Retinoic Acid-Induced CCR9 Expression Requires Transient TCR Stimulation and Cooperativity between NFATc2 and the Retinoic Acid Receptor/Retinoid X Receptor Complex

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The vitamin A metabolite retinoic acid (RA) plays a critical role in deploying lymphocytes into the gut tissue (1). Naïve T cells can migrate from the bloodstream into secondary lymphoid organs but not into nonlymphoid tissues (2–4). However, once they are activated with Ag, they acquire the ability to migrate into nonlymphoid tissues, preferentially into those associated with the secondary lymphoid organs where they encountered Ag (5, 6). This allows the dispatch of Ag-specific T cells to the place where the Ag-bearing pathogens invaded the body. T cells that are activated in the gut-related lymphoid organs, mesenteric lymph nodes, and Peyer’s patches preferentially migrate into gut tissues (7, 8). A subpopulation of dendritic cells (DCs) in these organs can produce RA from vitamin A (retinol) with retinaldehyde dehydrogenase (1, 9, 10).

RA exerts its physiological effects mostly through binding to the heterodimer of the nuclear receptors: RA receptor (RAR) and retinoic X receptor (RXR) (11–13). Three isoforms (α, β, and γ) of RAR and three isoforms (α, β, and γ) of RXR have been identified. These receptors are ligand-dependent transcription factors that bind to cis-acting DNA sequences, called RA response elements (RAREs), located in the promoter region of their target genes. The functional RAREs are composed of two repeats (also called half-sites), with one, two, or five nucleotides forming a space between the two half-sites. The consensus sequence of the RARE half-sites consists of PuG(T)/(T/A)CA. The major physiologic RA, all-trans-RA, binds to RAR but not to RXR, and it imprints naïve T cells with gut-homing specificity upon activation by specifically inducing the expression of the gut-homing receptors integrin α4β7 and chemokine receptor CCR9 (1). However, no typical RARE was found around the first exon of the mouse or human CCR9 gene or that of the integrin α4 or β7 gene. CCR9 expression seems to be more highly dependent on RA than is α4β7 expression (1, 10, 14).

It remained unclear how RA induced CCR9 expression. TCR-mediated signaling, as well as RA-dependent signaling, is essential for CCR9 expression. TCR-mediated signals with CD28-mediated costimulatory signals activate multiple signaling pathways, which converge on activation of transcription factors, such as NFAT, NF-kB, and AP-1 (Fos-Jun) (15). The NFAT family plays a pivotal role in the T cell activation-induced transcriptional responses (16, 17). The NFAT family contains five members: NFATc1 (NFAT2, NFATc4), NFATc2 (NFAT1, NFATp), NFATc3 (NFAT4, NFATx), NFATc4 (NFAT3), and NFAT5. NFATc1, NFATc2, and NFATc3 are expressed in lymphoid organs. NFAT is composed of several functional domains, including the Ca2+-regulatory domain, the DNA-
binding domain, and the N- and C-terminal transactivation domains (TAD1 and TAD2, respectively). NFAT proteins are primarily phosphorylated and found in the cytoplasm of resting cells. Increased intracellular Ca²⁺ levels activate calcineurin (CN), a Ca²⁺-dependent phosphatase, resulting in the dephosphorylation and translocation of NFAT into the nucleus. NFAT proteins bind to the core sequence (A/T)GGAA(A/N)A(T/C) (16) that is found in transcriptional regulatory regions of many genes inducible in immune cells. The transcriptional activity of NFAT is exerted depending on its binding partners, including AP-1, -Tet, GATA3, and Foxp3 (18–20). The immunosuppressants FK506 and cyclosporin A (CsA) target NFAT activity by inhibiting CN, which is required for nuclear translocation of cytoplasmic NFAT proteins (21).

In the current study, we observed that TCR stimulation induced nuclear translocation of NFATc1 and NFATc2 in murine naive CD4⁺ T cells. NFATc1 and NFATc2 interacted with RAR and RXR and bound to two NFAT-binding sites in the 5'-flanking region of the mouse Ccr9 gene. NFATc2 supported RA-induced Ccr9 promoter activity, whereas NFATc1 suppressed the NFATc2-dependent promoter activity. We found that transient TCR stimulation was essential for RA-induced CCR9 expression on naive CD4⁺ T cells and that NFATc2, but not NFATc1, remained in the nucleus after terminating TCR stimulation. Furthermore, we found that NFATc2 facilitated the binding of RAR to a RARE half-site located between the two NFAT-binding sites. Therefore, the functional cooperation between NFATc2 and the RAR/RXR complex on the promoter region seems to be critical for the transcriptional Ccr9 gene expression.

Materials and Methods

Mice

B10.D2 mice were obtained from Japan SLC. D011.10 TCR-transgenic/Rag2-deficient mice (B10.D2 background) were obtained from Taconic Farm. All animals were maintained in specific pathogen-free conditions in the animal facility of Tokushima Bunri University at Kagawa Campus. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Tokushima Bunri University.

Reagents

Recombinant mouse IL-2 was purchased from Peprotech. All-trans-RA and PMA were obtained from Sigma-Aldrich. AG490, CsA, FK506, ionomycin (IM), PD98059, and wortmannin were purchased from Merck-Calbiochem. LE135 and LE540 were kind gifts from Dr. H. Kagechika (Tokyo Medical and Dental University, Tokyo).

