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

Yoshiharu Ohoka,<sup>\*,†</sup> Aya Yokota,<sup>\*,†</sup> Hajime Takeuchi,<sup>\*,†</sup> Naoko Maeda,<sup>\*</sup> and Makoto Iwata<sup>\*,†</sup>

Retinoic acid (RA) imprints gut-homing specificity on T cells upon activation by inducing the expression of chemokine receptor CCR9 and integrin  $\alpha 4\beta 7$ . CCR9 expression seemed to be more highly dependent on RA than was the  $\alpha 4\beta 7$  expression, but its molecular mechanism remained unclear. In this article, we show that NFAT isoforms NFATc1 and NFATc2 directly interact with RA receptor (RAR) and retinoid X receptor (RXR) but play differential roles in RA-induced CCR9 expression on murine naive CD4<sup>+</sup> T cells. TCR stimulation for 6–24 h was required for the acquisition of responsiveness to RA and induced activation of NFATc1 and NFATc2. However, RA failed to induce CCR9 expression as long as TCR stimulation continued. After terminating TCR stimulation or adding cyclosporin A to the culture, *Ccr9* gene transcription was induced, accompanied by inactivation of NFATc1 and sustained activation of NFATc2. Reporter and DNA-affinity precipitation assays demonstrated that the binding of NFATc2 to two NFAT-binding sites and that of the RAR/RXR complex to an RA response element half-site in the 5'-flanking region of the mouse *Ccr9* gene were critical for RA-induced promoter activity. NFATc2 directly bound to RAR $\alpha$  and RXR $\alpha$ , and it enhanced the binding of RAR $\alpha$  to the RA response element half-site. NFATc1 also bound to the NFAT-binding sites and directly to RAR $\alpha$  and RXR $\alpha$ , but it inhibited the NFATc2-dependent promoter activity. These results suggest that the cooperativity between NFATc2 and the RAR/RXR complex is essential for CCR9 expression on T cells and that NFATc1 interferes with the action of NFATc2. *The Journal of Immunology*, 2011, 186: 733–744.

The vitamin A metabolite retinoic acid (RA) plays a critical role in deploying lymphocytes into the gut tissue (1). Naive 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 retinoid X receptor (RXR) (11–13). Three isoforms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of RAR and three isoforms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) 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(G/T)(T/A)CA. The major physiologic RA, all-*trans*-RA, binds to RAR but not to RXR, and it imprints naive T cells with gut-homing specificity upon activation by specifically inducing the expression of the gut-homing receptors integrin  $\alpha 4\beta 7$  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  $\alpha 4$  or  $\beta 7$  gene. CCR9 expression seems to be more highly dependent on RA than is  $\alpha 4\beta 7$  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- $\kappa$ B, 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, NFATc), 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 Ca<sup>2+</sup>-regulatory domain, the DNA-

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CN, calcineurin; CsA, cyclosporin A; DC, dendritic cell; DNAP, DNA-affinity precipitation; IM, ionomycin; MFI, mean fluorescence intensity; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid-response element; RXR, retinoid X receptor; TAD, transactivation domain; VD3, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>.

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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  $\text{Ca}^{2+}$  levels activate calcineurin (CN), a  $\text{Ca}^{2+}$ -dependent phosphatase, resulting in the dephosphorylation and translocation of NFAT into the nucleus. NFAT proteins bind to the core sequence (A/T)GGAAA(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, T-bet, 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  $\text{CD4}^+$  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  $\text{CD4}^+$  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. DO11.10 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  $\text{CD4}^+\text{CD62L}^{\text{high}}$  T cells were obtained from lymph nodes and spleens of DO11.10 TCR-Tg/Rag2-deficient mice by using Dynabeads Mouse  $\text{CD4}$  and DetachaBead Mouse  $\text{CD4}$  (Dyna) and subsequently using MACS  $\text{CD62L}$  Microbeads and MACS separation columns (Miltenyi Biotec), as previously described (1). In some experiments,  $\text{CD4}^+\text{CD44}^{\text{low}}$  T cells were isolated by negative selection using EasySep Mouse  $\text{CD4}^+$  T Cell Enrichment Kits (Stemcell Technologies), supplemented with biotinylated anti-mouse  $\text{CD44}$  Ab, and were used as naive T cells. More than 99% of the recovered cells were  $\text{CD4}^+\text{CD62L}^{\text{high}}$ . Naive  $\text{CD8}^+\text{CD62L}^{\text{high}}$  T cells were obtained from spleens of B10.D2 mice by negative selection using EasySep Mouse  $\text{CD8}^+$  T Cell Enrichment Kits (Stemcell Technologies), supplemented with biotinylated anti-mouse  $\text{CD44}$  Ab, and were used as naive  $\text{CD8}^+$  T cells. More than 95% of the recovered cells were  $\text{CD8}^+\text{CD62L}^{\text{high}}$ . 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  $\mu\text{M}$  2-ME, 20 mM HEPES-NaOH [pH 7.2], 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin).

### T cell culture

Naive  $\text{CD4}^+$  T cells were stimulated with plate-bound Abs to  $\text{CD3}$  and  $\text{CD28}$ , as described previously (1). The incubation period varied, depend-

ing 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  $\text{CD4}^+$  T cells ( $2 \times 10^4$  cells) in the presence of OVA peptide P323–339 (10  $\mu\text{M}$ ), at a ratio of 1:1 (DCs/T cells), in 200  $\mu\text{l}$  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  $\mu\text{M}$ ) and then cocultured with naive  $\text{CD4}^+$  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-FcR 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  $\Delta$ mean fluorescence intensity (MFI) calculated as: (MFI of cells stained with fluorophore-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 glycogen, 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 ( $\Delta\text{CT}$ ) method ( $\text{CCR9 CT} - \text{ARBP1 CT}$ ).

