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Efficient Induction of CCR9 on T Cells Requires Coactivation of Retinoic Acid Receptors and Retinoid X Receptors (RXRs): Exaggerated T Cell Homing to the Intestine by RXR Activation with Organotins

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The active vitamin A metabolite retinoic acid (RA) imprints gut-homing specificity on lymphocytes upon activation by inducing the expression of α4β7 integrin and CCR9. RA receptor (RAR) activation is essential for their expression, whereas retinoid X receptor (RXR) activation is not essential for α4β7 expression. However, it remains unclear whether RXR activation affects the RA-dependent CCR9 expression on T cells and their gut homing. The major physiological RA, all-trans-RRA, binds to RAR but not to RXR at physiological concentrations. Cell-surface CCR9 expression was often induced on a limited population of murine naive CD4+ T cells by all-trans-RRA or the RXR agonist Am80 alone upon CD3/CD28-mediated activation in vitro, but it was markedly enhanced by adding the RXR agonist PA024 or the RXR-binding environmental chemicals tributyltin and triphenyltin. Accordingly, CD4+ T cells treated with the combination of all-trans-RRA and tributyltin migrated into the small intestine upon adoptive transfer much more efficiently than did those treated with all-trans-RRA alone. Furthermore, naive TCR transgenic CD4+ T cells transferred into wild-type recipients migrated into the small intestinal lamina propria following i.p. injection of Ag, and the migration was enhanced by i.p. injection of PA024. We also show that PA024 markedly enhanced the all-trans-RRA–induced CCR9 expression on naturally occurring naive-like regulatory T cells upon activation, resulting in the expression of high levels of α4β7, CCR9, and Foxp3. These results suggest that RXR activation enhances the RAR-dependent expression of CCR9 on T cells and their homing capacity to the small intestine. The Journal of Immunology, 2010, 185: 000–000.

Abbreviations used in this paper: bkg, background; DBT, dibutyltin; DC, dendritic cell; LP, lamina propria; LXR, liver X receptor; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; PLN, peripheral lymph node; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RALDH, retinal dehydrogenase; RXR, retinoid X receptor; SNAPF-1, seminaphthenoidfluor-1-carboxylic acid succinimidyl ester; SPL, spleen; TBF, tributyltin; TPT, triphenyltin; Treg, regulatory T cell; wt, wild-type.

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Am80, as well as all-trans-RA, sufficiently induced α4β7 expression on naive T cells upon activation and that the RXR pan-agonist HX600 did not significantly enhance the Am80-dependent induction of α4β7 expression (8). In contrast, the effect of RXR-mediated stimulation on CCR9 expression has not been well studied. In the current study, we found that all-trans-RA induced various levels of CCR9 expression on T cells, depending on the stimulation conditions, and that, in most cases,RAR-mediated stimulation alone was much less efficient than stimulation via RAR and RXR. Thus, RXR-mediated stimulation seems to be critical for efficient CCR9 expression and homing of T cells to the small intestinal tissues. 9-cis-RA was initially considered the endogenous ligand for RXR, but it could not be detected in any tissues tested in later studies. A search for an endogenous RXR ligand showed that some unsaturated fatty acids only weakly bound to and activated RXR (21). Thus, we speculate that the availability or activity of endogenous RXR agonists may be limited or strictly regulated in vivo and that exogenous RXR agonists may easily disturb CCR9 expression on lymphocytes.

The persistent environmental pollutants and potent endocrine disruptors tributyltin (TBT) and triphenyltin (TPT) can activate RXR and PPAR-γ at nanomolar concentrations (22–25). It was recently shown that TBT activates RXR/PPAR heterodimers primarily through its binding to RXR (26). These compounds have been widely used as antifouling paints for ships and aquaculture nets, biocides, agricultural fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters (27, 28). Thus, they have become common contaminants of marine and freshwater ecosystems, exceeding acute and chronic toxicity levels. In mammals, exposure to TBT and TPT also induces deleterious effects, including immunosuppressive, metabolic, reproductive, or developmental effects (29–31). In this study, we found that these organotin signifi cantly enhanced the RXR-dependent induction of CCR9 expression and gut-homing specificity in naive T cells upon activation.

The result prompted us to test whether RXR-mediated stimulation might also enhance regulatory T cells (Tregs) to acquire gut tropism. It was shown that RA can induce α4β7 expression on naturally occurring naive-like CD62L<sup>high</sup>CD4<sup>+</sup>CD25<sup>+</sup> Tregs upon activation, but it only induced CCR9 expression on a minor fraction of the cells (32). In this study, we found that RXR-mediated stimulation significantly enhanced RA-dependent CCR9 expression without decreasing the expression of α4β7 and Foxp3.