T cell isolation

Naive CD4⁺CD62Lhigh T cells were obtained from lymph nodes and spleens of DO11.10 TCR-Tg/Rag2-deficient mice by using Dynabeads Mouse CD4 and DetachA bead Mouse CD4 (Dynal) and subsequently using MACS CD62L Microbeads and MACS separation columns (Miltenyi Biotec), as previously described (1). In some experiments, CD4⁺CD62L⁺ T cells were isolated by negative selection using EasySep Mouse CD4⁺ T Cell Enrichment Kits (Stemcell Technologies), supplemented with biotinylated anti-mouse CD44 Ab, and were used as naive T cells. More than 99% of the recovered cells were CD4⁺CD62Lhigh. Naive CD8⁺CD62Lhigh T cells were obtained from spleens of B10.D2 mice by negative selection using EasySep Mouse CD8⁺ T Cell Enrichment Kits (Stemcell Technologies), supplemented with biotinylated anti-mouse CD44 Ab, and were used as naive T cells. More than 99% of the recovered cells were CD8⁺CD62Lhigh. Naive T cells were suspended in complete medium (DMEM supplemented with 10% heat-inactivated FCS [Intergen], 3 mM l-glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 50 μM 2-ME, 20 mM HEPES-NaOH [pH 7.2], 100 U/ml penicillin, and 100 μg/ml streptomycin).

T cell culture

Naive CD4⁺ T cells were stimulated with plate-bound Abs to CD3 and CD28, as described previously (1). The incubation period varied, depending on the experiment, as indicated. All-trans-RA was added at the start of the culture or at the indicated time point.

DC-T cell coculture

DCs were prepared from splenocytes of B10.D2 mice, as described previously (10). DCs were cocultured with naive CD4⁺ T cells (2 × 10⁶ cells per well) in the presence of OVA peptide P323–339 (10 μM), at a ratio of 1:1 (DCs/T cells), in 200 μl complete medium in a round-bottom 96-well plate for 5 d with or without 10 nM all-trans-RA. Alternatively, DCs were pulse treated (2 h) with OVA peptide P323–339 (10 μM) and then cocultured with naive CD4⁺ T cells. Expression of CCR9 was measured by flow cytometry with a FACScalibur (BD Biosciences).

Flow cytometric analysis

The cells were stained with a PE-conjugated Ab to CCR9 (clone 242503; R&D Systems) in the presence of anti-Flt3 Ab (clone 2.4G2; BD Biosciences) and were analyzed by flow cytometry with a FACScalibur. In some experiments, surface protein-expression levels were expressed as mean fluorescence intensity (MFI) calculated as: (MFI of cells stained with fluorescein-conjugated Ab) – (MFI of the background staining cells).

Real-time PCR

Aliquots of cultured T cells were immediately lysed using a guanidine thiocyanate/phenol solution (RNAiso; TAKARA), and total RNA purified in the presence of carrier glycerogen, according to the manufacturer’s instructions; cDNA was generated using reverse transcriptase (TAKARA). The level of Ccr9 gene expression was determined by real-time PCR in triplicates with Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers (Supplemental Table I). PCR and analysis were performed on an Applied Biosystems 7500 Real-time PCR system. Quantitative normalization of cDNA in each sample was obtained by the cycle threshold (ΔCT) method (CCR9 CT – ARBPI CT).

Expression of RARs, RXRs, and NFATs in naive CD4⁺ T cells

Total RNA and cDNA from aliquots of cultured naive CD4⁺ T cells were prepared as described above. Expression levels of Rara, Rarb, Rarg, Rra, Rxrb, Nfatc1, Nfatc2, and Nfatc3 mRNA were determined by RT-PCR using the Taq DNA polymerase (TAKARA), with specific reverse and forward primers (Supplemental Table I). PCR products were analyzed by 1.2% agarose gel electrophoresis containing ethidium bromide.

EMSA

Nuclear extracts from T cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (PIERCE), according to the manufacturer’s instructions. The nuclear extracts were incubated with Alexa Fluor 780-labeled DR3 oligonucleotide (5'-Alexa Fluor 780-TCGGAGTGAGG-GTTCCACGAAAGTCTC-3' and 5'-Alexa Fluor 780-CAAGTAGAAC-TTTCGGTGAACCCTACCCTCGA-3'; Invitrogen) for 15 min at room temperature in 20 μl nuclear extract buffer (PIERCE) containing 5 μg poly-(deoxyinosinic-deoxycytidylic) acid (GE Healthcare). Unlabeled oligonucleotide competitor was added to the reaction mixture when indicated. The protein–DNA complexes were separated by 4% polyacrylamide gel and visualized on an ABI 3130 sequencer (Applied Biosystems). pSG5-RARα or pSG5-RXRα expression vector (Stratagene) was cloned into the pGEM-T Easy vector, according to the manufacturer’s instructions; cDNA was generated using reverse transcriptase (TAKARA). The PCR product was subcloned into the pGEM-T Easy vector (Promega), according to the manufacturer’s instructions. The PCR product was cloned into the promoterless firefly luciferase reporter plasmid pGL3-basic (Promega) at the KpnI and Xhol sites. The insert sequence of the resulting plasmid, named pGL3-CCR9, was identical to the sequence found under the National Center for Biotechnology Information’s GenBank accession number NW_001030922.1 (http://www.ncbi.nlm.nih.gov/Genbank). This 1852-bp gene region is located 65 bp upstream of the putative transcription start of the Ccr9 gene. The mouse RXRa was cloned by PCR with mouse kidney cDNA as a template, using specific reverse and forward primers (Supplemental Table II). Then, the PCR product was subcloned into the pGEM-T Easy vector (Promega), according to the manufacturer’s instructions. The PCR product was cloned into the pSG5 expression vector (Stratagene) at the EcoRI site. The insert sequence of the resulting plasmid, named pSG5-RA-RXa, was a kind gift from Drs. S. Kato (Tokyo University, Tokyo, Japan) and P. Chambon (Institute of Genetics and Molecular and Cellular Biology, University of Tokyo).
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tivity was normalized by the Renilla luciferase activity. Experiments were carried out in triplicates, and the firefly luciferase accessed by the dual-luciferase assay system (Promega) in a luminometer eluted in SDS-PAGE buffer. Samples were analyzed by SDS-PAGE, followed by western blot analysis with anti-Myc Ab (clone 9E19; Upstate) and then subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blot analysis using the ECL system (GE Healthcare).