### Expression of RARs, RXRs, and NFATs in naive $\text{CD4}^+$ T cells

Total RNA and cDNA from aliquots of cultured naive  $\text{CD4}^+$  T cells were prepared as described above. Expression levels of *Rara*, *Rarb*, *Rarg*, *Rxra*, *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 DR5 oligonucleotide (5'-Alexa Fluor 780-TCGAGGGTAGG-GTTTACCGGAAAGTTCACTCG-3' and 5'-Alexa Fluor 780-CGAGTGAAC-TTTCGGTGAACCTACCCTACCGTGA-3'; Invitrogen) for 15 min at room temperature in 20  $\mu\text{l}$  nuclear extract buffer (PIERCE) containing 5  $\mu\text{g}$  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 measured by Odyssey Infrared Imaging System (LI-COR Biosciences).

### Plasmid construction

The 5'-flanking region of the mouse *Ccr9* gene was cloned by PCR with mouse genomic DNA 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 promoterless firefly luciferase reporter plasmid pGL3-basic (Promega) at the KpnI and XhoI 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 RXR $\alpha$  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, according to the manufacturer's instructions. The PCR product was cloned into the pSG5 expression vector (Stratagene) at the EcoRI site. All constructs were checked by DNA sequencing using Big Dye Terminator (Applied Biosystems) on an ABI 3130 sequencer (Applied Biosystems). pSG5-RAR $\alpha$  was a kind gift from Drs. S. Kato (Tokyo University, Tokyo, Japan) and P. Chambon (Institute of Genetics and Molecular and Cellular Biology,

Universite Louis Pasteur, Illkirch, France) (22). The pME-NFATc1, -NFATc2, and -NFATc3 expression vectors were kind gifts from Dr. S. Miyatake (Metropolitan Institute of Medical Science, Tokyo) (23). pCMV-Myc-NFATc1 and -NFATc2 expression vectors were prepared with PCR fragments amplified from pME-NFATc1 and pME-NFATc2 using reverse and forward primers (Supplemental Table II); then, PCR products were subcloned into the pCMV-Myc vector at the EcoRI/SalI sites and ClaI/SalI sites, respectively. pGEX-5X-1-NFATc1 and -NFATc2 expression vectors were prepared with PCR fragments amplified from pSG5-RAR $\alpha$  and pSG5-RXR $\alpha$  using reverse and forward primers (Supplemental Table II). Then, PCR products were subcloned into the pGEX-5X-1 vector (GE Healthcare) at the EcoRI/SalI sites. The truncated forms of mouse *Ccr9* promoter were created using reverse and forward primers (Supplemental Table II). All were cloned into the pGL3-basic vector at the KpnI and XhoI sites. Site-directed mutagenesis of NFAT-binding sites,  $\kappa$ B-like site, and RARE half-site in the *Ccr9* promoter was introduced using the QuickChange kit (Stratagene), according to the manufacturer's instructions. The constructs were generated by PCR using pGL3-CCR9 as a template, with reverse and forward primers (Supplemental Table III).

#### DNA-affinity precipitation assay

The biotin-labeled DNA probes were purchased from Sigma-Aldrich and annealed to complementary oligonucleotides. COS cells were transfected with 2.5–4  $\mu$ g expression vectors using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Transfected COS cells or CD4<sup>+</sup> 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  $\mu$ g poly(deoxyinosinic-deoxycytidylic) acid and 2  $\mu$ g 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  $\times$  10<sup>5</sup> or 1  $\times$  10<sup>6</sup> cells/well, respectively) and transfected with 2.5–4  $\mu$ g pGL3-CCR9s, 0.5–1  $\mu$ g expression vectors, and 0.025  $\mu$ g 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 $\times$  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  $\mu$ g pCMV-Myc-NFATc1 or -NFATc2 using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were lysed in 0.5  $\mu$ l 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 RAR $\alpha$  or RXR $\alpha$  fixed on 20  $\mu$ l 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  $\times$  10<sup>6</sup> CD4<sup>+</sup> 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  $\mu$ g anti-NFATc1 (clone 7A6; Santa Cruz Biotechnology), anti-NFATc2 (clone 25A10.D6.D2; Thermo) or control IgG<sub>1</sub> overnight at 4°C with rotation. After 30  $\mu$ l 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 8 h. Proteins were digested with 10  $\mu$ g/ml proteinase K for 1 h at 45°C, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Specific DNA was amplified by PCR using primers specific for the mouse *Ccr9* promoter, forward: 5'-GACCCAGAACGGTACTTGGACTT-3' and reverse: 5'-CCCGGAGAATTAGTTTCTTGGTTC-3'. The binding of NFATc1 or NFATc2 to the *Ccr9* promoter site was estimated by real-time PCR. The binding levels were expressed as the percentage of input DNA and were calculated from  $\Delta CT$  ( $\Delta CT$  = input  $CT$  – ChIP  $CT$ ), according to the following equation: percentage total =  $2^{\Delta CT}$ .

#### Transcription factor-binding site analysis

Genomic DNA sequences were analyzed using the MatInspector computer program ([http://www.genomatix.de/online\\_help/help\\_matinspector/matinspector\\_help.html](http://www.genomatix.de/online_help/help_matinspector/matinspector_help.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

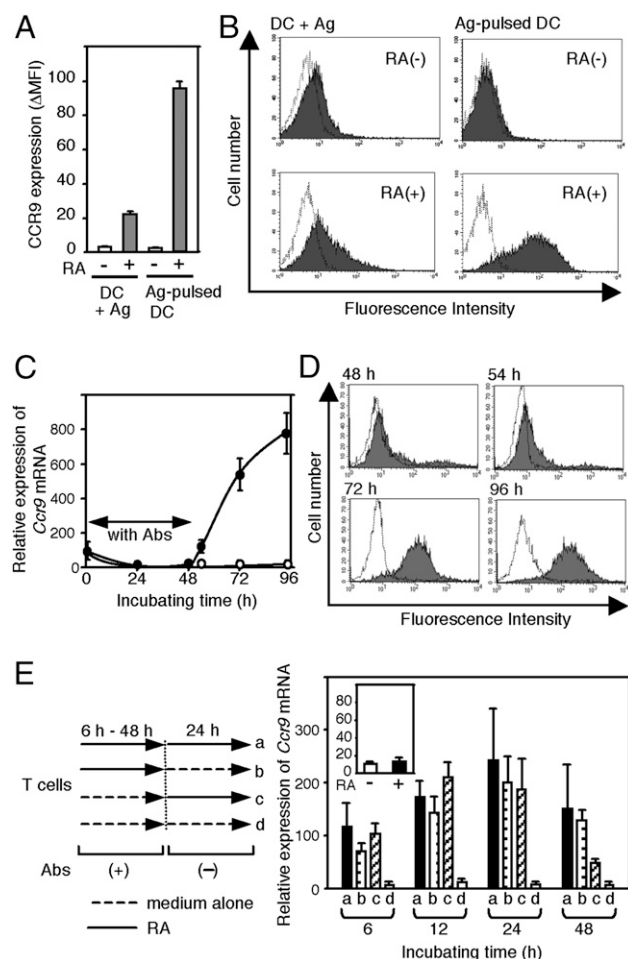
### Transient TCR stimulation is essential for RA-induced CCR9 expression in CD4<sup>+</sup> T cells

We often noticed that CCR9 expression was induced by RA on naive CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells (Supplemental Fig. 1).