Materials and Methods

**Mice**

B10.D2 mice were from Japan SLC (Hamamatsu, Japan). BALB/c mice were from CLEA Japan (Tokyo, Japan). TCR-D011.10/Rag2<sup>−/−</sup> (B10.D2 or BALB/c background [bkg]) mice were from Taconic Farms (Hudson, NY). All animals were maintained in specific-pathogen-free conditions in our animal facility. All animal experiments were performed according to the protocols approved by the Animal Care and Use Committee of Tokushima Bunri University.

**Reagents**

Recombinant murine IL-2 and IFN-γ were from PeproTech (Rocky Hill, NJ). All-trans-RA, 9-cis-RA, all-trans-retinol, and TPT were from Sigma-Aldrich (St. Louis, MO). Dibutyltin (DBT) and TBT were from Wako Pure Chemical Industries (Osaka, Japan). CellTracker Green 5-chloromethylfluorescein diacetate (CFSE) and seminaphtothalindione-<i>carboxylic acid</i> acetate succinimidylderiv (SNARF-1) were from Invitrogen (Tokyo, Japan). Am80, HX600, PA024, and LE540 were prepared as described (33–35).

**Cell purification and culture**

Naive CD<sup>+</sup>CD62L<sup>high</sup>DO11.10/Rag2<sup>−/−</sup> T cells were purified from spleen (SPL) and lymph nodes (inguinal, axillary, brachial, and MLN), as previously described (36). In some experiments, CD<sup>+</sup>CD4<sup>+</sup> T cells were isolated by negative selection, using an EasySep Mouse CD4<sup>+</sup> T Cell Enrichment Kit (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with biotinylated anti-mouse CD44 mAb (BioLegend, San Diego, CA), and were used as naive T cells. Naive CD4<sup>+</sup> T cells were suspended in the culture medium (DMEM supplemented with 100 μM nonessential aa, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 20 mM HEPEs [pH 7.2], 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS) and were stimulated with mAbs to CD3 and CD28 with a slight modification (36). Briefly, T cells were stimulated in plates coated with 5 μg/ml anti-CD3 mAb (145-2C11) and 1 μg/ml anti-CD28 mAb for 2 d. The cells were then resuspended in three times as much volume of fresh medium containing recombinant murine IL-2 and were transferred into new wells and cultured for 2 d. Retinoids, organotins, and/or other reagents were added at the start of each culture, unless otherwise indicated. In some experiments, supernatants were harvested at the first 2-d culture and assessed for the presence of IFN-γ, IL-2, IL-4, IL-6 (BD Biosciences, San Jose, CA), and IL-21 (R&D Systems, Minneapolis, MN) by ELISA. For isolation of SPL-DCs, SPL was digested with 400 Mandl U/ml collagenase type VIII (Sigma-Aldrich) in culture medium for 45–90 min at 37°C. Cells were incubated with anti-CD11c microbeads (Milenyi Biotec, Bergisch Gladbach, Germany) and then immunomagnetically sorted by sequential passage over two MACS separation columns (Milenyi Biotec). For DC–T cell cocultures, 10<sup>7</sup> naive CD4<sup>+</sup> DO11.10/Rag2<sup>−/−</sup> T cells were stimulated with Ag (OVA peptide 332–339, 1 μM) and 10<sup>5</sup> SPL-DCs in 200 μl culture medium in a well of a 96-well round-bottom plate for 5 d. For the isolation of naturally occurring naive-like Tregs, CD4<sup>+</sup> T cells from SPL and lymph nodes were isolated by immunomagnetic cell sorting using an EasySep Mouse CD4<sup>+</sup> T Cell Enrichment Kit. The enriched cells were incubated with PE-Cy7– conjugated anti-CD4 mAb (RM4-5), PE-conjugated anti-CD62L mAb (ME-L14), and allopbycycin-conjugated anti-CD25 mAb (PC61.5) and were sorted on a FACSaria (BD Biosciences) to isolate CD62L<sup>high</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells. The purity was >98%. The purified cells were stimulated in plates coated with 5 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb in the presence of 1000 U/ml IL-2 for 3 d. The cells were then resuspended in three times as much volume of fresh medium containing 1000 U/ml IL-2 and were transferred into new wells and cultured for 2 d. All-trans-RA or PA024 was added, as indicated, to the culture at the start of each culture.