DNA-affinity precipitation assay

The biont-labeled DNA probes were purchased from Sigma-Aldrich and annealed to complementary oligonucleotides. COS cells were transfected with 2.5–4 μg expression vectors using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. Transfected COS cells or CD4+ T cells were lysed with DNA-affinity precipitation (DNAP) binding buffer (25 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.25% Nonidet P-40, 1 mM DTT, and complete protease inhibitor mixture [Nacalai Tesque]). Cell debris was removed by centrifugation for 10 min. Lysates were first incubated with streptavidin-Sepharose beads (GE Healthcare) for 30 min to eliminate nonspecific binding and then were incubated with 1.5 μg poly(dexoyinosinic-deoxycytidylic) acid and 2 μg biotinylated DNA probe for 1 h at 4°C. Streptavidin-Sepharose beads were then added and incubated with these mixtures for an additional 30 min at 4°C. After washing the beads three times in DNAP binding buffer, precipitated proteins were eluted in SDS-PAGE buffer. Samples were analyzed by SDS-PAGE, followed by Western blot analysis.

Transfection and luciferase assay

EL4 lymphoma cells were maintained in complete medium. Cells were seeded into 12- or six-well plates (5 × 105 or 1 × 106 cells/well, respectively) and transfected with 2.5–4 μg pGL3-CCR9s, 0.5–1 μg expression vectors, and 0.025 μg pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. After 24 h, the cells were transferred into new 48-well plates and stimulated with IM and PMA in the presence or absence of 100 nM RA. Fourteen hours after transfection, the cells were washed in PBS and lysed in 1× passive lysis buffer (Promega). The firefly and Renilla luciferase activities were measured by the dual-luciferase assay system (Promega) in a luminometer (Turner TD-20/20), according to the manufacturer’s instructions. All experiments were carried out in triplicates, and the firefly luciferase activity was normalized by the Renilla luciferase activity.

Pull-down assay

COS cells were transfected with 2.5 μg pCMV-MyoN-FATc1 or -NFATc2 using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were lysed in 0.5 μl lysis buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1% [v/v] Triton X-100, and complete protease inhibitor mixture). Cell debris was removed by centrifugation for 20 min. Lysates were incubated with the GST-fused RARα or RXRα fixed on 20 μl glutathione beads (GE Healthcare). After the beads were washed extensively with the lysis buffer, they were eluted with SDS-PAGE sample buffer. Each fraction was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blot analysis with anti-Myc Ab (clone 9E19; Upstate) and then visualized using the ECL system (GE Healthcare).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed on 1 × 106 CD4+ T cells, essentially according to the manufacturer’s instructions (Upstate Biotechnology), with modifications. Briefly, aliquots of cultured T cells were fixed with 1% formaldehyde at 37°C for 10 min. Cross-linking reactions were quenched with 250 mM glycine. Cells were washed, suspended in SDS lysis buffer, and sonicated to shear the chromatin into 200–500-bp fragments using a sonicator (Bioruptor UCW-201). After centrifugation to remove debris, aliquots were incubated with 5 μg anti-NFATc1 (clone 7A6; Santa Cruz Biotechnology), anti-NFATc2 (clone 25A10.D6.D2; Thermo) or control IgG overnight at 4°C with rotation. After 30 μl protein G beads (Cell Signaling Technology) was added and incubated for 1 h at 4°C with rotation, the immunoprecipitates were sequentially washed with low-salt buffer, high-salt buffer, and LiCl buffer and twice with TE buffer. The DNA–protein complex was eluted with elution buffer, and cross-links were reversed at 65°C for 4 h. Proteins were digested with 10 μg/ml proteinase K for 1 h at 45°C, and DNA was re-replenished by phenol/chloroform extraction and ethanol precipitation. Specific DNA was amplified by PCR using primers specific for the mouse Ccr9 promoter, forward: 5′-GACCCCAAGCGTACCTGGACCTT-3′ and reverse: 5′-CCCGGAGAATTGATTCTCGTTGC-3′. The binding of NFATc1 or NFATc2 to the Ccr9 promoter site was estimated by real-time PCR. The binding levels, R²-expressed as the percentage of input DNA and were calculated from ΔCT (ΔCT = input CT − ChIP CT), according to the following equation: percentage total = 2−ΔCT.

Transcription factor-binding site analysis

Genomic DNA sequences were analyzed using the MatInspector computer program (http://web.biocomp.unibo.it/matinsearch/db/TFSEARCH.html) or TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) to find specific sequences, including some transcription factor-binding sites.

Statistical analysis

Statistical comparisons were carried out using the two-tailed unpaired Student t test. The p values <0.05 were considered significant.

Results

Transient TCR stimulation is essential for RA-induced CCR9 expression in CD4+ T cells

We often noticed that CCR9 expression was induced by RA on naive CD4+ T cells more efficiently when DCs had been pulsed with Ag peptide before culturing with T cells than when the same concentration of Ag peptide was directly added to the culture of DCs and T cells (Fig. 1A, 1B). It was reported that a low Ag dose was required for efficient CCR9 induction on naive CD8+ T cells (24). The Ag dose on our Ag-pulsed DCs was likely to decrease along with the culture. However, it might be possible that the duration of antigenic stimulation affected CCR9 expression.

Thus, we performed kinetic analysis of Ccr9 expression in naive CD4+ T cells activated with Abs to CD3 and CD28 for 48 h in the presence of RA. After the culture, the cells were cultured in fresh medium containing IL-2 without Abs in the presence of RA (Fig. 1C). CD28-mediated stimulation is required for the full activation of naive T cells (15). IL-2 was not essential for CCR9 expression, but it supported the cell survival (data not shown). Freshly isolated naive CD4+ T cells expressed a low level of Ccr9 mRNA but lost it within 24 h of stimulation in the presence or absence of RA. RA failed to induce mRNA and cell surface expression of CCR9 as long as the Ab stimulation continued, but it dramatically induced it after the removal of Abs (Fig. 1C, 1D). Similar results were obtained with naive CD8+ T cells (Supplemental Fig. 1).

