

BRIEF COMMUNICATION

Retinoic acids up-regulate functional eosinophil-driving receptor CCR3

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To cite this article: Ueki S, Nishikawa J, Yamauchi Y, Konno Y, Tamaki M, Itoga M, Kobayashi Y, Takeda M, Moritoki Y, Ito W, Chihara J. Retinoic acids up-regulate functional eosinophil-driving receptor CCR3. *Allergy* 2013; DOI: 10.1111/all.12175.

Keywords

CCR3; eosinophils; retinoic acids; vitamin A.

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Accepted for publication 24 March 2013

DOI:10.1111/all.12175

Edited by: Hans-Uwe Simon

Abstract

Eotaxins and their receptor CCR3 have a definitive role for tissue accumulation of eosinophils both under homeostatic and pathologic conditions. However, physiological stimuli that can up-regulate CCR3 in blood-derived human eosinophils have not been recognized. As a prior gene microarray study revealed up-regulation of CCR3 in eosinophils stimulated with retinoic acids (RAs), the expression of functional CCR3 was examined. We found that 9-cis RA and all-trans RA (ATRA) significantly induced surface CCR3 expression regardless of the presence of IL-3 or IL-5. Pharmacological manipulations with receptor-specific agonists and antagonists indicated that retinoic acid receptor- α activation is critical for CCR3 up-regulation. RA-induced CCR3 was associated with its functional capacity, in terms of the calcium mobilization and chemotactic response to eotaxin-1 (CCL11). Our study suggests an important role of vitamin A derivatives in the tissue accumulation of eosinophils.

Eosinophils are bone-marrow-derived multifunctional leukocytes involved in initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity. Under homeostatic conditions, most eosinophils traffic into the gastrointestinal tract where they normally reside within the lamina propria (1). Although IL-5 is important in regulating circulating levels of eosinophils, their accumulation in the gastrointestinal tract depends on a synergistic effect with eotaxins (2). Indeed, intestinal cells constitutively express eotaxin-1 (CCL11) (3, 4) and mice deficient for either eotaxin-1 or its precise receptor CCR3 deficient mice have a marked decrease in recruitment of gastrointestinal eosinophils (1, 5). Furthermore, the impact of CCR3 on pathogenesis of allergic diseases such as asthma has been demonstrated both in human and in mouse studies

(6). Thus, CCR3-eotaxin axis has a critical role for tissue assignment and accumulation of eosinophils.

CCR3, highly expressed on circulating eosinophils, is a seven transmembrane-spanning, G-coupled receptor. Studies using blood-derived human eosinophils revealed that expression of CCR3 can be regulated by receptor recycling and transcription mechanisms. Upon ligand stimulation, CCR3 undergoes rapid internalization that is required for both cellular functions and receptor desensitization (7). Notably, eosinophilopoietic cytokines do not up-regulate CCR3 on mature eosinophils. IL-3 decreases CCR3 mRNA levels, which is associated with continuous down-regulation of surface CCR3 expression (8). IL-5 and GM-CSF induce transient down-regulation of surface expression of CCR3, but do not affect the mRNA levels (8, 9). To our knowledge, physiological stimuli that can up-regulate CCR3 in eosinophils have not been recognized.

Retinoic acids (RAs) are active metabolites of vitamin A that participate in a variety of biological processes, including granulocyte differentiation and immunity (10). Accumulating evidence indicates the critical roles of RAs for imprinting gut-homing capacity on lymphocytes (11). In a prior study, we found up-regulation of CCR3 transcription

Abbreviations

ATRA, all-trans retinoic acid; FCS, fetal calf serum; MFI, mean fluorescence intensity; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

in RA-stimulated eosinophils using gene microarray (data were deposited in the public Gene Expression Omnibus database under accession no. GSE12926) (12). Here, we report that RAs mediate functional CCR3 expression in human eosinophils.

