic stimulation with APC. Furthermore, AGN194204 also enhanced T_h2 development in $CD4^+CD62L^{high}$ lymph node cells from normal C57BL/6 mice upon stimulation with antibodies to CD3 and CD28 (13), suggesting that RXR-mediated stimulation may directly enhance T_h2 development. However, $CD4^+CD62L^{high}$ cells from normal mice or even from TCR-Tg mice are likely to contain not only naive T cells, but also memory and other types of T cells (14–16). Thus, it remains unclear if RA exerts a direct effect on the development of T cells into T_h1 and T_h2 cells, and if RXR or RAR is involved in its effect.

We focused on a possible direct effect of RA on T_h cell differentiation, and used two independent culture systems to induce the functional differentiation of T cells into T_h1 and T_h2 cells in the absence of APC or other types of cells. We found that all-*trans*-RA, 9-*cis*-RA and the RAR agonists, but not the RXR agonists, suppressed T_h1 development and enhanced T_h2 development. The enhancement of T_h2 development was, however, dependent on the timing of RA addition to the culture. Furthermore, RAR antagonists, but not a RXR antagonist, canceled the effect of RA on T_h1 and T_h2 development. These results clearly indicate that, through RAR, RA can directly inhibit T_h1 development and enhance T_h2 development with their timely addition.

Methods

Mice and reagents

DO-11.10 αβTCR-Tg mice with a RAG-2-deficient and B10.D2 background, and male MHC class I and II double-knockout (DKO) mice (C57BL/6 deficient in $A\beta^b$ and β_2-microglobulin) were obtained from Taconic (Immuno-Biological Laboratories, Gunma, Japan). The mice were kept in our animal facility for at least 1 week before use. All-*trans*-RA, 9-*cis*-RA and phorbol myristate acetate (PMA) were obtained from Sigma (St Louis, MO). RA were dissolved in ethanol at 1 mM before dilution with medium and were handled under a small yellow light. Ionomycin was obtained from Calbiochem-Novabiochem (San Diego, CA). Mouse rIL-2, rIL-4 and rIL-12 were obtained from GenzymeTechne (Cambridge, MA). To express IL-2 and IL-4 activities, U was used by calculating 1 U/ml = 1 ED_{50} according to the manufacturer's definition of ED_{50}. Anti-transforming growth factor-β mAb (clone 1D11) was obtained from R & D systems (Minneapolis, MN).

Thymocyte culture

DKO mouse thymocytes were used for *in vitro* induction of the differentiation of thymocytes into CD4 T cells and T_h1/T_h2 cells. T cell differentiation is arrested at the $CD4^+CD8^+$ stage in the thymus of these mice and most of the thymocytes are $CD4^+CD8^+$ (17). Further differentiation of T cells was induced *in vitro* as previously described (18). Briefly, $CD4^+CD8^+$ thymocytes were stimulated with 0.2 μg/ml ionomycin and 0.2 ng/ml PMA for 20 h at 37°C in 24-well tissue culture plates (25820; Corning, Corning, NY). The stimulated cells were washed and further cultured in the same volume of medium without the stimuli to obtain $CD4^+CD8^{low/-}$ cells committed to the CD4 T cell lineage (19). For the secondary stimulation, the cells were cultured with 0.2 μg/ml ionomycin and 3 ng/ml PMA for 16 h. After culture, the cells were washed twice and further cultured at 1×10^6 cells/ml in medium containing 10–50 U/ml IL-2 in the presence of the indicated concentrations of IL-4 and/or IL-12 with RA and/or antagonists or agonists of RAR or RXR for 4 days (Step D culture) (Fig. 1A) (20). For inducing exclusively T_h1 development, 10 ng/ml IL-12, 1 μg/ml anti-IL-4 mAb and 50 U/ml IL-2 were added into the Step D culture (T_h1 condition for cultured thymocytes). For inducing T_h2 development, 100 U/ml IL-4, 1 μg/ml anti-IFN-γ, 1 μg/ml anti-IL-12 and 10 U/ml IL-2 were added (T_h2 condition for cultured thymocytes). For inducing both T_h1 and T_h2 development,

0.01 ng/ml IL-12, 100 U/ml IL-4 and 50 U/ml IL-2 were added (T_h1/T_h2 condition for cultured thymocytes). To assess the functional differentiation of the cells to T_h1 or T_h2, cytokines secreted upon stimulation were detected as previously described (18) using OptEIA mouse cytokine kits (BD PharMingen, San Diego, CA).