The duration of Ab stimulation also affected Ccr9 expression (Fig. 1E). Naive CD4+ T cells were stimulated for various periods (6–48 h) with Abs and then cultured without Abs for 24 h. Six to forty-eight hours of stimulation resulted in significant induction of CCR9 expression in the presence of RA in the primary or secondary culture. RA was not necessarily required for the entire culture period. However, 0–3 h of Ab stimulation failed to induce expression in the presence or absence of RA (Fig. 1E, inset, data not shown). Twenty-four hours of Ab stimulation was optimal for expression. In contrast, when RA was added only after the 48-h Ab stimulation, the cells expressed a significantly lower level of Ccr9.
mRNA than did those treated with RA during Ab stimulation. These results suggested that TCR stimulation is essential for naive CD4+ T cells to acquire responsiveness to RA, but it has to be terminated after a proper period to induce CCR9 expression.

TCR-mediated activation of MAPKs and Ca2+/CN-NFAT is essential for sensitizing naive CD4+ T cells to RA

To clarify the TCR signals essential for sensitizing naive CD4+ T cells to RA, we examined the effects of various inhibitors on Ccr9 expression. To minimize any toxicity of inhibitors on T cells, we shortened the stimulation period in the presence of inhibitors to 6 h. The cells were then cultured without Abs and inhibitors in the presence of RA for 12 h. RA-induced Ccr9 expression was markedly inhibited by the addition of PD98059, a MEK1 inhibitor, or FK506, an immunosuppressant (Fig. 2A). Another immunosuppressant (CsA) also inhibited the expression (data not shown). Wortmannin, a PI3K inhibitor, moderately inhibited RA-induced Ccr9 expression, whereas AG490, a JAK inhibitor, failed to inhibit it. The inhibitors did not significantly affect the cell viability under these conditions (Fig. 2B). The combination of PMA and the Ca2+ ionophore IM, but neither one alone, mimicked the Ab stimulation to induce Ccr9 expression in the presence of RA (Fig. 2C). These results suggested that an increase in the intracellular Ca2+ level and PKC activation induced in naive CD4+ T cells upon TCR stimulation trigger them to acquire the responsiveness to RA through the activation of Ca2+/CN-NFAT and MAPK pathways.

TCR stimulation enhances the expression of RXRs and NFAT in naive CD4+ T cells

We previously suggested that RARα and/or RARβ, but not RARγ, are involved in RA-induced gut-homing receptor expression (1). To determine the RA isoforms in charge, we first evaluated the expression levels of the RAR and RXR genes in naive CD4+ T cells and in those stimulated with DCs and CD28 in the presence or absence of RA. Rara was expressed in naive CD4+ T cells, and its expression level remained unchanged by the stimulation (Fig. 3A). Rarb expression was undetectable with or without the stimulation. Expressions of Rarg, Rxra, and Rxrb were undetectable or marginal in naive CD4+ T cells, but they became detectable after 6–12 h of stimulation. RA did not significantly affect the expression of some isoforms might be slightly downregulated in the presence of RA. Next, we analyzed RARE-specific DNA-binding activity in nuclear extracts from naive and activated CD4+ T cells by EMSA. The activity was only weakly detected in the nuclear extracts of naive CD4+ T cells, but it was strongly detected in those obtained after 24 h of stimulation (Fig. 3B). Because the DNA-binding activity of RARs usually depends on the formation of the RAR/RXR complex, the present observations suggested that the induction of RXRα or RXRβ expression by TCR signals may contribute to the acquisition of RA responsiveness. We then assessed the expression levels of the NFAT genes in naive CD4+ T cells and in those stimulated with Abs to CD3 and CD28 for 48 h in the presence or absence of RA (Fig. 3C). The mRNA transcripts of Nfatc1, Nfatc2, and Nfatc3 were detected in naive CD4+ T cells. The expression levels of Nfatc1 and Nfatc2 increased significantly after 12–48 h of stimulation, whereas the expression level of Nfatc3 increased moderately. RA did not significantly affect the expression of Nfat isoforms.

Negative transcriptional control of CCR9 expression by TCR signaling

Because CCR9 expression was not induced during TCR stimulation, even in the presence of RA (Fig. 1C), we wondered whether TCR stimulation might also deliver suppressive signals to CCR9 expression. Thus, after we stimulated naive CD4+ T cells with Abs.
for 24 h, we restimulated the cells with Abs in the successive culture in the presence of various inhibitors and RA for 6 h (Fig. 4A). RA-induced Ccr9 expression was low, but it was significantly enhanced in the presence of FK506 or CsA. In contrast, PD98059 or wortmannin failed to affect the expression. Next, we examined whether FK506 or CsA also canceled the suppressive signals to CCR9 expression in continuously stimulated CD4+ T cells with Abs to CD3 and CD28. Increasing concentrations of FK506 and CsA progressively canceled the suppressive effect of CD3/CD28 stimulation on Ccr9 expression (Fig. 4B). These results suggested that TCR signaling prevents RA-induced transcription of the Ccr9 gene in CD4+ T cells by activating a Ca2+/CN-NFAT–signaling pathway. Similar results were obtained with naive CD8+ T cells, suggesting that NFAT activation is involved in the regulation of CCR9 expression on CD8+ T cells as well, although higher concentrations of CsA were required for its effects compared with those for CD4+ T cells (Supplemental Fig. 2).

However, unlike Ccr9 mRNA expression, CsA failed to induce CCR9 protein expression on the CD4+ T cell surface during CD3/CD28 stimulation (Fig. 4B). After removal of Ab stimulation, the CsA-treated T cells expressed higher levels of CCR9 protein on their surfaces than did T cells stimulated without CsA. Similar results were obtained with FK506 (data not shown). The addition of CsA or FK506 to the recovery culture without Abs did not affect CCR9 expression (data not shown). These results suggested that CD3/CD28-mediated stimulation downregulates RA-induced transcription of the Ccr9 gene in an NFAT-dependent fashion, as well as downregulates translation into or translocation of CCR9 protein in a CsA/FK506-resistant fashion in T cells.