Materials and methods

Materials

9-cis RA and all-trans RA (ATRA) were purchased from Sigma-Aldrich. TTNPB and Ro41-5253 were purchased from

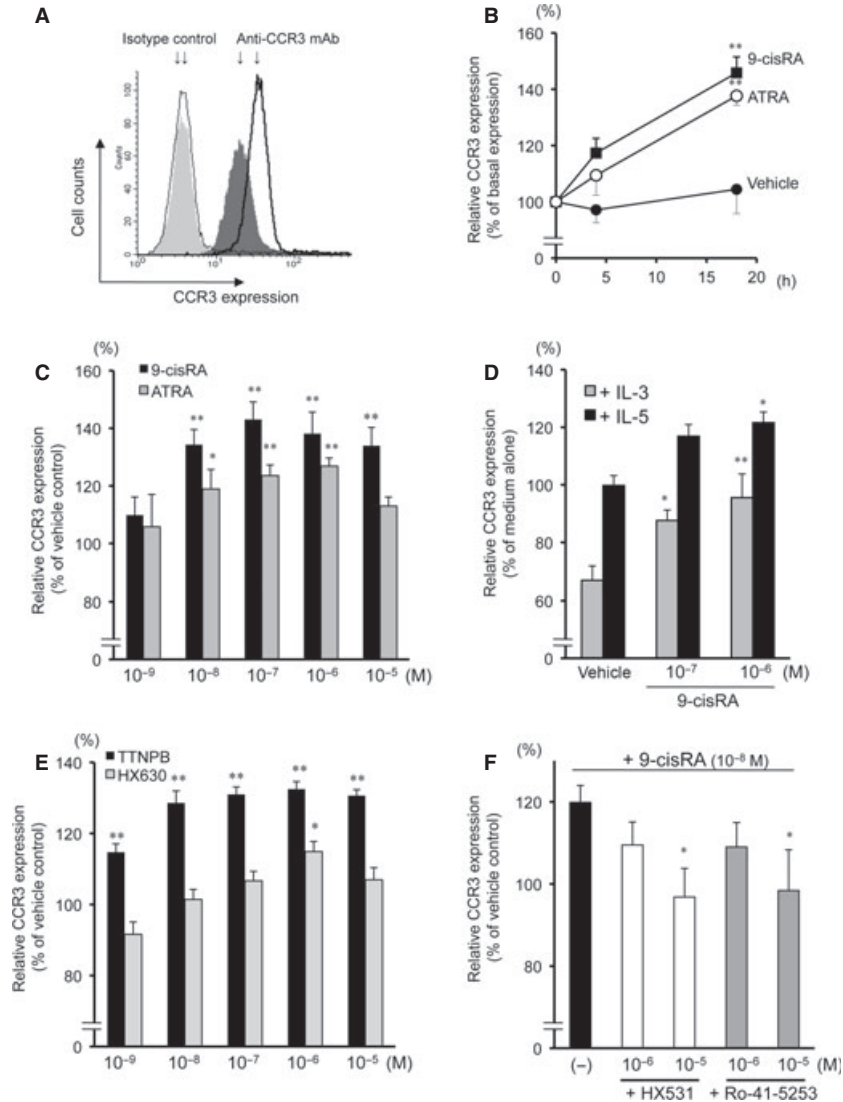


Figure 1 Retinoic acids up-regulate surface CCR3 expression on blood-derived human eosinophils through ligation of RA receptors (RAR) and retinoid X receptors (RXRs). (A) Purified eosinophils were incubated with vehicle or 10⁻⁷ M 9-cis RA for 18 h. Representative histograms of flow cytometric analysis for CCR3 expression are shown (filled histograms; baseline expression in vehicle-treated control cells, open histograms; 9-cis RA-stimulated cells). (B) Time-course response of CCR3 expression by 9-cis RA and ATRA (10⁻⁷ M). The MFI ratio in each sample was measured and the data are expressed as the % of basal expression ($n = 4-5$, different donors). **, $P < 0.01$ vs vehicle control. (C) Concentration response of CCR3 expression by 9-cis RA and ATRA. Cells were incubated with indicated concentrations of RAs for 18 h. The data are expressed as the % of vehicle control ($n = 4-5$, different donors). *, $P < 0.05$, **, $P < 0.01$ vs vehicle control. (D) The