Naive T cells and culture conditions

Splenic and lymph node CD4 T cells were obtained from DO-11.10 TCR-Tg/RAG-2-deficient mice by using Dynabeads Mouse CD4 and DetachaBead Mouse CD4 (Dynal, Oslo, Norway) according to the manufacturer's instructions, and were incubated with MACS CD62L Microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). The cells were washed and applied to MS columns to obtain $CD62L^{high}$ cells according to the manufacturer's instructions. More than 99% were $CD4^+CD62L^{high}$, indicating highly purified naive CD4 T cells were obtained. Naive CD4 T cells were suspended in DMEM supplemented with 10% heat-inactivated FCS (Intergen, Purchase, NY), 3 mM L-glutamine, 1 mM sodium pyruvate, 1 × MEM non-essential amino acids, 50 μM 2-mercaptoethanol, 20 mM HEPES (pH 7.2), 20 U/ml penicillin and 20 μg/ml streptomycin, and were plated at a density of 1.5×10^5 cells/0.4 ml in 48-well suspension culture plates (MS-8048R; Sumitomo Bakelite, Tokyo, Japan) that had been coated with 3 μg/ml anti-CD3 mAb (145-2C11) (21) and 3 μg/ml anti-CD28 mAb (37.51) (BD PharMingen). Cytokines were added to wells at the initiation of culture as indicated. For inducing exclusively T_h1 development, 4 ng/ml IL-12 and 5 μg/ml anti-IL-4 mAb were added (T_h1 condition for naive T cells). For inducing exclusively T_h2 development, 50 U/ml IL-4 and 2 μg/ml anti-IL-12 mAb were added (T_h2 condition for naive T cells). For inducing both T_h1 and T_h2 development, 4 ng/ml IL-12 and 50 U/ml IL-4 were added (T_h1/T_h2 condition for naive T cells). After 2 days, cells were harvested and transferred to new 24-well culture plates (BD Falcon, Franklin Lakes, NJ) without antibodies, and were expanded 3 times with medium containing the same concentrations of cytokines and 50 U/ml IL-2 for 4 days. One milliliter of fresh medium containing 20 U of IL-2 with or without RA was added for the last 24 h to avoid cell death from starvation if necessary. In some experiments, supernatants were removed after the initial 2 days of culture with antibodies, and the cells were resuspended and cultured in 1.2 ml of fresh medium containing the same concentrations of cytokines and 50 U/ml IL-2. RA and/or antagonists or agonists of RAR or RXR were added as indicated. After culture, cells were harvested and washed twice, and re-stimulated for the measurement of cytokine production in the culture supernatant or intracellular cytokine production as previously described (18).

Semi-quantitative RT-PCR

Total RNA was prepared from cultured cells by using the lysis buffer Isogen (Wako Pure Chemical, Tokyo, Japan) as previously described (22). Oligo-dT primed first-strand cDNA was prepared from 5 μg total RNA using Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) and the resulting cDNA was serially diluted. PCR was performed in a total volume of 30 μl containing 0.5 μl of appropriately diluted cDNA, 0.2 mM of dNTP, 0.6 μM of each primer and 1 × PCR buffer (Roche Molecular Systems, Branchburg, NJ), and 0.5 U of AmpliTaq DNA polymerase (Roche Molecular Systems) by using a Takara PCR thermal cycler (TP480). After the first denaturation step (94°C for 3 min), the amplification was performed for 25 or 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min (25 cycles for β-actin, IL-12Rβ2, GATA-3 and IL-4Rα; 30 cycles for T-bet and c-*maf*). The final cycle was followed by an extension step of 5 min at 72°C. Sequences of the sense and anti-sense primers were as follows: β-actin, 5′-CGTGGGCCGCCCTAGGCACCA-3′ and 5′-TTGGCCTTAGGGTTCAGGGGGG-3′ (35); T-bet, 5′-TGCCTGCAGTGCTTCTAACA-3′ and 5′-GCTGTGAGATCATATCCTTGGG-3′; 12Rβ2, 5′-TCTGCGAAATTCAGTACCGACG-3′ and 5′-CATGCCATCAGGAGATTATCCG-3′; GATA-3, 5′-CTCCAAGTGTGCGAAGAGTTCCTC-3′ and 5′-GTGCCCATTTGGACATCAGACT-3′; c-*maf*, 5′-GACGAGCAGTTGGTGACCATG-3′ and 5′-GCTCACCAGCTTCTCGTATTTC-3′; IL-4Rα, 5′-GCTCCGTGCCCTTATTTACTTTCG-3′ and 5′-GGTTGGCTTCTGGTGGTATTCC-3′. PCR products were resolved by electrophoresis on a 1.3% agarose gel containing ethidium bromide. T-bet, IL-12Rβ2, GATA-3, c-*maf*, IL-4Rα, and β-actin cDNA yielded PCR products of 305, 376, 423, 273, 382 and 243 bp respectively. Fluorescence images were captured by a video monitor system (Image Freezer; ATTO, Tokyo, Japan) and band intensities were quantified using the analytical program Densitograph 4.0 (ATTO). The bands that corresponded to the exponential amplification phases were analyzed, and the intensities of cDNA-derived bands were normalized with those of β-actin cDNA-derived bands.

Statistical analysis

The significance of mean values was determined by Student's *t*-test. Values of $P > 0.05$ were considered insignificant.