**Flow-cytometric analysis**

The cells were stained with PE-conjugated mAbs to CCR9 (clone 242503; R&D Systems) or α4β7 (clone DATK32; eBioscience, San Diego, CA) in the presence of anti-CD16/CD32 mAb (2.4.G2). Some cells were stained with E-selectin/human IgG Fc chimera (R&D Systems) and then PE-conjugated goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories, West Grove, PA). Intracellular staining of Foxp3 was performed using allophycocyanin mouse/rat Foxp3 staining sets (eBioscience). Stained cells were analyzed with a FACS caliber flow cytometer (BD Biosciences). In some experiments, expression levels were expressed as the mean fluorescence intensity (MFI), which was calculated as: (MFI of the cells over FCS and extensively washed. An aliquot (3 × 10<sup>7</sup>) of the cells was injected i.v. into each BALB/c mouse 1 d before Ag challenge. The mice were given an i.p. injection of OVA (300 μg) (Wako Pure Chemical Industries) with alum (1 mg, Imject Alum; Pierce, Rockford, IL) on day 0 and an i.p. injection of PA024 (30 nmol/kg) in corn oil or corn oil alone on days 0, 2, and 4. The mice were sacrificed on day 5, and the transferred cells were recovered from lymphoid organs and the small intestinal lamina propria (ILP). For recovery from the small intestinal LP, after removal of dust and mesenteric lymph nodes, the small intestine was flushed with serum HBSS, the resulting tissue was digested twice for 45 min in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The digest mixture was then filtered through 400 Mandl U/ml collagenase type VIII (Sigma-Aldrich) in culture medium for 30 min at 37°C. For recovery of LP cells, the resulting mixture was digested twice for 45 min in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The digest mixture was then filtered through 400 Mandl U/ml collagenase type VIII (Sigma-Aldrich) in culture medium for 30 min at 37°C. After washing with HBSS, the resulting tissue was digested twice for 45 min in DMEM containing collagenase type VIII (400 Mandl U/ml; Sigma-Aldrich) and DNase I 250 Unit/ml; Invitrogen) in a shaking incubator at 37°C. LP cells were washed, resuspended, layered on a 40% Percoll gradient, and centrifuged. The recovered cells were stained with allopbycycin-conjugated anti-CD4 mAb (GK1.5) and PE-conjugated anticytotoxic
mAb (KJ1-26) specific for DO11.10 TCR and were analyzed with a FACSCalibur flow cytometer.

**Homing assay: the in vitro effect of TBT**

Naive CD4⁺ T cells from B10.D2 mice were cultured with all-trans-RA alone or with all-trans-RA and TBT. The cells were then labeled with 2.5 μM SNARF-1 or 0.85 μM CFSE for 15 min at 37°C. Thereafter, cells were centrifuged over FCS and extensively washed. Five million cells from each preparation were mixed and injected i.v. into each recipient B10.D2 mouse. An aliquot was saved to assess the input cell population by flow cytometry. Recipients were sacrificed 16 h after the injection, and the transferred cells were recovered from lymphoid organs and the small intestinal LP, as described above. To assess the migration efficiency of the transferred cells, the ratios of CFSE+ cells/SNARF-1+ cells in recipient tissues were analyzed by flow cytometry. To confirm the presence of the transferred cells in the small intestinal LP, in some experiments, blocks of small intestines with or without PPs were fixed and observed using a confocal laser-scanning microscope (FV1000, Olympus, Tokyo, Japan) for histological analyses, as previously described (8).

**Real-time PCR**

Total RNA was isolated from cells using an RNeasy Mini Kit, and cDNA was generated using a QuantiTect Reverse Transcription Kit (both from Qiagen, Hilden, Germany). cDNA was used as a template for real-time PCR to address the expression of CCR9 and other genes in RA-treated cells. The results are expressed as mean ± SD of triplicate cultures. Data are representative of three independent experiments.

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**FIGURE 1.** 9-cis-RA induces CCR9 expression on T cells more efficiently than does all-trans-RA at 100 nM. Naive CD4⁺ T cells derived from DO11.10/Rag2⁻²⁻/B10.D2 bkg mice (A, B) or from B10.D2 mice (C, D) were stimulated with mAbs to CD3 and CD28 in the presence of graded concentrations of 9-cis-RA (dotted lines) or all-trans-RA (solid lines) for 2 d and were further cultured with IL-2 and the same RA concentration without mAbs for 2 d. The cells were analyzed for the expression of CCR9 (A, C) or α4β7 (B) by flow cytometry (A–C) or for that of Ccr9 mRNA by real-time PCR (D). The results are expressed as mean percentage ± SD of CCR9⁺ cells (A, C) and ΔMFI ± SD (B) of triplicate cultures. D. The relative expression levels of Ccr9 mRNA in RA-treated cells are shown as the mean fold induction ± SD relative to that of untreated cells (cultured without all-trans-RA or 9-cis-RA) SD of triplicate cultures. Data are representative of three independent experiments.