The duration of Ab stimulation also affected *Ccr9* expression (Fig. 1E). Naive CD4<sup>+</sup> 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*





**FIGURE 1.** Transient TCR stimulation is essential for the RA-dependent induction of CCR9 expression in CD4<sup>+</sup> T cells. **A** and **B**, Naive CD4<sup>+</sup> T cells were cultured with DCs in the presence of OVA peptide P323–339 (10  $\mu$ M) (left panels) or with DCs prepulsed with the OVA peptide (10  $\mu$ M) (right panels) for 5 d. RA (10 nM) or its control vehicle was added at the start of the culture. After the culture, the cells were analyzed for the expression of CCR9 by flow cytometry. **A**, The CCR9 expression levels are presented as  $\Delta$ MFI  $\pm$  SD of triplicate cultures. **B**, Representative flow cytometric profiles for CCR9 expression of the cells cultured with (lower panels) or without (upper panels) RA. Dotted lines indicate staining isotype controls. **C** and **D**, Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 in the presence (●) or absence (○) of 10 nM RA for 48 h. The cells were then cultured in fresh medium containing 20 U/ml IL-2 in the presence (●) or absence (○) of 10 nM RA. At the indicated time points, aliquots of the cells were analyzed for CCR9 expression by real-time PCR (**C**) or flow cytometry (**D**). **C**, The relative *Ccr9* expression levels are shown as the mean  $\pm$  SD of triplicate cultures. The *Ccr9* expression level in the cells stimulated with the Abs in the absence of RA for 24 h was set as 1. **D**, Representative flow cytometric profiles for CCR9 expression of the cells harvested at the indicated time points. Dotted lines represent the staining isotype controls. **E**, Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 for 6, 12, 24, or 48 h in the presence (solid line) or absence (dotted line) of 10 nM RA. The cells were then cultured in fresh medium containing 20 U/ml IL-2 for 24 h in the presence (solid line) or absence (dotted line) of 10 nM RA. Inset: naive CD4<sup>+</sup> T cells were cultured with or without 10 nM RA for 12 h and were analyzed for *Ccr9* expression by real-time PCR. The relative *Ccr9* expression levels were calculated as above and are shown as mean  $\pm$  SD of triplicate cultures. Results are representative of three independent experiments.

mRNA than did those treated with RA during Ab stimulation. These results suggested that TCR stimulation is essential for naive CD4<sup>+</sup> 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 Ca<sup>2+</sup>/CN-NFAT is essential for sensitizing naive CD4<sup>+</sup> T cells to RA

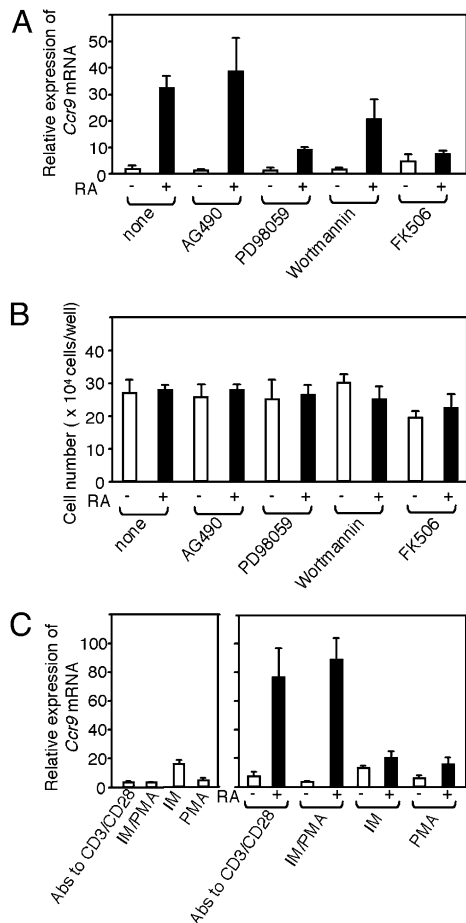
To clarify the TCR signals essential for sensitizing naive CD4<sup>+</sup> T cells to RA, we examined the effects of various inhibitors on *Ccr9* expression. To minimize any toxicity of inhibitors on T cells, we shortened the stimulation period in the presence of inhibitors to 6 h. The cells were then 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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> level and PKC activation induced in naive CD4<sup>+</sup> T cells upon TCR stimulation trigger them to acquire the responsiveness to RA through the activation of Ca<sup>2+</sup>/CN-NFAT and MAPK pathways.

#### TCR stimulation enhances the expression of RXRs and NFAT in naive CD4<sup>+</sup> T cells

We previously suggested that RAR $\alpha$  and/or RAR $\beta$ , but not RAR $\gamma$ , are involved in RA-induced gut-homing receptor expression (1). To determine the RAR isoforms in charge, we first evaluated the expression levels of the RAR and RXR genes in naive CD4<sup>+</sup> T cells and in those stimulated with Abs to CD3 and CD28 in the presence or absence of RA. *Rara* was expressed in naive CD4<sup>+</sup> 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<sup>+</sup> T cells, but they became detectable after 6–12 h of stimulation. RA did not significantly affect the expression of *Rar* or *Rxr* isoforms, although in the secondary culture without Abs, 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<sup>+</sup> T cells by EMSA. The activity was only weakly detected in the nuclear extracts of naive CD4<sup>+</sup> 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 RXR $\alpha$  or RXR $\beta$  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<sup>+</sup> 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<sup>+</sup> 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.