Results

RA directly influence the functional differentiation of T cells into T_h1 and T_h2 in vitro

To examine the direct effect of RA on T cell differentiation, we first used our *in vitro* T cell differentiation system starting from $CD4^+CD8^+$ thymocytes of DKO mice. Thymocyte differentiation was induced by 2 times transient stimulation with ionomycin/PMA in the absence of other types of cells such as APC and thus provided $>10^8$ T cells per mouse ready for differentiation into T_h1 or T_h2 (Fig. 1A) (18,20). The thymocytes stimulated twice with ionomycin/PMA were washed, and cultured with IL-2 and IL-4 and/or IL-12 in the presence of all-*trans*-RA or 9-*cis*-RA for 4 days. The cultured cells were re-stimulated with plate-bound antibodies to CD3 and CD28 to assess their ability to produce IFN-γ, IL-4, IL-5 and IL-10. In the presence of both IL-12 and IL-4 (T_h1/T_h2 condition), all-*trans*-RA and 9-*cis*-RA suppressed IFN-γ production and enhanced IL-4 and IL-5 production dose-dependently (Fig. 1B). The effect of all-*trans*-RA at 1 nM on IFN-γ and IL-4 production was more significant than that of 9-*cis*-RA at 1 nM, but the two RA exerted similar effects on the cytokine production. Thus, all-*trans*-RA at physiological or near-physiological concentrations (23) appears to affect T_h1/T_h2 development, although the effective concentrations of RA *in vitro* and *in vivo* should be

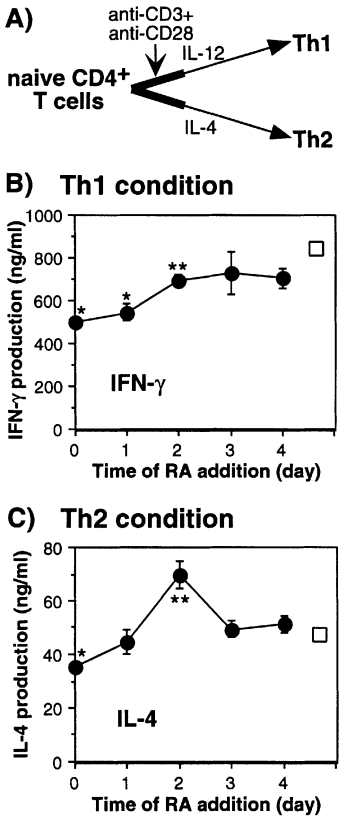

Fig. 2. All-*trans*-RA directly suppresses naive T cell differentiation into IFN-γ-producing cells, but enhances differentiation into IL-4-producing cells with its timely addition. (A) Naive CD4$^+$CD62L^{high} T cells were purified from male DO-11.10 TCR-Tg and RAG-2-deficient mice, and stimulated with plate-bound antibodies to CD3 and CD28 for 2 days in the presence of IL-12 and anti-IL-4 antibody (T_h1 condition) or IL-4 and anti-IL-12 antibody (T_h2 condition). After stimulation, the cells were resuspended in 3 times as much volume of fresh medium containing IL-12 and IL-2 (T_h1 condition) or IL-4 and IL-2 (T_h2 condition) without antibodies, and cultured for 4 days (thus 6 days in total) to induce T_h1 and T_h2 development. (B and C) All-*trans*-RA (10 nM) was added at the start of culture or 1, 2, 3 or 4 days after the start of the first culture. The cells were then re-stimulated with plate-bound antibodies to CD3 and CD28 for 48 h to analyze their ability to produce IFN-γ (B) and IL-4 (C). Open squares in (B) and (C) indicate the cytokine levels produced by the cells differentiated without addition of RA [850.3 ± 25.2 ng/ml IFN-γ in (B) and 47.6 ± 0.8 ng/ml IL-4 in (C)]. Vertical bars represent SD in triplicate cultures. Asterisks indicate that the inhibition or enhancement by RA is statistically significant (*$P < 0.001$, **$P < 0.01$).

different. IL-10 production was slightly enhanced by RA at high concentrations (Fig. 1B). In the presence of IL-12 without IL-4 (T_h1 condition), all-*trans*-RA also suppressed the production of IFN-γ (Fig. 1C), and in the presence of IL-4 without IL-12 (T_h2 condition), all-*trans*-RA enhanced IL-4 production (Fig. 1D). These results suggest that both all-*trans*-RA and 9-*cis*-RA inhibit T_h1 development and enhance T_h2 development.

T_h1 and T_h2 development from naive T cells is affected variably depending on the timing of RA addition

To confirm the results obtained with the thymocyte differentiation system, we chose DO-11.10 TCR-Tg mice to prepare