NFAT plays a crucial role in CCR9 promoter activity

The mouse Ccr9 gene consists of three exons separated by two introns (25) (Fig. 5A). The first exon encodes a 5’-untranslated region. The second exon encodes the N-terminal extracellular domain, and the third exon encodes the seven-transmembrane...
domain and C-terminal domain. The 5’-flanking region of the first exon contains typical mammalian promoter consensus elements, a TATA box (−312), several SP1-binding sites, three putative NFAT-binding sites (−322, −1083, and −1583), and an κB-like site (−426). Thus, we obtained a 1.85-kb fragment (−1787 to +65) of the 5’-flanking sequence that contained these elements. To examine whether NFAT is directly involved in the induction of Ccr9 expression, EL4 lymphoma cells were transfected with an NFAT expression vector and a pGL3 reporter vector containing the 5’-flanking region of the Ccr9 gene (pGL3-CCR9).

**FIGURE 4.** Successive TCR stimulation prevents the RA-dependent induction of Ccr9 expression in CD4+ T cells but fails to prevent it in the presence of immunosuppressants. A, Naïve CD4+ T cells were stimulated with Abs to CD3/CD28 for 24 h without RA. The cells were resuspended in fresh medium containing 20 U/ml IL-2 and cultured further in plates coated with or without Abs to CD3/CD28 for 6 h, in the presence (black bars) or absence (white bars) of 10 nM RA, together with 10 nM FK506 (left panel) or with 200 nM CsA, 50 μM PD98059, or 100 nM wortmannin (right panel). The relative Ccr9 expression levels are shown as the mean ± SD of triplicate cultures. The Ccr9 expression level in the cells stimulated with the Abs only in the first culture. B, Naïve CD4+ T cells were stimulated with Abs to CD3/CD28 for 24 h in the presence of 10 nM RA. Graded concentrations of FK506 or CsA were added during the last 12 h of the culture. Aliquots of the cells were analyzed for the expression of Ccr9 by real-time PCR (left lower panel). The relative Ccr9 expression levels are shown as the mean ± SD of triplicate cultures. The Ccr9 expression level in the cells stimulated with the Abs in the absence of the immunosuppressants was set as 1. Other aliquots of the cells were further cultured without Abs for 24 h in the presence of 10 nM RA. At the indicated time, cells were analyzed for the surface expression of CCR9 by flow cytometry (right lower panel). The CCR9 expression levels are shown as the mean ± SD of triplicate cultures. Statistical significance was determined by the Student t test; *p < 0.01. Results are representative of three independent experiments.

Ccr9 expression, EL4 lymphoma cells were transfected with an NFAT expression vector and a pGL3 reporter vector containing the 5’-flanking region of the Ccr9 gene (pGL3-CCR9). Ccr9 promoter
activities were calculated by arbitrarily defining the activity of pGL3-RA for 16 h; luciferase activities were measured. The relative promoter stimulated with 0.2 μm RA. Relative luciferase activity in each sample was represented as the ratio to luciferase activity without RA and IM/PMA.

FIGURE 6. Expression of NFATc2 and RARα/RXRα is required for the RA-dependent induction of Ccr9 promoter activity. A, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid with or without the expression vectors pME-NFATc2 and/or pSG5-RARα and pSG5-RXRα. One day after transfection, cells were stimulated or not with 0.2 μg/ml IM and 50 ng/ml PMA and with or without 100 nM RA for 16 h; luciferase activities were measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9 alone (without RA and IM/PMA) as 1. C, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid in combination with pME-NFATc2 and either pSG5-RARα or pSG5-RXRα. One day after transfection, cells were treated with IM/PMA and graded concentrations of RA. Relative luciferase activity in each sample was represented as the ratio to luciferase activity without RA and IM/PMA. B, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid in combination with empty vector pME-NFATc1, pME-NFATc2, or pME-NFATc3, together with pSG5-RARα and pSG5-RXRα. One day after transfection, cells were stimulated with 0.2 μg/ml IM and 50 ng/ml PMA with or without 100 nM RA for 16 h; luciferase activities were measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9 alone (without RA and IM/PMA) as 1. C, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid in combination with pME-NFATc2 and either pSG5-RARα or pSG5-RXRα. One day after transfection, cells were stimulated as described in B, and the relative promoter activities were calculated as in B. D, Serial-deletion constructs derived from the mouse Ccr9 5′-flanking region were inserted in the reporter plasmid pGL3-basic. EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid or the deletion mutants in combination with pME-NFATc2, pSG5-RARα, pSG5-RXRα, and pME-NFATc1 expression vectors. One day after transfection, cells were stimulated with IM/PMA and graded concentrations of RA. Relative luciferase activity in each sample was represented as the ratio to luciferase activity without RA and IM/PMA.

The ectopic NFATc2 expression induced promotor activity slightly. IM/PMA stimulation alone failed to induce Ccr9 promoter activity. In contrast, II22 promoter activity was significantly induced upon IM/PMA stimulation alone in EL4 cells, and it was moderately enhanced by ectopic expression of NFATc1, NFATc2, or NFATc3 (data not shown). Among the three putative NFAT-binding sites in the 5′-flanking region of the Ccr9 gene, the site at −322, but not the other two sites, seemed to be essential, because mutation in the site at −322 alone resulted in a dramatic decrease in NFATc2-dependent promoter activity (Supplemental Fig. 3).