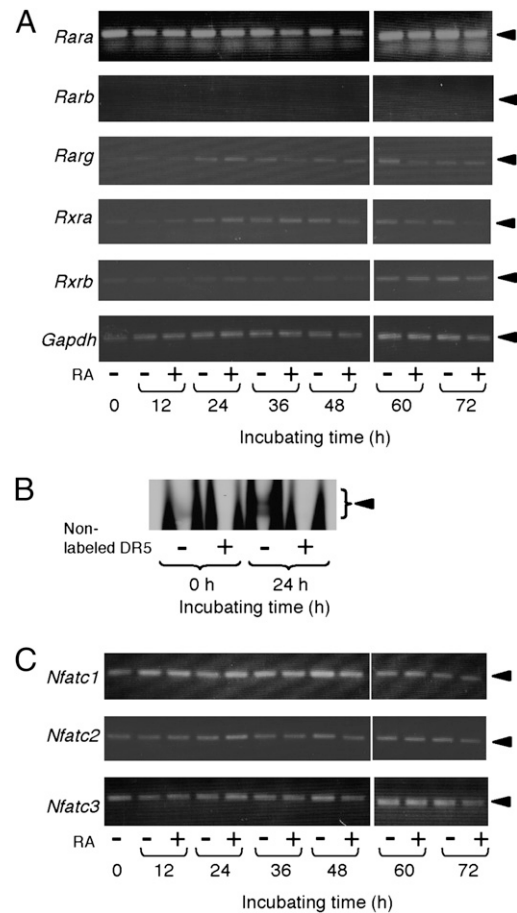
#### Negative transcriptional control of CCR9 expression by TCR signaling

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<sup>+</sup> T cells with Abs



**FIGURE 2.** Pharmacological analyses of TCR signals required for the RA-dependent induction of *Ccr9* expression in naive CD4<sup>+</sup> T cells. *A* and *B*, Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 for 6 h in the presence or absence of 50  $\mu$ M AG490, 50  $\mu$ M PD98059, 100 nM wortmannin, or 10 nM FK506. The cells were then cultured in fresh medium containing 20 U/ml IL-2 in the presence or absence of 10 nM RA without the inhibitors for 12 h. After the culture, *Ccr9* expression was assessed by real-time PCR (*A*), and the recovery of viable cells per culture was determined (*B*). *C*, Naive CD4<sup>+</sup> T cells were stimulated with 0.2  $\mu$ g/ml IM and/or 50 ng/ml PMA or with Abs to CD3/CD28 for 6 h without RA. Aliquots of the cells were analyzed for *Ccr9* expression by real-time PCR (*left panel*), and other aliquots of the cells were further cultured in fresh medium containing 20 U/ml IL-2 with or without 10 nM RA for 12 h and then analyzed for *Ccr9* expression (*right panel*). Data are shown as the mean relative *Ccr9* expression  $\pm$  SD (*A*, *C*) or the mean recovery of viable cells per culture  $\pm$  SD (*B*) of triplicate cultures. The *Ccr9* expression level in the cells stimulated with Abs in the absence of RA for 6 h was set as 1. Results are representative of three independent experiments.

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<sup>+</sup> 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<sup>+</sup> T cells by activating a Ca<sup>2+</sup>/CN-NFAT-signaling pathway. Similar results were obtained with naive CD8<sup>+</sup> T cells, suggesting that NFAT activation is involved in the regulation of



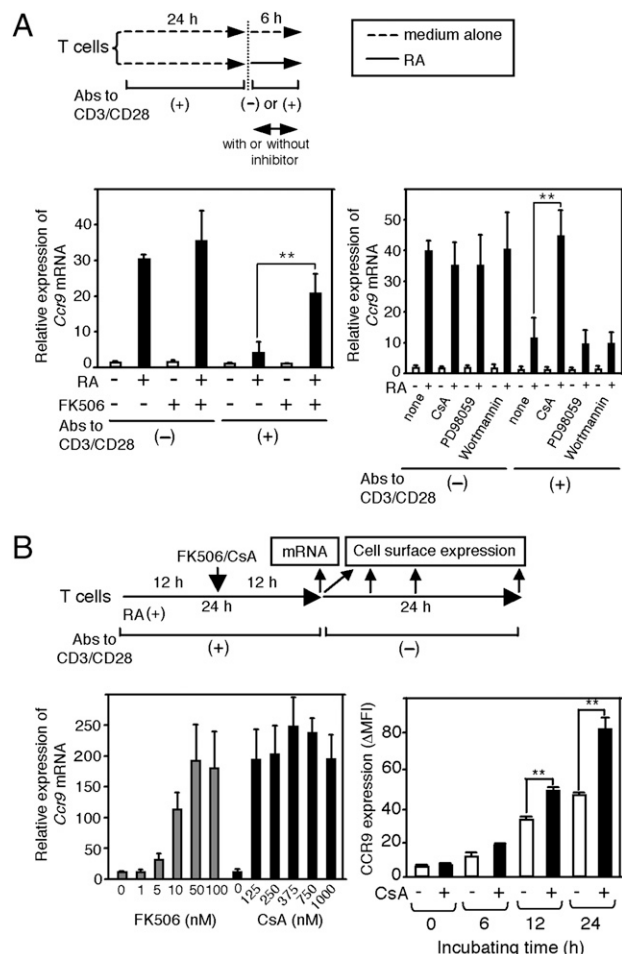
**FIGURE 3.** Kinetic changes in the expression of the RAR, RXR, and NFAT isoforms in naive CD4<sup>+</sup> T cells during TCR stimulation. Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 in the presence or absence of RA. *A* and *C*, At the indicated times, cells were harvested and analyzed for the expression levels of *Rara*, *Rarb*, *Rarg*, *Rxra*, *Rxrb*, and *Gapdh* as a loading control (*A*) and those of *Nfatc1*, *Nfatc2*, and *Nfatc3* (*C*) by RT-PCR. The arrows indicate the mRNA expression of the RAR, RXR or NFAT isoforms. *B*, Nuclear extracts (5  $\mu$ g each) of naive CD4<sup>+</sup> T cells or those stimulated for 24 h were analyzed by EMSA. Nuclear extracts were pretreated with or without a 10-fold excess of unlabeled DR5 oligonucleotide. Results are representative of three independent experiments.