**FIGURE 2.** RXR agonists enhance RAR agonist-induced expression on T cells. Naive CD4⁺ T cells from B10.D2 mice were stimulated with mAbs to CD3 and CD28 in the presence of indicated reagents for 2 d and were further cultured with IL-2 and the same concentration of the reagents without mAbs for 2 d. The cells were analyzed for the expression of CCR9 (A, B, E, F), α4β7 (C), or E-selectin ligands (D) by flow cytometry. A. Effects of Am80 (1 nM), HX500 (100 nM), or both on CCR9 expression. Effects of Am80 (1 nM), HX600 (100 nM), or both on CCR9 expression. Effects of Am80 (1 nM), PA024 (100 nM), or both on the expression of CCR9 (B), α4β7 (C), and E-selectin ligands (D), E. Effects of graded concentrations of PA024 on CCR9 expression in the presence (solid line) or absence (dotted line) of 1 nM all-trans-RA. F. Effects of PA024 (100 nM) with or without LE540 (1 μM) on CCR9 expression. Data are mean ± SD of triplicate cultures and are representative of three independent experiments. Statistical significance was determined by the Student t test. *p < 0.05; **p < 0.001.
PCR in triplicates with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers. Ccr9-specific primers were 5'-TGC CAT GTG CAT CTC CAA CTG-3' (forward) and 5'-GAA CTG GGT TCA GAC AAC TGT GG-3' (reverse). PCR and analysis were performed on an ABI 7500 Real-time PCR system. The expression of each gene was normalized with Rplp0.

Statistical analysis

The unpaired two-tailed Student t test was used. Values of p < 0.05 were considered significant.

Results

RXR agonists markedly enhance the RAR-mediated induction of CCR9 expression on T cells

CCR9 expression is induced in naive CD4+ T cells upon stimulation with anti-CD3 and anti-CD28 mAbs in the presence of all-trans-RA (8). However, we noticed that CCR9 expression was often induced in only a small proportion of cells. Under the present culture conditions, <50% of the cells became CCR9+ with various all-trans-RA concentrations, with the exception of an unphysiologically high concentration (1000 nM) (Fig. 1A). 9-cis-RA did not efficiently induce CCR9 expression at 0.1 or 1 nM, but it induced the expression in ~70% of the cells at 100 nM. With regard to inducing α4β7 expression, all-trans-RA and 9-cis-RA were equally efficient at 10–1000 nM, although all-trans-RA was much more potent than 9-cis-RA at lower concentrations (Fig. 1B). The affinities of 9-cis-RA to RAR isotypes, with the exception of RARγ, seem to be equivalent to those of all-trans-RA (37). However, at low concentrations, the availability of 9-cis-RA for RAR binding may be lower than that of all-trans-RA, because RXR can heterodimerize with RAR, as well as with other subfamily 1 nuclear receptors, including liver X receptor (LXR), PPAR, and vitamin D receptor (20). The present results were obtained with naive CD4+ T cells from DO11.10/Rag2−/− mice. Similar results were obtained with naive CD4+ T cells from wild-type (wt) mice, but an even smaller population of the cells became CCR9+ with all-trans-RA (Fig. 1C). The mRNA expression levels of CCR9 corresponded well with the cell-surface expression, with the exception of the expression induced by all-trans-RA at 1000 nM (Fig. 1D). These results suggest the possibility that activation of RAR and RXR is required for the maximal induction of CCR9 expression on T cells.

To test the possibility, we assessed the effect of the RARα,β agonist Am80 with or without an RXR agonist. As shown in Fig. 2A, a low concentration of Am80 (1 nM) alone induced CCR9 expression in a small proportion of T cells. The RXR pan-agonist HX600 (100 nM) by itself failed to induce CCR9 expression, but it significantly upregulated Am80-induced CCR9 expression. PA024 is an RXR pan-agonist that is more potent than HX600 (35). PA024 alone (100 nM) induced CCR9 expression in a small proportion of T cells, but the combination of Am80 and PA024 markedly and synergistically upregulated CCR9 expression (Fig. 2B). PA024, as well as Am80, enhanced α4β7 expression and suppressed E-selectin ligand expression, but the coordinated actions of these two agonists on these expressions were moderate at best (Fig. 2C, 2D). Even at lower concentrations (1 and 10 nM), PA024 significantly upregulated all-trans-RA (1 nM)-induced CCR9 expression, but it induced CCR9 expression minimally at these concentrations (Fig. 2E). The weak effect of 100 nM PA024 alone on CCR9 expression was inhibited by the RXR antagonist LE540 (Fig. 2F), suggesting that PA024 might also exert a weak RAR-activating effect by itself or that weak RAR-stimulating molecules, such as retinol in the culture medium (38), might synergize with PA024. The latter possibility might be involved, because LE540 by itself suppressed basal α4β7 expression and enhanced basal E-selectin ligand expression (Supplemental Fig. 1). Furthermore, in the presence of high concentrations (100 and 1000 nM) of all-trans-retinol, PA024 markedly enhanced CCR9 expression (Supplemental Fig. 2). These results indicate that the full or efficient induction of CCR9 expression is dependent on the activation of RAR and RXR with respective ligands. The results also suggest that the induction of α4β7 expression and the suppression of E-selectin ligand expression may require much lower levels of RAR- and/or RXR-mediated signals than does the induction of CCR9 expression.