The sequence (5′-CGGAAA-3′) of the kB-like site (−426) in the 5′-flanking region of the Ccr9 gene was closely related to the sequences of the reported NFAT-binding sites (16). To test whether the kB-like site also contributes to the NFATc2-dependent Ccr9 promoter activity, we introduced a mutation in the kB-like site (−426) or the NFAT site (−322) (Fig. 5C) within the 0.68-kb fragment (−615 to +65) of the 5′-flanking region (pGL3-CCR9S), which contained the minimal essential elements for the CCR9 expression (see later discussion). The mutation in either site markedly reduced the promoter activity (Fig. 5D). Furthermore, NFATc1 and NFATc2 bound to an oligonucleotide DNA containing the kB-like site (−426), as well as to that containing the NFAT site (−322), but they bound only weakly to those containing the mutated sites (Fig. 5E). The results indicated that the binding of NFATc2 to the kB-like site (−426), as well as the NFAT site (−322), is essential for Ccr9 promoter activity. The results also raised the possibility that NFATc1 competes with NFATc2 to bind to these sites.

Cooperation between NFATc2 and RAR/RXR is essential for RA-induced CCR9 expression

Although NFATc2 induced Ccr9 promoter activity in EL4 cells transfected with pGL3-CCR9, RA with or without IM/PMA exerted little effect on the activity (Fig. 6A). The NFATc2-induced promoter activity was not dependent on the residual serum retinoids in the culture, because the activity was also induced in the serum-free culture medium in the presence or absence of RAR antagonists, although IM/PMA moderately enhanced the NFATc2-induced activity under these conditions (Supplemental Fig. 4). Ectopic expression of RARαs and RXRα alone did not induce promoter activity, even in the presence of RA; however, a marked RA-dependent increase in promoter activity was observed upon IM/PMA stimulation in EL4 cells that had been transfected with the
expression vectors of NFATc2, RARα, and RXRα (Fig. 6A). RA enhanced the promoter activity in a dose-dependent manner (Fig. 6A, inset). However, the enhancement was not significant when NFATc1 or NFATc3 was ectopically expressed instead of NFATc2 (Fig. 6B) or when either RARα or RXRα was transfected instead of both RARαs and RXRαs (Fig. 6C). Cooperation between activated NFATc2 and the RARα/RXRα complex seemed to be required for RA to enhance the Ccr9 promoter activity. To define the RA-responding sites within the 5′-flanking region of the Ccr9 promoter, we made a series of truncations within the region (Fig. 6D). Deletion of the 5′-flanking region up to nucleotide −615 did not markedly reduce the RA-induced Ccr9 promoter activity. However, further deletion, including the kB-like site (−426), resulted in a dramatic loss of reporter activity. These data suggested that the NFAT site (−322) and the kB-like site (−426) were necessary for the RA-dependent promoter activity.

Computational analysis of the Ccr9 promoter region showed a putative RARE half-site (−329) located adjacent to the NFAT site (−322) (Fig. 7A). To analyze its possible role, we introduced mutations in the RARE half-site (−329), the NFAT site (−322), and the kB-like site (−426) within pGL3-CCR9S (Fig. 7A). Mutation in any one of these sites resulted in a pronounced reduction in the RA-induced promoter activity (Fig. 7B). We also found that RARα could bind to an oligonucleotide DNA containing the RARE half-site, but it failed to bind to one containing the mutated RARE half-site (Fig. 8A). Interestingly, when NFATc2 was coexpressed with RARαs, the binding of RARα to the DNA containing the RARE half-site (−329) was apparently enhanced. The binding of RXRαs to the same DNA probe was also observed but was weaker than that of RARαs. Thus, we examined whether NFATc2 also directly interacted with RARα or RXRαs. Indeed, NFATc2 bound to RARα and RXRαs (Fig. 8B). The DNA-binding domains of NFATc2 and RXRα were required for their binding (Supplemental Figs. 5, 6). These observations suggested that cooperation between activated NFATc2 and the RARα/RXRα complex and their binding to the NFAT site (−322), the kB-like site (−426), and the RARE half-site (−329) are involved in activation of the Ccr9 promoter. However, NFATc1 also bound to RARα and RXRαs (Fig. 8B), which raised the possibility that NFATc1 might fail to form a proper transcription-initiation complex or rather actively obstruct RA-induced CCR9 expression.

**Differential roles of NFATc1 and NFATc2 in RA-induced CCR9 gene expression in CD4⁺ T cells**

We found that overexpression of NFATc1 markedly inhibited the RA-induced Ccr9 promoter activity with ectopic expression of NFATc2 and RARα/RXRαs (Fig. 9A).

Because NFATc1 and NFATc2 during the process of RA-dependent induction of CCR9 expression (Fig. 9B). The naïve CD4⁺ T cells were stimulated with Abs to CD3 and CD28 in the absence of RA for 48 h, and aliquots of the cells were further cultured without Abs in the

**FIGURE 7.** The RARE half-site is essential for the RA-dependent induction of Ccr9 promoter activity. A, Localization of the RARE half-site (half-RARE) adjacent to the NFAT site (−322) in the mouse CCR9 5′-flanking region and nucleotide sequences around the kB-like site (−426). NFAT site (−322), and RARE half-site (−329). B, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid or pGL3-CCR9S-reporter plasmids containing mutated (indicated by X) NFAT site (−322), kB-like site (−426), or RARE half-site (−329) with or without the expression vectors pME-NFATc2 and/or pSG5-RARαs and pSG5-RXRαs. One day after transfection, cells were stimulated as described in the legend of Fig. 6, and luciferase activities were measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9S alone (without RA and IM/PMMA) as 1. Error bars represent SD. Results are representative of three independent experiments.
FIGURE 9. Differential roles of NFATc1 and NFATc2 in the RA-dependent induction of Ccr9 expression. A, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid in combination with the expression vectors pSG5-RAα, pSG5-RXα, pCMV-Myc-NFATc2 (0 or 0.1 μg), and pCMV-Myc-NFATc1 (0, 1.0, or 2.5 μg). One day after transfection, cells were stimulated as described in the legend for Fig. 7, and luciferase activities were measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9 alone as 1. Error bars represent SD. B, Naive CD4+ T cells were stimulated with Abs to CD3 and CD28 for 48 h in the presence or absence of RA for 12 h to induce CCR9 expression. Immediately after the first culture with the Abs, NFATc1 and NFATc2 were detected in the nuclear fraction, significantly and weakly, respectively. However, after the secondary culture, a marked decrease in NFATc1 and a moderate increase in NFATc2 in the nuclear fraction were observed. RA did not significantly affect the expression or localization of NFATc1 and NFATc2.