CCR9 expression on CD8<sup>+</sup> T cells as well, although higher concentrations of CsA were required for its effects compared with those for CD4<sup>+</sup> T cells (Supplemental Fig. 2).

However, unlike *Ccr9* mRNA expression, CsA failed to induce CCR9 protein expression on the CD4<sup>+</sup> 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.

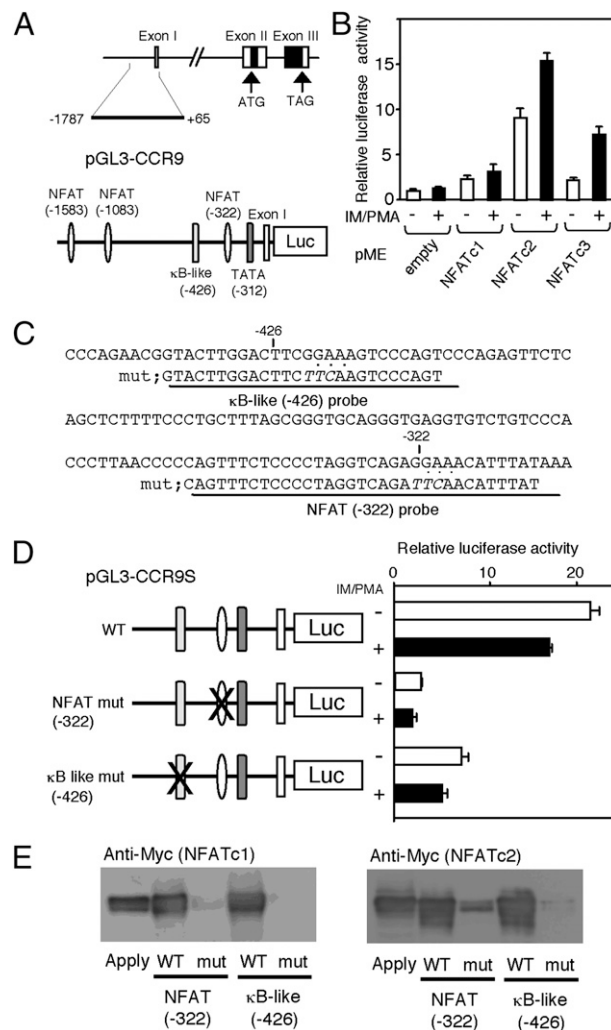
#### NFAT plays a crucial role in CCR9 promoter activity

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



**FIGURE 4.** Successive TCR stimulation prevents the RA-dependent induction of *Ccr9* expression in CD4<sup>+</sup> T cells but fails to prevent it in the presence of immunosuppressants. **A**, Naive CD4<sup>+</sup> 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**, Naive CD4<sup>+</sup> 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.

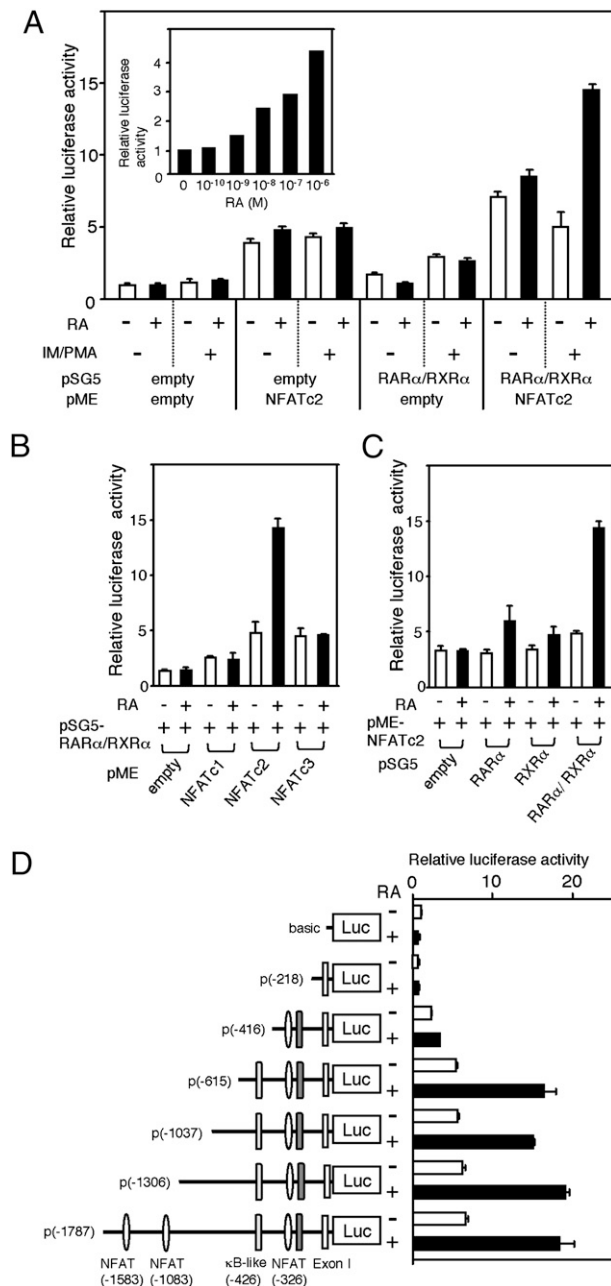
domain and C-terminal domain. The 5'-flanking region of the first exon contains typical mammalian promoter consensus elements, a TATA box (-312), several SP1-binding sites, three putative NFAT-binding sites (-322, -1083, and -1583), and an κB-like site (-426). Thus, we obtained a 1.85-kb fragment (-1787 to +65) of the 5'-flanking sequence that contained these elements. To examine whether NFAT is directly involved in the induction of