The RXR agonist PA024 enhances T cell homing to the small intestinal LP in vivo

To examine the effect of RXR activation on T cell homing in vivo, we used an i.p. injection of Ag because it induces Ag presentation by MLN-DCs that can produce all-trans-RA. First, BALB/c mice received adoptive transfer of CFSE-labeled CD4+ T cells from DO11.10/Rag2−/− mice and then received an i.p. injection of alum-precipitated OVA followed by an i.p. injection of PA024 or vehicle control every other day. Without the OVA injection, almost no CFSE+ T cells migrated into the small intestinal LP, with or without PA024 administration (data not shown). However, after OVA injection, the migration of CFSE+ T cells into the small intestinal LP was detected without PA024 administration, and it was markedly enhanced by PA024 administration (Fig. 3). In contrast, PA024 administration did not significantly affect the numbers of CFSE+ T cells in MLNs, PPs, peripheral lymph nodes (PLNs), and mediastinal lymph nodes. The results indicate that...
RXR stimulation enhances T cells to acquire gut-homing capacity in vivo as well.

The organotins TBT and TPT markedly enhance the all-trans-RA–induced expression of CCR9 on T cells and their gut-homing capacity.

The organotins TBT and TPT are known to bind to RXR at nanomolar concentrations (22–25). They also were suggested to activate an RAR-dependent signaling pathway in mouse monocytic RAW264.7 cells (39). Thus, we examined whether these organotins enhanced CCR9 expression on T cells. We found that 10–30 nM of TBT alone, but not TPT or DBT alone, induced CCR9 expression in a small population of the cells and that 3–30 nM of TBT and TPT markedly enhanced the all-trans-RA–dependent expression of CCR9 at the cell-surface protein levels (Fig. 4A, 4C, 4E, upper panels, Supplemental Fig. 3) and mRNA levels (Fig. 4B). Accordingly, the ability of DBT to interact with RXR is known to be much lower than that of TBT or TPT (40). α4β7 expression was only moderately enhanced by 30 nM TBT (Fig. 4D, 4E, lower panels).

The synergistic effect of all-trans-RA and TBT on CCR9 expression was also observed when naive CD4⁺ T cells were stimulated with Ag and SPL-DCs (Fig. 5A). The RAR antagonist LE540 almost completely inhibited the all-trans-RA– or TBT-
induced expression of CCR9, and it significantly inhibited the effect of 1 nM all-trans-RA plus 10 nM TBT (Fig. 5B).

We then examined whether TBT treatment might also enhance the all-trans-RA-dependent acquisition of T cells with gut-homing capacity. Naïve CD4⁺ T cells were activated and expanded in the presence of a low concentration (1 nM) of all-trans-RA, with or without 30 nM TBT, and were labeled with CFSE, a green fluorophore, or SNARF-1, a red fluorophore, respectively. Equal numbers of cells from the two populations were mixed (Fig. 6A) and adoptively transferred into normal recipients. The homing profiles of the transferred cells were analyzed 16 h later. In the small intestinal LP, the RA+TBT-treated green cells migrated two to three times more efficiently than did the RA-treated red cells (Fig. 6B, 6C). The RA+TBT-treated T cells homed poorly to PLNs, and equivalently to SPL, compared with the RA-treated T cells (Fig. 6C). Typical examples of immunohistochemical staining of transferred cells in LP and PP are also shown (Fig. 6D–I). These results indicate that treatment of naïve T cells with TBT, together with all-trans-RA, enhances their capacity to migrate into the small intestine.

TBT enhances RAR-mediated induction of CCR9 expression via RXR

CCR9 expression induced by TBT alone was inhibited by the RAR antagonist L6540 (Fig. 7A). However, the combination of TBT and the RAR agonist Am80 markedly and synergistically induced CCR9 expression (Fig. 7B–E), whereas the combination of TBT and RXR agonist PA024 failed to exert a synergistic effect (Fig. 7B), suggesting that TBT-mediated signals synergize with RAR-mediated signals, but not RXR-mediated signals, for efficient CCR9 expression. Thus, without the addition of Am80 or all-trans-

RA, TBT might synergize with weak RAR-stimulating molecules, including retinol in the culture medium, rather than with its own weak RAR-stimulating signals. Accordingly, in the presence of high concentrations of all-trans-retinol, TBT markedly enhanced CCR9 expression as did PA024 (Supplemental Fig. 2). Nonetheless, the results indicate that TBT can synergize with the RAR ligands and agonists to enhance CCR9 expression.