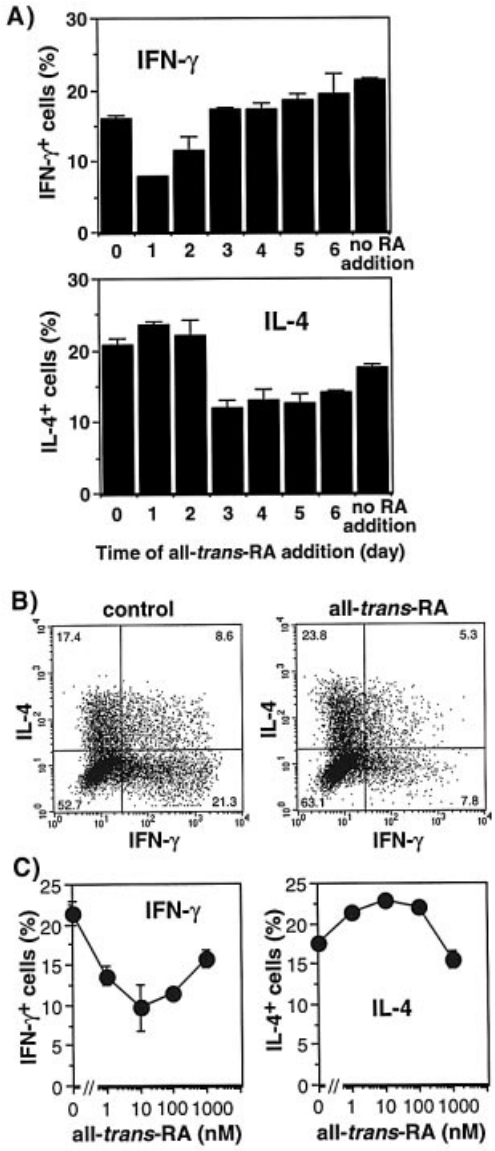

Fig. 3. Intracellular expression of cytokines in T cells cultured with or without all-*trans*-RA. Differentiation was induced in naive T cells *in vitro* as shown in Fig. 2, except that cells were stimulated in the presence of both IL-12 and IL-4, and expanded in the presence of the same cytokines and IL-2 (T_h1/T_h2 condition). (A) All-*trans*-RA (10 nM) was added at the start of culture or 1–6 days after the start of culture. 'Day 6' indicates that RA was added 30 min before harvesting cells. The cells were then re-stimulated with ionomycin/PMA, and the intracellular production of IFN-γ and IL-4 was determined. (B) FACS analysis of intracellular IFN-γ and IL-4 expression. Vehicle (left panel) or all-*trans*-RA (10 nM) (right panel) was added 1 day after the start of culture. A representative result of three experiments is shown. (C) Graded concentrations of all-*trans*-RA were added 1 day after the start of culture and the intracellular cytokine levels were determined. IFN-γ$^+$IL-4$^-$ cells are shown as IFN-γ$^+$ cells and IFN-γ$^-$IL-4$^+$ cells are shown as IL-4$^+$ cells. Vertical bars represent SD in triplicate cultures.

naive CD4$^+$ T cells. Since these mice were RAG-2-deficient, they lacked endogenous TCRα chains and expressed the transgenic TCRαβ exclusively. Thus, few memory or other types of cells should contaminate the CD4$^+$CD62L^{high} naive T cell preparation in the absence of ovalbumin peptide.

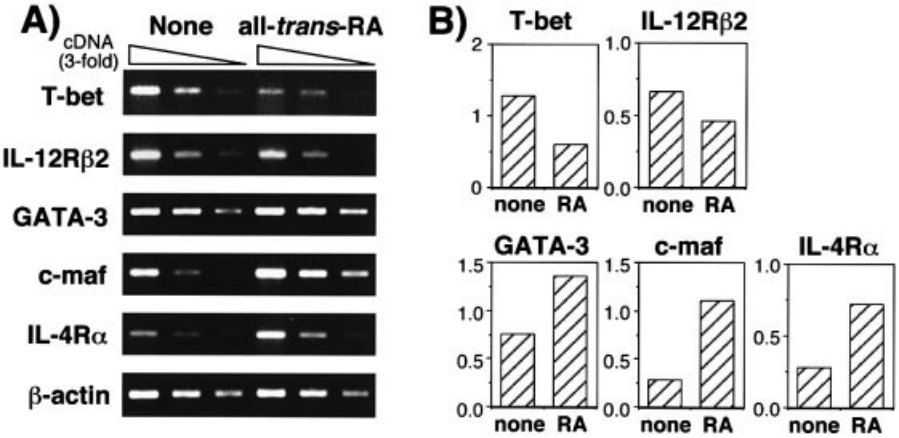

Fig. 4. All-*trans*-RA affects the mRNA expression levels of T_h1-associated genes and T_h2-associated genes during T_h cell differentiation. DKO mouse thymocytes were stimulated twice as shown in Fig. 1 and vehicle or 10 nM all-*trans*-RA was added to the Step D culture in the T_h1/T_h2 condition for 4 days. The reverse-transcribed cDNA from each sample was serially diluted, and each diluted sample was subjected to PCR amplification for T-bet and c-*maf* for 30 cycles, and IL-12Rβ2, GATA-3 and β-actin for 25 cycles. (A) The PCR products were resolved by electrophoresis on a 1.3% agarose gel containing ethidium bromide. (B) Relative expression levels of mRNA of each gene were calculated. A representative result of three experiments is shown.

Purified $CD4^+CD62L^{high}$ naive T cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-12 or IL-4 to induce differentiation into T_h1 and T_h2 cells respectively. After 2 days of stimulation, the cells were further cultured in the presence of the same cytokine plus IL-2, but without antibodies, for 4 days, and were assessed for their ability to produce IFN-γ and IL-4 (Fig. 2A). When 10 nM of all-*trans*-RA was added at the beginning of culture, both IFN-γ production in the T_h1 condition and IL-4 production in the T_h2 condition were suppressed (Fig. 2B and C). However, when all-*trans*-RA was added after the initial stimulation, IL-4 production in the T_h2 condition was significantly enhanced (Fig. 2C). The effect of all-*trans*-RA was not significant when it was added 3 or 4 days after the start of culture. In the absence of exogenous cytokines, IFN-γ production was suppressed by 10 nM of all-*trans*-RA, but an increase in IL-4 production was not detected even with a delayed addition of RA (data not shown).