Because FK506 and CsA canceled the suppressive effect of the successive TCR stimulation on CCR9 expression (Fig. 4), we examined whether the addition of CsA for the last 12 h of the 48-h culture with Abs to CD3 and CD28 might induce similar changes in NFAT translocation. Indeed, upon CsA treatment, NFATc1 disappeared from the nuclear fraction, whereas NFATc2 in the nuclear fraction remained unchanged or increased (Fig. 9C). Next, we used DNAP assay to examine whether NFATc1 or NFATc2 in the cytosolic and nuclear fractions (Fig. 9B) could bind to the NFAT site (−322) and the κB-like site (−426) (Fig. 9D). Immediately after the stimulation with Abs to CD3 and CD28, NFATc1 and NFATc2 in the nuclear fraction bound to both sites. However, after the secondary culture without Abs, NFATc1 in the nuclear fraction minimally bound to either one of the NFAT-binding sites, whereas NFATc2 in the nuclear fraction still bound to both. Finally, we performed ChIP assay to confirm the results (Fig. 9E). Indeed, after the secondary culture, the binding of NFATc1 to the NFAT-binding sites was significantly reduced, whereas the binding of NFATc2 was sustained, even in the absence of RA. These results suggested that transient TCR stimulation of naive CD4+ T cells induced the activation of NFATc1 and NFATc2, followed by an enhanced NFATc2 activation and the inactivation or degradation of NFATc1, and, thus, differentially recruit NFATc1 and NFATc2 to the two NFAT-binding sites within the Ccr9 promoter in a RA-independent manner. Therefore, as found in the EL4 cells, the cooperation between activated NFATc2 and the RAα/RXα complex through binding to the NFAT site (−322), the κB-like (−426) site, and the RARE half-site (−329) is likely to be involved in RA-induced CCR9 expression in T cells (Supplemental Fig. 7). Thus, depending on the binding of these molecules to the Ccr9 promoter, RA may exert its role through binding to RAR in the complex.

Discussion

In this study, we showed that transient TCR stimulation was required for RA-induced expression of the chemokine receptor CCR9 on naive CD4+ and CD8+ T cells. TCR signaling seems to play dual conflicting roles in the regulation of CCR9 expression. One of the roles is to confer responsiveness to RA in naive T cells. Because RA
exerts a variety of biological effects on various cells, it is conceivable that the responsiveness to RA is tightly regulated, depending on the cell type and the biological process. The type II nuclear receptors, such as RAR and 1α,25-dihydroxyvitamin D₃ (VD₃) receptor, require heterodimerization with RXR for high-affinity binding to the target DNA and their transcriptional activities (11, 26, 27). It was reported that the responsiveness to RA or VD₃ depended on the localization, ectopic expression, or stability of RXRs in a breast cancer cell line (28–30). Thus, the available RXR protein levels might define the RA or VD₃ sensitivity in certain cell types. In this study, we demonstrated that the naive CD4⁺ T cells expressed Rara, but they did not significantly express Rtra or Rthr, and they failed to respond to RA without TCR stimulation. However, upon CD₃- and CD28-mediated stimulation, the expression of Rtra and Rthr was induced (Fig. 3). Furthermore, recent results from our laboratory indicated that RXR activation enhanced the RA-dependent induction of CCR9 expression on naive CD4⁺ T cells, especially when RA levels were low (31). Thus, the expression of Rtra or Rthr seemed to be required for the induction of RA responsiveness in naive T cells. However, a RARE half-site, but no typical RARE, was found in the 5′-flanking region of the first exon of the mouse Ccr9 gene. Accordingly, the ectopic expression of RARα and RXRα without NFATc2 in EL4 cells in the presence of RA showed no effect on the promoter activity of the luciferase reporter gene containing the 5′-flanking region of the Ccr9 gene.

The activation of Ccr9 promoter required two NFAT-binding sites located at −322 and −246 (κB-like site). NFATc1 and NFATc2 bound to these sites, but there is no typical AP-1-binding site around the NFAT-binding sites. In contrast, IL2 promoter activity is regulated by the cooperation of activated NFAT and AP-1 (32). NFAT induces the promoter activity of TNF-α or IL-13, without any necessity to cooperate with AP-1, but it may use κB-like sites, to which NFATc2 proteins bind as a dimer or a heterodimer with another nuclear partner distinct from AP-1 (33). In the current study, we found that coexpression of RARα/RXRα with NFATc2 in EL4 cells significantly induced Ccr9 promoter activity in response to RA upon IM/PMA stimulation. This suggested that cooperation between NFATc2 and RARα/RXRα induces RA responsiveness. Indeed, we found that the RARE half-site located close to the NFAT-binding site within the Ccr9 promoter played an important role in RA-induced Ccr9 promoter activation. The role of RARE half-sites has been a puzzle. A pair of RARE half-sites, separated by 10–200 bp, was considered to function as a nonclassical RARE for the gene expression (34, 35). Recently, it was reported that a single RARE half-site plays an important role in the Foxp3 gene promoter but without demonstrating the actual binding of RARα/RXRα (36). We demonstrated that RARα and RXRα could bind to the RARE half-site (−329), and coexpression of NFATc2 with RARα increased binding of RARα to the RARE half-site. In addition, we showed that NFATc2 directly bound to RARα. Our results suggested that RARα and NFATc2 bound to the respective binding sites in the Ccr9 promoter region interact, even in the absence of RA, and that the cooperation between them plays a significant role in the RA-dependent CCR9 expression. Taken together, the formation of a multimolecular complex containing NFATc2, RARα, and RXRα, with footholds at the two NFAT-binding sites and the RARE half-site, may be induced upon transient TCR stimulation. The formation of the multimolecular complex may bring about the responsiveness to RA by T cells and, thus, allow RA to trigger the Ccr9-transcription process. It was reported that RA-induced CXCR5 expression is dependent on the interaction of RARα/RXRα with other transcription factors (37). RARα and RXRα bind to a novel RARE containing two GT boxes in the distal portion of the 5′-flanking region of the Cxcr5 gene. The RA-induced trans-activating capacity of this element depends on downstream sequences containing Oct1-, NFAT-, and CREB-binding sites. This suggests that RARα binds to Oct1, NFATc3, and CREB, and it may form a large complex with them to initiate RA-induced transcription activation of the Cxcr5 gene. However, in the Ccr9-promoter region, there is no other RAR- or RXR-binding site next to the RARE half-site. Nonetheless, TCR stimulation or IM/PMA also induces or activates transcription factors other than NFATc2 and RAR/RXR; some of them may participate in the complex formation with NFATc2 and RAR/RXR for the Ccr9 transcription. This is in agreement with the fact that IM/PMA stimulation was required, in addition to the ectopic expression of NFATc2, RARα, and RXRα, for the Ccr9 promoter-driven reporter in EL4 cells to respond to RA, although IM/PMA might contribute to the responsiveness, in part, by keeping NFATc2 active.