Some nuclear receptors, such as PPARs and LXR, can form permissive heterodimers with RXR, whereas RXR/RAR is non-permissive or conditionally permissive (41). Permissive heterodimers can be activated with an RXR agonist alone, whereas a full response of the RXR/RAR heterodimer to RXR agonist occurs only in the presence of an RAR agonist. However, up to 10 μM of the PPARγ agonist rosiglitazone, the PPARγ agonist WY-14643, or the LXR agonist T0901317 alone did not induce CCR9 expression on T cells, and none of them enhanced Am80-dependent induction of CCR9⁺ cells in the presence or absence of the RXR agonist RXR (Hx600 (Supplemental Fig. 4A). Furthermore, up to 1 μM of the PPARγ antagonist T0070907 did not significantly affect CCR9 expression induced by Am80, TBT, or PA024 (Supplemental Fig. 4B). Therefore,
PPARγ, PPARα, and LXR do not seem to be involved in the RXR activator-dependent enhancement of CCR9 expression on T cells.

All-trans-RA and TBT synergistically suppress IFN-γ production, but it does not contribute significantly to enhanced CCR9 expression

Under the present culture conditions, T cells produced IL-2 (20–40 ng/ml) and IFN-γ (0.2–2.0 ng/ml) during the 2 d of culture with anti-CD3 and anti-CD28 mAbs (Fig. 8A, 8B). Other cytokines, including IL-4, IL-6, IL-10, and IL-21, were not detected in any of these culture supernatants. IFN-γ production was significantly suppressed by TBT or all-trans-RA, and it was further suppressed by the combination of the two agents (Fig. 8A). We wondered whether the IFN-γ produced by T cells by themselves might, in turn, curb their CCR9 expression. The addition of 1.5 ng/ml IFN-γ did not significantly affect the TBT- and/or all-trans-RA-dependent induction of CCR9 expression (Fig. 8C), although the addition of a neutralizing mAb to IFN-γ for the first 2 d of culture with anti-CD3 and anti-CD28 mAbs moderately enhanced all-trans-RA– or TBT-dependent induction of CCR9 expression (Fig. 8D).
However, the anti–IFN-γ mAb failed to affect CCR9 expression in the presence or absence of the combination of all-trans-RA and TBT (Fig. 8D). All-trans-RA enhanced IL-2 production in the presence or absence of TBT in naive CD4+ T cells from DO11.10/Rag2−/− mice but only slightly in those from wt mice (Fig. 8B). The addition of 100 ng/ml IL-2 did not affect CCR9 expression in the presence or absence of RA and TBT (data not shown). These results indicate that all-trans-RA and TBT synergistically enhance CCR9 expression without significant dependence on the cytokine production.

**Efficient induction of CCR9 expression on Tregs with costimulation via RAR and RXR**

If Tregs can be efficiently imprinted with organ-selective homing specificities, it would allow efficient targeting of Tregs into distinct tissues for the repression of immune responses. Thus, we isolated naturally occurring naive-like CD62LhighCD4+CD25+ Tregs (42) and activated them with mAbs to CD3 and CD28 in the presence of all-trans-RA and/or PA024. All-trans-RA alone induced significant levels of α4β7 expression, but it only induced CCR9 expression in a minor fraction of the cells, as reported (32). In contrast, PA024 markedly enhanced CCR9 expression in the presence of all-trans-RA (Fig. 9). PA024 also enhanced α4β7 expression, but it did not affect Foxp3 expression. Therefore, costimulation via RAR and RXR may provide an efficient method to obtain Tregs with small intestine-selective homing specificity.

**Discussion**

The ligation of RAR is essential for the induction of expression of small intestine-specific homing receptors α4β7 and CCR9. The RARα,β agonist Am80 mimics the role of all-trans-RA (8). Because T cells usually do not express RARβ, RARα is likely to be responsible for these effects. However, our present study indicates that RXR ligation exerts differential effects on their expression. RXR-mediated stimulation enhanced the RAR-mediated induction of α4β7 expression on T cells only moderately, but it mark-

**FIGURE 9.** The combination of all-trans-RA and the RXR agonist PA024 efficiently induces the expression of CCR9 and α4β7 on naive-like CD62LhighCD4+CD25+ Tregs upon activation. CD62LhighCD4+CD25+ T cells were stimulated with mAbs to CD3 and CD28 in the presence of all-trans-RA (10 nM), PA024 (100 nM), or a combination of them and IL-2 for 3 d and were further cultured with IL-2 and the same concentrations of all-trans-RA and PA024 without mAbs for 2 d. The cells were analyzed for the expression of α4β7, CCR9, and Foxp3 by flow cytometry. Representative flow-cytometric profiles for the expression of CCR9 and α4β7 (A) and Foxp3 (B). Dotted lines in B represent the isotype controls. α4β7 expression (C) and CCR9 expression (D) are shown as mean ± SD of triplicate cultures. Data are representative of three independent experiments.
edly enhanced the induction of CCR9 expression. Thus, costimulation of RAR and RXR efficiently induced small intestine-seeking T cells. Indeed, the migration of CD4⁺ T cells into the small intestinal LP was significantly enhanced by the in vivo or in vitro administration of the RXR agonist PA024 or TBT, respectively (Figs. 3, 6). However, endogenous RXR ligands with high affinities have not been found. The following possibilities can be considered: a yet-to-be discovered endogenous RXR ligand is present; 9-cis-RA is produced and catabolized in a strictly regulated fashion; and RXR ligands with high affinity, such as 9-cis-RA, are produced only spontaneously from physiological retinoids by isomerization and/or dehydrogenation. If one of the latter two possibilities were true, direct RXR stimulation could be limited in vivo, and exogenous RXR agonists might easily exaggerate CCR9 expression on T cells in the presence of low levels of RA or physiological levels of retinol that could weakly bind to RAR (38).