We then studied the effect of RA on T_h1 and T_h2 development in naive CD4 T cells in the presence of both IL-12 and IL-4 (T_h1/T_h2 condition) by determining their ability to produce intracellular IFN-γ and IL-4. All-*trans*-RA at 10 nM enhanced the induction of $IL\text{-}4^+$ cells, but suppressed the induction of $IFN\text{-}\gamma^+$ cells, when it was added during the first 3 days of culture (Fig. 3). The enhancement of $IL\text{-}4^+$ cells was most efficient when all-*trans*-RA was added 1 or 2 days after the start of culture in repeated experiments (Fig. 3A and data not shown). The suppression of $IFN\text{-}\gamma^+$ cells was most efficient when all-*trans*-RA was added 1 day after the start of culture, not at the start of culture (Fig. 3A and B). However, we found that, with RA added at the start of culture, the majority of the induced $IFN\text{-}\gamma^+$ cells bore low levels of IFN-γ and that the ability to produce IFN-γ into the supernatant upon re-stimulation was equally low if all-*trans*-RA was added within 2 or 3 days after the start of culture (data not shown). The cells that received RA 1 day after the start of culture produced 151 ± 11 ng/ml IFN-γ and 63 ± 5 ng/ml IL-4 upon re-stimulation, while those without RA addition produced 393 ± 52 ng/ml IFN-γ and 44 ± 5 ng/ml IL-4 in triplicate cultures, indicating that the delayed addition of RA also modulated the ability of T cells to produce IFN-γ and IL-4 in this culture condition. The optimal concentrations of all-*trans*-RA to increase $IL\text{-}4^+$ cells and to decrease $IFN\text{-}\gamma^+$ cells were 10–100 nM when it was added 1 day after the start of culture (Fig. 3C). These results indicate that all-*trans*-RA suppresses T_h1 development if it is added during or immediately after the TCR-mediated stimulation period and that all-*trans*-RA enhances T_h2 development if it is added 1 day after the start of culture or immediately after the stimulation period. A delayed addition of 10 nM 9-*cis*-RA also suppressed T_h1 development and enhanced T_h2 development (data not shown).

All-trans-RA inhibits the expression of T_h1-associated genes and enhances that of T_h2-associated genes

To examine if all-*trans*-RA also regulates the expression of genes essential for T_h1 and T_h2 development, mRNA levels of T-bet, IL-12Rβ2, GATA-3, c-*maf* and IL-4Rα were semi-quantitatively assessed by RT-PCR. $CD4^+CD8^+$ thymocytes of DKO mice were treated with ionomycin/PMA 2 times and cultured in the T_h1/T_h2 condition. Both T_h1- and T_h2-associated genes were expressed with or without RA (Fig. 4). However, all-*trans*-RA at 10 nM suppressed the expression of the T_h1-associated genes, T-bet (24) and IL-12Rβ2 (25), and enhanced the expression of the T_h2-associated genes, c-*maf* (26) and IL-4Rα (27) (Fig. 4). The suppression of IL-12Rβ2 was consistently observed, but was mostly moderate. The expression of GATA-3, a gene expressed in naive T cells and T_h2 cells, but marginally in T_h1 cells (28), was enhanced by RA (Fig. 4). The results further support the notion that all-*trans*-RA directly regulates T_h1/T_h2 development.

RA regulate T_h1/T_h2 development through RAR

9-*cis*-RA binds to both RAR and RXR, whereas all-*trans*-RA binds to RAR and may bind to RXR at high concentrations. The dose–response analysis with all-*trans*-RA and 9-*cis*-RA in Fig. 1 suggested that RAR rather than RXR might be