Another role of TCR signaling is to inhibit RA-induced expression of CCR9. FK506 and CsA canceled the TCR-mediated inhibition of Ccr9 gene transcription, suggesting that NFAT activation is involved in the inhibition. TCR stimulation may also inhibit translocation into or translocation of CCR9 protein but in a CsA/FK506-resistant fashion. It is still possible that an NFAT-independent mechanism may be involved in the TCR-mediated regulation of surface CCR9 protein expression, because FK506 or CsA could not induce surface CCR9 protein expression in the presence of TCR stimulation and RA. We showed that NFATc1 minimally induced Ccr9-promoter activity, even in the presence of RA, IM/PMA, and the ectopic expression of RARα and RXRα in EL4 cells, although the DNA-binding domains of NFATc1 and NFATc2 are highly conserved. Similarly, it was shown that NFATc1 and NFATc2 bound to the NFAT-binding region in the Tfna gene, but only NFATc2 induced Tfna-α expression (38). Because NFATc1 and NFATc2 bound to the NFAT sites and RARα, their functional differences may depend on the TAD2 in their C-terminal ends. NFATc1 may play an essential role in the inhibition of CCR9 expression by prolonged TCR stimulation. Indeed, although NFAT is an important mediator for the expression of various cytokines, evidence has accumulated that NFAT also represses the transcription of some genes. NFATc1 and NFATc2 repress the expression of osteocalcin and Cdk4, respectively, by increased recruitment of histone deacetylase (39, 40). Thus, NFAT1 may inhibit CCR9 expression by recruiting histone deacetylase to the Ccr9 promoter or by preventing the complex formation of NFATc2 with RARα/RXRα and other transcriptional factors.

We cannot deny that NFATc1 may also play a positive role in CCR9 expression. In the early stage of TCR stimulation, the increase and activation of NFATc1 may be required for the sufficient expression of RARs or other molecules, which may contribute to induce the RA responsiveness in naive T cells. In the late stage, sustained NFATc1 activation may inhibit RA-induced CCR9 expression. After terminating TCR stimulation or adding CsA, NFATc1 disappeared from the nucleus; in contrast, NFATc2 was still present. ChIP assays further indicated that NFATc1 dissociated from the Ccr9 promoter, but NFATc2 remained bound to the promoter after terminating TCR stimulation. These results suggested that NFATc2 is the predominant isoform involved directly in RA-induced CCR9 expression. The mechanism of the differential localization of NFATc1 and NFATc2 is not clear. It was suggested that NFATc2 is activated preferentially by reduced calcium signaling compared with NFATc1 in CD8⁺ anergic T cells, although these responses occurred in minutes (41). Thus, selective activation of NFATc2 and inactivation of NFATc1 after transient stimulation via TCR may be regulated, in part, by the intracellular Ca²⁺ level. However, in EL4 cells, ectopic NFATc2 expression and IM/PMA
addition were required for RA responsiveness. Depending on the cell type, additional factors, such as the expression levels of CN, NFAT kinases, or degradation enzymes, may affect the activation status of NFAT members.

The physiological meaning of the negative regulation of CCR9 expression by TCR stimulation is not clear. One possibility is that it reduces Th1 differentiation in the gut. Sustained NFAT signaling promotes a Th1-like pattern of gene expression including the skin-homing receptors (P-selectin ligands) in primary CD4+ T cells (42). These Th1-like cells might be undesirable in the normal gut tissues. Recent data from our laboratory (A. Yokota Y. Ohoka, unpublished observations) suggested that the expression of many chemokine receptors other than CCR9 is induced more efficiently by DCs with the persistent presence of Ag than by Ag-pulsed DCs. The expression of another gut-homing receptor (α4β7) was also enhanced after terminating TCR stimulation, although α4β7 expression began before the termination (data not shown). Accordingly, it was reported that high Ag doses suppressed CCR9 expression significantly and α4β7 expression moderately (24). The maximal gut-homing specificity might be induced only when the Ag supply is limited or temporal in the gut. In contrast, inflammation may be accompanied by a sustained presence of Ag. T cells activated under inflammatory conditions instead may express inflammatory site-homing receptors, which resemble skin-homing receptors, even in the presence of RA.

It was shown that RA enhances the TGF-β-dependent differentiation of naive CD4+ T cells into Foxp3+ regulatory T cells that may be anti-inflammatory and contribute to oral tolerance (43–46). Th1-like cells might be undesirable in the normal gut. NFAT kinases, or degradation enzymes, may affect the activation of retinoid X receptor alpha coincides with loss of retinoid responsiveness in skin-homing T cells by Peyer’s patch dendritic cells. The Journal of Immunology 743

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