Weak agonistic activities on RXR were found in some environmental chemicals, including bisphenol A, alkylphenols, and organochlorine pesticides (43). However, organotins, including TBT and TPT, can bind to RXR with even greater affinity than 9-cis-RA (23). It was recently reported that a covalent bond is formed between the tin atom of TBT and residue Cys 432 of the human RXRα ligand-binding domain and largely accounts for the high binding affinity of TBT (26). Although the International Maritime Organization called for a global treaty that bans the application of harmful organotin compounds on ships starting January 1, 2003 and a complete prohibition by January 1, 2008, TBT and TPT have frequently been detected at high concentrations in marine sediments, fish, and mollusks (28, 44). The biomagnification of organotins occurs mainly through the food web (28, 44–46). TBT and TPT have even been detected in human blood and liver (28).

These organotin compounds markedly enhanced CCR9 expression on T cells in the presence of low levels of all-trans-RA, and they induced moderate levels of CCR9 expression, even without exogenous RA, in vitro. Similar results were obtained with the RXR agonist PA024. The effect of TBT or PA024 alone was inhibited by the RAR antagonist LE540. Potential RAR ligands might be brought into the culture with FCS. Fresh normal sera usually contain 1–2 μM retinol, most of which is bound to retinol-binding protein. The addition of 0.1 or 1 μM all-trans-retinol with TBT or PA024 markedly enhanced CCR9 expression (Supplemental Fig. 2). TBT or PA024 by itself might also activate RAR directly or indirectly through the conformational change in RXR. However, RXR/RAR is known to be nonpermissive or conditionally permissive (41). Nonpermissive heterodimers are unable to release corepressors in response to RXR ligands and, thus, cannot activate transcription. Corepressors are released upon ligand binding by the heterodimerizing partner receptor (47). It is believed that PA024 does not directly activate RARs (35). It was shown that TBT activates the RXRα/PPARγ heterodimer primarily through its binding to RXR (26). The RXRα/PPARγ heterodimer is asymmetric, allowing the activation function-2 helix of PPARγ to interact with helices 7 and 10 of RXRα and providing a structural basis for permissiveness (48). In contrast, the RXR/RAR heterodimer is symmetric, and no significant interaction between the activation function-2 helix of RXRα and RARα was found (49). Nonetheless, the expression of RAR and RXR seems to be necessary for the efficient expression of CCR9. We found that overexpression of RAR and RXR in a T cell line enhanced the RA-dependent CCR9 promoter activity far more efficiently than that of RAR or RXR alone (Y. Ohoka, A. Yokota, H. Takeuchi, N. Maeda, M. Iwata, submitted for publication). The forced expression of a dominant negative form of RXR in naïve T cells may clarify the role of RXR-mediated signals in CCR9 expression. It is possible that TBT or PA024 activates some nuclear receptors other than those that we and other investigators examined.

The efficiency of CCR9 induction on T cells was affected by various culture conditions, including Ag doses (50), RA doses, RA species, and the presence of RXR ligand. The duration of TCR stimulation seems to be another key for CCR9 expression (Y. Ohoka et al., submitted for publication). However, RXR agonists more or less enhanced CCR9 expression under nearly every condition that we examined. We also noticed that naïve CD4⁺ T cells from DO11.10/Rag2⁻/⁻ mice were more sensitive to all-trans-RA alone for the expression of CCR9 than were those from wt mice (Fig. 1). The difference may be due, in part, to the fact that the wt naïve CD4⁺ T cells were heterogeneous in TCR and might contain some central memory-type cells; however, it might suggest the importance of RXR-mediated signals for CCR9 expression in the in vivo scenario. Upon antigenic stimulation of naïve CD4⁺ T cells with SPL-DCs, TBT also enhanced all-trans-RA–induced CCR9 expression. Thus, SPL-DCs from normal BALB/c mice may not be capable of converting all-trans-RA to 9-cis-RA to provide enough RXR ligands to T cells. However, it is known that CD103⁺ MLN-DCs that express high levels of RALDH2 are highly capable of inducing CCR9 expression on T cells in vitro. Thus, we tried to test the possibility that these DCs might also provide RXR-activating signals by using RXR antagonists. The potent RXR antagonist HX531 (1 μM) significantly suppressed CCR9 expression, but it is also known to inhibit RAR activation at high concentrations (51). In contrast, the specific, but relatively weak, RXR antagonist PA452 (52) did not significantly suppress CCR9 expression, even at 10 μM (Supplemental Fig. 5). Thus, the possibility that MLN-DCs provide RXR-activating signals remains to be investigated.