**FIGURE 5.** NFAT modulates *Ccr9* promoter reporter activity. **A**, The organization of mouse *Ccr9* gene and its fragment containing exon 1 and its 5'-flanking region from -1787 to +65. The NFAT-binding sites (at -322, -1083, and -1583) in the fragment are indicated. **B**, EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid together with empty vector, pME-NFATc1, pME-NFATc2, or pME-NFATc3. One day after transfection, cells were stimulated with 0.2 μg/ml IM and 50 ng/ml PMA for 16 h, and reporter luciferase activities were measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9 alone without IM/PMA as 1. Error bars represent SD. **C**, Nucleotide sequences of NFAT site (-322), κB-like site (-426), and their mutants in the 5'-flanking region of the *Ccr9* gene are shown in italics. The DNA probes used for DNAP assay are underlined. **D**, EL4 cells were transfected in triplicate with pGL3-CCR9S reporter plasmid (WT) or pGL3-CCR9S reporter plasmids containing mutated (indicated by X) NFAT site (-322) and κB-like site (-426), together with empty vector (pME-NFATc2). One day after transfection, cells were stimulated with 0.2 μg/ml IM and 50 ng/ml PMA for 16 h, and the relative promoter activities were assessed. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-CCR9S alone (without IM/PMA) as 1. Error bars represent SD. **E**, COS cells were transfected with pCMV-myc-NFATc1 or pCMV-myc-NFATc2. One day after transfection, cell lysates were analyzed for DNA-binding activity by DNAP assay using the indicated biotinylated DNA probes, whose nucleotide sequences are shown in C, and with anti-Myc Ab. 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





**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. Inset: EL4 cells were transfected in triplicate with pGL3-CCR9 reporter plasmid in combination with pME-NFATc2, pSG5-RARα, and pSG5-RXRα expression vectors. 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

activity was significantly induced by the ectopic expression of NFATc2, and it was enhanced by IM/PMA (Fig. 5B), although the effect of IM/PMA was often smaller or even suppressive in our other experiments under similar conditions (Fig. 5D, data not shown). The ectopic NFATc3 expression induced promoter activity slightly. IM/PMA significantly enhanced it but to a lesser extent than ectopic NFATc2 expression alone. Ectopic NFATc1 expression showed only a weak effect with or without IM/PMA. IM/PMA stimulation alone failed to induce *Ccr9* promoter activity. In contrast, *Il2* 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 κB-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 κB-like site also contributes to the NFATc2-dependent *Ccr9* promoter activity, we introduced a mutation in the κB-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 κB-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 κB-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

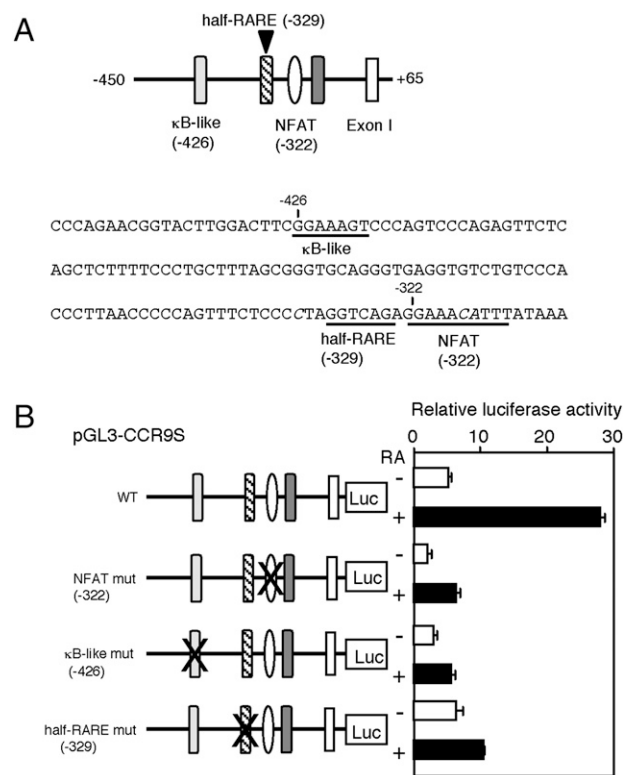
Although NFATc2 induced *Ccr9* promoter activity in EL4 cells transfected with pGL3-CCR9, RA with or without IM/PMA exerted little effect on the activity (Fig. 6A). The NFATc2-induced promoter activity was not dependent on the residual serum retinoids in the culture, because the activity was also induced 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 RARα 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

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 pSG5-RARα, pSG5-RXRα, and pME-NFATc2 expression vectors. One day after transfection, cells were stimulated as above, and luciferase activity was measured. The relative promoter activities were calculated by arbitrarily defining the activity of pGL3-basic alone (without RA and IM/PMA) as 1. Error bars represent SD. Results are representative of three independent experiments.

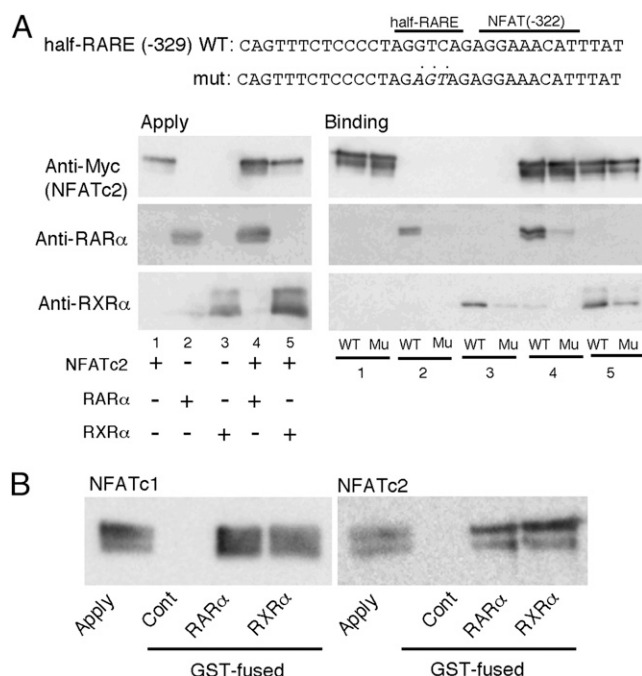


expression vectors of NFATc2, RAR $\alpha$ , and RXR $\alpha$  (Fig. 6A). RA enhanced the promoter activity in a dose-dependent manner (Fig. 6A, inset). However, the enhancement was not significant when NFATc1 or NFATc3 was ectopically expressed instead of NFATc2 (Fig. 6B) or when either RAR $\alpha$  or RXR $\alpha$  was transfected instead of both RAR $\alpha$  and RXR $\alpha$  (Fig. 6C). Cooperation between activated NFATc2 and the RAR $\alpha$ /RXR $\alpha$  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  $\kappa$ B-like site (-426), resulted in a dramatic loss of reporter activity. These data suggested that the NFAT site (-322) and the  $\kappa$ B-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  $\kappa$ B-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 RAR $\alpha$