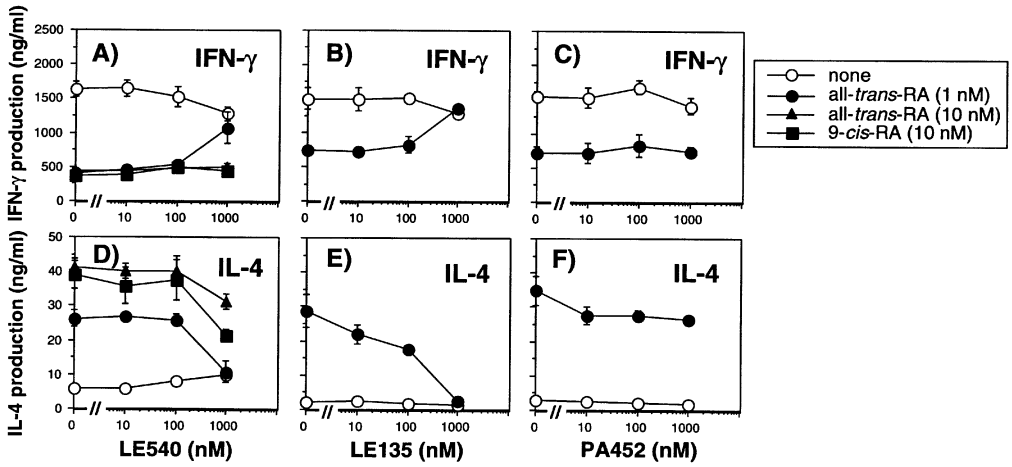

Fig. 5. Effects of antagonists of RAR and RXR on RA-induced modulation of T_h1 and T_h2 development. DKO mouse thymocytes were transiently stimulated twice as shown in Fig. 1, and cultured with graded concentrations of the RAR pan-antagonist LE540 (A and D), the RARβ (and α) antagonist LE135 (B and E) or the RXR pan-antagonist PA452 (C and F) in Step D for 4 days. The cells were then re-stimulated with plate-bound antibodies to CD3 and CD28, and the production of IFN-γ (A–C) and IL-4 (D–F) determined. Vertical bars represent SD in triplicate cultures.

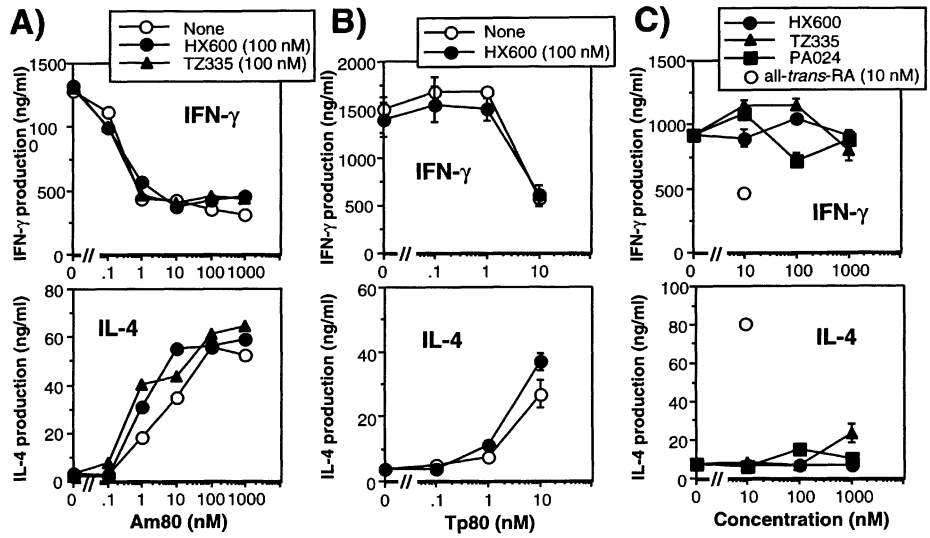

Fig. 6. Effects of agonists of RAR and RXR on T_h1 and T_h2 development. DKO mouse thymocytes were transiently stimulated twice as shown in Fig. 1, and cultured with graded concentrations of the RARα (and β) agonist Am80 (A), the RAR pan-agonist Tp80 (B), or the RXR pan-agonists HX600, TZ335 or PA024, or 10 nM all-*trans*-RA (C) in Step D for 4 days. In some cultures, 100 nM of HX600 (A and B) or TZ335 (A) was added with a RAR agonist. The cells were then re-stimulated for 48 h with plate-bound antibodies to CD3 and CD28, and the production of IFN-γ and IL-4 was determined. Vertical bars represent SD in triplicate cultures. A representative result of three experiments is shown.

responsible for the effect of RA on T_h1/T_h2 development. However, isomerization of all-*trans*-RA to 9-*cis*-RA might occur within the cells (29). We thus examined the effect of antagonists of RAR and RXR on T_h1/T_h2 development. $CD4^+CD8^+$ thymocytes of DKO mice were treated with ionomycin/PMA 2 times and cultured with an antagonist or agonist in the presence or absence of RA. The cells were collected, and their ability to produce IFN-γ and IL-4 was determined. As shown in Fig. 5(A and D), 1 μM of the RAR pan-antagonist LE540 (30) partially canceled the suppressive effect of 1 nM all-*trans*-RA on IFN-γ production, and partially or completely canceled the enhancing effect of all-*trans*-RA and 9-*cis*-RA on IL-4 production, although LE540 by itself at 1 μM slightly inhibited IFN-γ production and slightly enhanced IL-4 production. LE135 is a potent antagonist of RARβ and a less potent antagonist of RARα (30). LE135 also canceled the effect of 1 nM all-*trans*-RA on IL-4 and IFN-γ production (Fig. 5B and E). On the other hand, the RXR pan-antagonist PA452 (31) at up to 1 μM failed to cancel the effects of 1 nM all-*trans*-RA (Fig. 5C and F).