There may still be a risk for incorporating the organotins with RXR-activating capacity through the diet. Although many studies showed that these organotins disrupt immune functions in vivo (29, 53–55), some of the effects, such as the induction of T cell apoptosis, may be induced by rather high concentrations of the compounds (56). However, their effects on CCR9 expression and homing specificity of T cells could be exerted at nanomolar concentrations and might result in the perturbation of some immune responses.

We also showed in this study that RXR-mediated stimulation significantly enhanced RA-induced CCR9 expression on naïve-like Tregs, retaining the expression of e4β7 and Foxp3. Naturally occurring Tregs are more effective than inductive Tregs from conventional CD4⁺ T cells (57, 58). Furthermore, CD45RA⁺CD4⁺ CD25high naïve human Tregs maintain their Treg phenotype and function upon in vitro expansion (59). These Tregs may be efficiently targeted to the small intestine upon stimulation via RAR and RXR and may be useful for the regulation or prevention of food allergy or inflammatory diseases in the small intestine, including ileitis or Crohn’s disease.

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Disclosures

The authors have no financial conflicts of interest.

References

10 EFFICIENT CCR9 EXPRESSION REQUIRES RXR ACTIVATION


SUPPLEMENTAL FIGURE 1. The RXR agonist PA024 modulates the basal expression of α4β7 and E-selectin ligands on T cells in vitro, and the effects are inhibited by the RAR antagonist LE540. Naïve CD4+ T cells from B10.D2 mice were stimulated and expanded as described in the legend of Fig. 1 in the presence or absence of PA024 (100 nM), LE540 (1 μM) or both. The cells were analyzed for the expression of α4β7 (A) and E-selectin ligands (B) by flow cytometry. The results are expressed as ΔMFI ± SD of triplicate cultures. Data are representative of three independent experiments.
SUPPLEMENTAL FIGURE 2. The RXR agonists PA024 and TBT markedly enhance the CCR9 expression on T cells in the presence of high concentrations of all-trans-retinol. Naïve CD4+ T cells from B10.D2 mice were stimulated and expanded as described in the legend of Fig. 1 with or without PA024 (100 nM) or TBT (30 nM) in the presence of all-trans-retinol (0, 100 or 1,000 nM). The cells were analyzed for the CCR9 expression by flow cytometry. The results are expressed as the mean percentage of CCR9+ cells ± SD of triplicate cultures. Data are representative of three independent experiments.
SUPPLEMENTAL FIGURE 3. TBT enhances the all-trans-RA-induced expression of CCR9 in a dose-dependent fashion. Naïve CD4+ T cells from B10.D2 mice were stimulated and expanded as described in the legend of Fig. 1 in the presence of graded concentrations of all-trans-RA and TBT. The cells were analyzed for the CCR9 expression by flow cytometry. The results are expressed as the mean percentage of CCR9+ cells ± SD of triplicate cultures. Data are representative of three independent experiments.
SUPPLEMENTAL FIGURE 4. Agonists of PPARα, PPARγ or LXR fail to enhance the TBT-induced CCR9 expression on T cells. Naïve CD4+ T cells from B10.D2 mice were stimulated and expanded as described in the legend of Fig. 1 in the presence of indicated reagents, and were analyzed for their CCR9 expression by flow cytometry. A, Effects of Am80 (1 nM), HX600 (100 nM), TBT (30 nM), Rosiglitazone (10 μM), T0901317 (10 μM), WY-14643 (10 μM) or their combinations. B, Effects of Am80 (1 nM), TBT (30 nM), PA024 (100 nM), T0070907 (1 μM) or their combinations. The results are expressed as the mean percentage of CCR9+ cells ± SD of triplicate cultures. Data are representative of three independent experiments.
SUPPLEMENTAL FIGURE 5. The RXR antagonist HX531 but not PA452 suppresses the CD103+ MLN-DC-induced CCR9 expression on T cells. CD103+ DC were obtained from MLN of BALB/c mice as described previously (16). The DC were pulsed with OVA peptide P323-339 (1 μM) and co-cultured with naïve DO11.10 CD4+ T cells at a ratio of 1:1 in the presence or absence of an antagonist. After 5 days of culture, cells were analyzed for the CCR9 expression by flow cytometry. Effects of PA452 (1 or 10 μM), HX531 (0.1 or 1 μM), or the RAR antagonist LE540 (1 μM) are shown. The results are expressed as the mean percentage of CCR9+ cells ± SD of triplicate cultures.