**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  $\kappa$ B-like site (-426), NFAT site (-322), and RARE half-site (-329). **B**, EL4 cells were transfected in triplicate with pGL3-CCR9S reporter plasmid or pGL3-CCR9S reporter plasmids containing mutated (indicated by X) NFAT site (-322),  $\kappa$ B-like site (-426), or RARE half-site (-329) with or without the expression vectors pME-NFATc2 and/or pSG5-RAR $\alpha$  and pSG5-RXR $\alpha$ . 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/PMA) as 1. Error bars represent SD. Results are representative of three independent experiments.



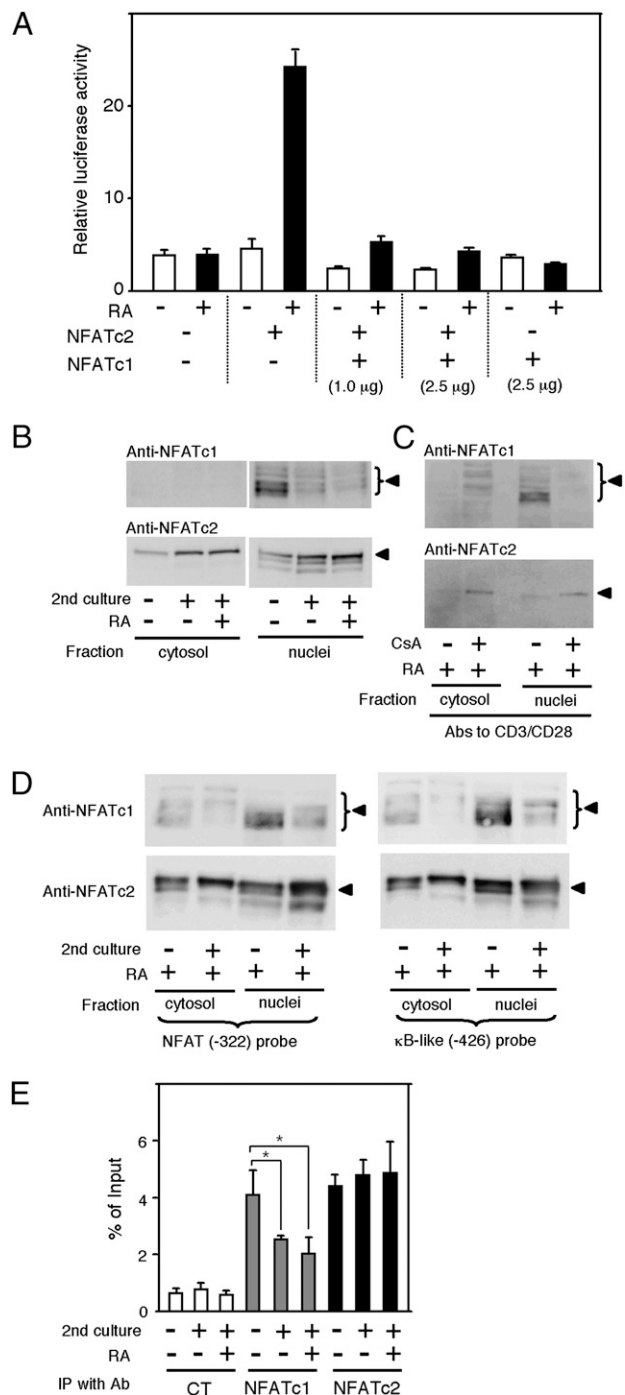
**FIGURE 8.** Interactions of NFATc2 with RAR $\alpha$  and RXR $\alpha$ . **A**, COS cells were transfected with pCMV-Myc-NFATc2 and pSG5-RAR $\alpha$  or pSG5-RXR $\alpha$ . One day after transfection, cell lysates were subjected to DNAP assay using biotinylated DNA probes, whose nucleotide sequences are shown at the top of the figure, and with anti-Myc, anti-RAR $\alpha$ , or anti-RXR $\alpha$  Abs. **B**, COS cells were transfected with pCMV-Myc-NFATc1 or pCMV-Myc-NFATc2. One day after transfection, cell lysates were incubated with glutathione beads containing GST-RAR $\alpha$  or RXR $\alpha$ . The binding proteins on beads were subjected to Western blot analysis with anti-Myc Ab. Results are representative of three independent experiments.

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 RAR $\alpha$ , the binding of RAR $\alpha$  to the DNA containing the RARE half-site (-329) was apparently enhanced. The binding of RXR $\alpha$  to the same DNA probe was also observed but was weaker than that of RAR $\alpha$ . Thus, we examined whether NFATc2 also directly interacted with RAR $\alpha$  or RXR $\alpha$ . Indeed, NFATc2 bound to RAR $\alpha$  and RXR $\alpha$  (Fig. 8B). The DNA-binding domains of NFATc2 and RAR $\alpha$  were required for their binding (Supplemental Figs. 5, 6). These observations suggested that cooperation between activated NFATc2 and the RAR $\alpha$ /RXR $\alpha$  complex and their binding to the NFAT site (-322), the  $\kappa$ B-like site (-426), and the RARE half-site (-329) are involved in activation of the *Ccr9* promoter. However, NFATc1 also bound to RAR $\alpha$  and RXR $\alpha$  (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<sup>+</sup> T cells

We found that overexpression of NFATc1 markedly inhibited the RA-induced *Ccr9* promoter activity with ectopic expression of NFATc2 and RAR $\alpha$ /RXR $\alpha$  (Fig. 9A).