We then determined the effect of agonists of RAR and RXR on T_h1/T_h2 development in the same culture system. Am80 is a potent RARα agonist, and is a less potent agonist of RARβ (32). Am80 dose-dependently suppressed IFN-γ production and enhanced IL-4 production (Fig. 6A). The RAR pan-agonist Tp80 (33) suppressed IFN-γ production and enhanced IL-4

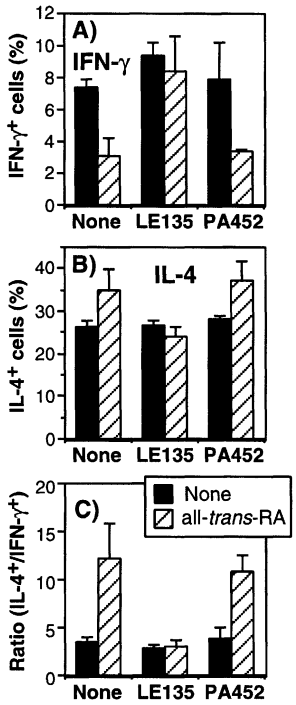

Fig. 7. Effects of antagonists of RAR and RXR on RA-induced modulation of T_h1 and T_h2 development in naive T cells. $CD4^+$ naive T cells from DO-11.10 mice were stimulated and cultured in the T_h1/T_h2 condition as shown in Fig. 3. All-*trans*-RA (10 nM) and/or LE135 (1 μM) or PA452 (1 μM) were added 24 h after the start of culture. The cells were then re-stimulated with ionomycin/PMA, and the intracellular expression of IFN-γ (A) and IL-4 (B) determined. (C) The ratio of $IL\text{-}4^+$ cells (%)/$IFN\text{-}\gamma^+$ cells (%) is shown. Vertical bars represent SD in triplicate cultures.

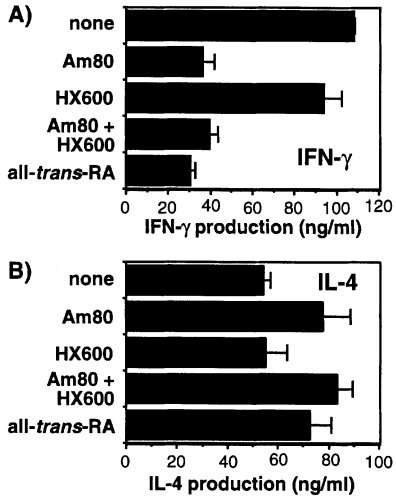

Fig. 8. Effects of agonists of RAR and RXR on T_h1 and T_h2 development in naive T cells. $CD4^+$ naive T cells from DO-11.10 mice were stimulated and cultured in the T_h1/T_h2 condition as shown in Fig. 3. All-*trans*-RA (10 nM), Am80 (100 nM) and/or HX600 (100 nM) were added 24 h after the start of culture. The cells were then re-stimulated for 48 h with plate-bound antibodies to CD3 and CD28, and the production of IFN-γ (A) and IL-4 (B) was determined. The data are expressed as means ± SD of triplicate cultures.

production at 10 nM (Fig. 6B). On the other hand, the RXR pan-agonists HX600 (30), TZ335 (34) and PA024 (35) only slightly affected the production of IFN-γ and IL-4 (Fig. 6C). The RXR pan-agonists at 100 nM exerted little effect if any on the Am80- or Tp80-induced modulation of IFN-γ and IL-4 production (Fig. 6A and B). These results collectively indicate that RARα and/or β are involved in the direct effect of RA on T_h1/T_h2 development, but that RXR activation may not play an important role in the effect.

RAR, but not RXR, is critical for RA to exert their effects on the differentiation of naive T cells to T_h1 and T_h2 cells

Effects of antagonists and agonists of RAR and RXR on T_h1 and T_h2 development in naive $CD4^+$ T cells from DO-11.10 mice were also examined. An antagonist, an agonist and/or 10 nM all-*trans*-RA were added 1 day after the start of culture in the T_h1/T_h2 condition. The RARβ (and α) antagonist LE135, but not the RXR pan-antagonist PA452, canceled the effect of all-*trans*-RA on the induction of $IL\text{-}4^+$ cells and $IFN\text{-}\gamma^+$ cells (Fig. 7). On the other hand, the RARα (and β) agonist Am80, but not the RXR pan-agonist HX600, exerted effects similar to those of all-*trans*-RA on IL-4 and IFN-γ production (Fig. 8). HX600 failed to enhance the effects of Am80. Similar results were obtained in the analyses of intracellular cytokine production (data not shown). These results confirm that RARα and/or β, but not RXR, are involved in the RA-induced regulation of T_h1/T_h2 development.

Discussion

RA modulates T_h1/T_h2 responses *in vivo*. Previous reports indicate that APC is one of the target sites of RA. RA also directly down-regulates IFN-γ production in an established T_h1 cell line upon stimulation with antibodies to CD3 and CD28 (36). However, it remained unclear whether RA directly exerts its effect on naive T cells to influence T_h1/T_h2 development. In the present study, we demonstrated that both all-*trans*-RA and 9-*cis*-RA directly suppressed T_h1 development and enhanced T_h2 development in naive T cells in the absence of APC or other types of cells. For the enhancement of T_h2 development, however, the timing of RA addition was critical. RA can be suppressive of T_h2 development if it is given at the initiation of TCR stimulation. This phenomenon may contribute to the lack of a primary IgE response in mice treated with RA together with antigen in spite of a potentiated secondary IgE response in these mice (37). TCR-mediated T cell activation involves AP-1 activity and RA negatively regulate AP-1-responsive genes in a retinoid receptor-dependent fashion (38). Thus, the suppression of both T_h1 and T_h2 development by RA given at the initiation of culture may involve the retinoid receptor-mediated inhibition of AP-1 activity.

9-*cis*-RA binds to both RAR and RXR, whereas all-*trans*-RA binds well to RAR, but not to RXR (8,9). Since isomerization of all-*trans*-RA to 9-*cis*-RA might occur within the cells (29), to clarify which receptor family is critically involved in the direct effect of RA on the functional differentiation of T cells, we used several antagonists and agonists of RAR and RXR. The results indicated that RARα and/or β are involved in the direct effect of RA on T_h1/T_h2 development. Although RAR function as ligand-inducible transcription factors as RXR/RAR heterodimers (9),

RXR activation does not appear to play an important role in the direct effect of RA on T_h1/T_h2 development. In contrast to our results, Stephensen *et al*. (13) have recently suggested that the RXR agonist AGN194024 enhanced T_h2 development upon stimulation with antibodies to CD3 and CD28 without exogenous IL-4. However, they used $CD4^+CD62L^{high}$ lymph node cells from normal C57BL/6 mice as naive T cells. It is likely that these cell preparations not only contained naive T cells, but also contained memory and other types of T cells (14–16). Thus, an indirect effect through the activation of RXR in the latter cell populations might be involved in the enhanced T_h2 development. Nonetheless, some RXR agonists may induce weak effects on T_h cell differentiation, since TZ335, one of the RXR agonists we used in Fig. 6(C), exerted a weak effect on IL-4 production at a high concentration (1 μM) in the presence of IL-4 and IL-12. It might be possible that heterodimers of RXR with its permissive partners including peroxisome proliferator activator receptors, LXRα and NGFI-B (9) directly affect T_h1/T_h2 development through the ligation of RXR under some conditions.

Kang *et al*. (10) reported that pretreatment with the RAR agonist 4-[*E*-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) inhibited IL-12 production by macrophages stimulated with lipopolysaccharide or heat-killed *Listeria monocytogenes*, although pretreatment with all-*trans*-RA or 9-*cis*-RA was more effective. TTNPB- or retinoid-pretreated macrophages reduced their ability to induce IFN-γ and increased the ability to induce IL-4 in antigen-primed $CD4^+$ T cells. However, the same group (39) also suggested a possibility that retinoids inhibit IL-12 production in macrophages through physical association of RXR and NF-κB. Indeed, Stephensen *et al*. (13) have shown that the RXR agonist AGN194204 enhanced T_h2 development upon antigenic stimulation with APC in repeated experiments and that TTNPB exerted little effect. Thus, retinoid-induced modulation of the APC function may largely involve the regulation of RXR activity.

RA has been shown to induce or enhance transforming growth factor (TGF)-β activity in some types of cells (40). TGF-β blocks T_h1 development by inhibiting the expression of IL-12Rβ2 or T-bet (41,42). It may be possible that RA-induced TGF-β suppresses T_h1 development, although TGF-β also blocks T_h2 development through inhibition of GATA-3 expression (43,44). RA, however, did not enhance TGF-β production during the stimulation of DO-11.10 mouse-derived naive T cells (data not shown). Furthermore, addition of an anti-TGF-β mAb enhanced the induction of both T_h1 and T_h2 development, and failed to cancel the effect of RA (data not show). We noticed, however, that RA enhanced T_h2 development in naive $CD4^+$ T cells more efficiently when the cells were resuspended in fresh medium after 2 days of stimulation with antibodies than when simply expanded with fresh medium, suggesting that some soluble factor(s) produced by the cells themselves may disturb the differentiation. The suppression of IL-12Rβ2 expression by RA was consistently observed, but was mostly moderate (Fig. 4), suggesting that it may not be a main cause of the suppression of T_h1 development.

RA down-regulates IFN-γ production in an established T_h1 cell line (36). We found that RA also down-regulated IFN-γ production in naive T cells after 2 days of stimulation with antibodies to CD3 and CD28 (data not shown). It may partly contribute to the suppression of T_h1 development. The molecular mechanism of the RAR-dependent effect of RA on T_h1/T_h2 development still remains to be elucidated.

The modernization of life styles is often accompanied by an increase in allergic diseases. Because the increase has been so rapid, it is likely that changes in environmental rather than genetic factors are mainly involved. Although vitamin A deficiency still causes serious problems in many developing areas of the world (45), vitamin A intake levels in modernized areas may tend to skew the T_h cell balance toward T_h2. It may be also possible that some environmental chemicals mimic the effect of RA through binding to RAR. Hind-limb malformations found in amphibians across North America within the last decade can be mimicked with an exposure of embryos to RA, although xenobiotic disturbance has not been proved to cause the malformations in the field (46). RA may be beneficial, however, in the treatment of multiple sclerosis or experimental allergic encephalomyelitis (2) and other T_h1-mediated diseases. By clarifying the mechanism by which RA modulates T_h1/T_h2 development, better methodologies or the cure of various immune diseases may be discovered.

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Abbreviations

APC	antigen-presenting cell
DKO	MHC class I and class II double knockout
PMA	phorbol myristate acetate
RA	retinoic acid
RAR	retinoic acid receptor
RXR	retinoic X receptor
Tg	transgenic
TGF	transforming growth factor
TTNPB	4-[*E*-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid

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