Because NFAT activation is accompanied by its translocation from the cytosol to the nucleus, we analyzed the cellular localization of NFATc1 and NFATc2 during the process of RA-dependent induction of CCR9 expression (Fig. 9B). The naive CD4<sup>+</sup> 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 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-RAR $\alpha$ , pSG5-RXR $\alpha$ , pCMV-Myc-NFATc2 (0 or 0.1  $\mu$ g), and pCMV-Myc-NFATc1 (0, 1.0, or 2.5  $\mu$ g). 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 (without RA and IM/PMA) as 1. Error bars represent SD. **B**, Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 for 48 h in the presence or absence of 10 nM RA. Aliquots of the cells were collected, and other aliquots of the cells were further cultured in fresh medium containing 20 U/ml IL-2 in the presence of 10 nM RA for 12 h and collected. Ten micrograms of cytoplasmic proteins or 5  $\mu$ g of nuclear proteins from these cells were fractionated on SDS-PAGE, followed by Western blotting with anti-NFATc1 or anti-NFATc2 Abs. Arrowheads indicate the locations of NFATc1 or NFATc2 proteins. **C**, Naive CD4<sup>+</sup> T cells were stimulated with

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  $\kappa$ B-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<sup>+</sup> 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 an RA-independent manner. Therefore, as found in the EL4 cells, the cooperation between activated NFATc2 and the RAR $\alpha$ /RXR $\alpha$  complex through binding to the NFAT site (-322), the  $\kappa$ B-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<sup>+</sup> and CD8<sup>+</sup> 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

Abs to CD3/CD28 for 48 h in the presence of 10 nM RA. CsA (0 or 200 nM) was added during the last 12 h of the culture. Nuclear and cytoplasmic proteins from the cells were analyzed for the presence of NFATc1 and NFATc2 proteins, as described above. **D**, The nuclear proteins prepared in **B** were analyzed for DNA-binding activity with the biotinylated DNA probes containing the NFAT-binding site (-322) or the  $\kappa$ B-binding site (-426) and with anti-NFATc1 or anti-NFATc2 Abs. Arrowheads indicate the locations of NFATc1 or NFATc2 proteins. **E**, Naive CD4<sup>+</sup> T cells were stimulated with Abs to CD3/CD28 for 48 h. Aliquots of the cells were collected, and other aliquots of the cells were further cultured in fresh medium containing 20 U/ml IL-2 in the presence of 10 nM RA without Abs for 16 h and collected. These cells were subjected to ChIP assay with control IgG<sub>1</sub>, anti-NFATc1, or anti-NFATc2. Binding of NFAT proteins to the *Ccr9* promoter site was estimated by real-time PCR. Statistical significance was determined by the Student *t* test; \**p* < 0.05. Results are representative of three independent experiments.

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  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> (VD<sub>3</sub>) 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 VD<sub>3</sub> depended on the localization, ectopic expression, or stability of RXR $\alpha$  in a breast cancer cell line (28–30). Thus, the available RXR protein levels might define the RA or VD<sub>3</sub> sensitivity in certain cell types. In this study, we demonstrated that the naive CD4<sup>+</sup> T cells expressed *Rara*, but they did not significantly express *Rxra* or *Rxrb*, and they failed to respond to RA without TCR stimulation. However, upon CD3- and CD28-mediated stimulation, the expression of *Rxra* and *Rxrb* was induced (Fig. 3). Furthermore, recent results from our laboratory indicated that RXR activation enhanced the RAR-dependent induction of CCR9 expression on naive CD4<sup>+</sup> T cells, especially when RA levels were low (31). Thus, the expression of *Rxra* or *Rxrb* 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 RAR $\alpha$  and RXR $\alpha$  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 -426 ( $\kappa$ B-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, *Ii2* promoter activity is regulated by the cooperation of activated NFAT and AP-1 (32). NFAT induces the promoter activity of TNF- $\alpha$  or IL-13, without any necessity to cooperate with AP-1, but it may use  $\kappa$ B-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 RAR $\alpha$ /RXR $\alpha$  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 RAR $\alpha$ /RXR $\alpha$  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 RAR $\alpha$ /RXR $\alpha$  (36). We demonstrated that RAR $\alpha$  and RXR $\alpha$  could bind to the RARE half-site (-329), and coexpression of NFATc2 with RAR $\alpha$  increased binding of RAR $\alpha$  to the RARE half-site. In addition, we showed that NFATc2 directly bound to RAR $\alpha$ . Our results suggested that RAR $\alpha$  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, RAR $\alpha$ , and RXR, 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 RAR $\alpha$ /RXR $\alpha$  with other transcription factors (37). RAR $\alpha$  and RXR $\alpha$  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 transactivating capacity of this element depends on downstream sequences containing Oct1-, NFAT-, and CREB-binding sites. This suggests that RAR $\alpha$  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, RAR $\alpha$ , and RXR $\alpha$ , 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 translation 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 RAR $\alpha$  and RXR $\alpha$  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 *Tnfa* gene, but only NFATc2 induced TNF- $\alpha$  expression (38). Because NFATc1 and NFATc2 bound to the NFAT sites and RAR $\alpha$ , 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 RAR $\alpha$ /RXR $\alpha$  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 RXRs 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<sup>+</sup> 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 Ca<sup>2+</sup> 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<sup>+</sup> T cells (42). These Th1-like cells might be undesirable in the normal gut tissues. Recent data from our laboratory (A. Yokota Y. Ohoka, H. Takeuchi, and A. Iwata, 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 ( $\alpha 4\beta 7$ ) was also enhanced after terminating TCR stimulation, although  $\alpha 4\beta 7$  expression began before the termination (data not shown). Accordingly, it was reported that high Ag doses suppressed CCR9 expression significantly and  $\alpha 4\beta 7$  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- $\beta$ -dependent differentiation of naive CD4<sup>+</sup> T cells into Foxp3<sup>+</sup> regulatory T cells that may be anti-inflammatory and contribute to oral tolerance (43–46). TCR-NFAT signaling also plays a determinant role in the differentiation of these cells (47, 48). In this study, we showed that TCR-NFAT signaling plays an essential role in regulating RA-induced gut-homing receptor expression. The cooperation between RA-mediated signaling and TCR-NFAT signaling may play important roles in regulating T cell trafficking and immune responses.

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## Disclosures

The authors have no financial conflicts of interest.

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