effect of 9-cis RA in the presence of cytokines. Cells were incubated with IL-3 or IL-5, and indicated concentrations of 9-cis RA for 18 h. The data are expressed as % of medium control without cytokines ($n = 5$, different donors). *, $P < 0.05$, **, $P < 0.01$ vs medium control. (E) The effect of RA receptor agonists on CCR3 expression. Cells were incubated with indicated concentrations of RAR-selective agonist TTNPB or RXR-selective agonist HX630 for 18 h. The data are expressed as % of vehicle control ($n = 7$, different donors). *, $P < 0.05$, **, $P < 0.01$ vs vehicle control. (F) The effect of receptor antagonists on 9-cis RA-induced CCR3 up-regulation. Cells were preincubated with indicated concentrations of RAR/RXR antagonist HX531 or RAR α antagonist Ro41-5253 for 45 min, and then stimulated with 10⁻⁸ M 9-cis RA for 18 h ($n = 4$, different donors). The data are expressed as % of vehicle control. *, $P < 0.05$ vs 9-cis RA.

BioMol Research Laboratories (Plymouth Meeting, PA, USA). HX630 and HX531 were a kind gift from Dr. H. Kagechika, University of Tokyo, Japan.

Cell preparation and culture

Peripheral venous blood was obtained from normal and mildly atopic subjects. Informed consent was obtained from each subject, and the study protocol was approved by the Ethics Committee of Akita University School of Medicine. Eosinophils were purified by a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (12). Purity and viability were >98% and >99%, respectively. Cells were resuspended in RPMI1640 with 10% FCS and incubated with indicated concentrations of RAs, IL-3 (10 ng/ml; Genzyme), or IL-5 (1 ng/ml; R&D Systems) in a CO₂ incubator.

Expression of surface CCR3 on eosinophils

Nonpermeabilized eosinophils were stained with FITC-conjugated anti-human CCR3 mAb (mouse IgG; DAKO) or isotype-matched control. Cells were analyzed using a flow cytometer (FACScan, BD Immunocytometry, San Jose, CA, USA). Expression was assessed as the ratio of mean fluorescence intensity (MFI) of the sample and the isotype-matched control.

Measurement of intracellular calcium

After incubation, cells were loaded with 2 mM fura 2-AM and resuspended at a concentration of 3×10^6 cells/ml in HBSS with Ca²⁺. Changes in fluorescence were measured each 2.5 s as previously described (13). During the experiments, CCL11 (50 nM; R&D) was added 45 s after commencing recording.

Migration assay

Migration assays toward medium or eotaxin (10 nM) were conducted using Boyden chambers as previously described (13).

Statistical analysis

Results were expressed as mean $\pm$ SEM. Differences of groups were evaluated using two-tailed Student's *t*-test.

Results and discussion

To examine RA-induced surface expression of CCR3, purified eosinophils were incubated with 10^{-7} M 9-cis RA for 18 h and subjected to flow cytometry. Remarkably, the histograms indicated that the whole population of eosinophils responded to increased CCR3 expression by 9-cis RA and ATRA (Fig. 1A, ATRA: data not shown). Donor histories of allergies were not related to the response to RAs (data not shown). Over time, the effect of RAs was not significant at 4 h (Fig. 1B), but was observed at 18 h. Taken together with prior gene microarray data, these results indicate that RAs up-regulate CCR3 transcription and following *de novo*

protein synthesis. As shown in Fig. 1(C), RAs induced CCR3 expression from a physiologically available concentration (10^{-8} M) (14) in a bell-shaped response. We also tested the effect of 9-cis RA in the presence of IL-3 or IL-5 (Fig. 1D). In line with previous reports (8, 9), IL-3 significantly down-regulated CCR3 expression ($67.1 \pm 2.6\%$ of medium control, $P < 0.01$), but IL-5 did not ($99.7 \pm 3.4\%$). CCR3 was up-regulated by 9-cis RA regardless of the

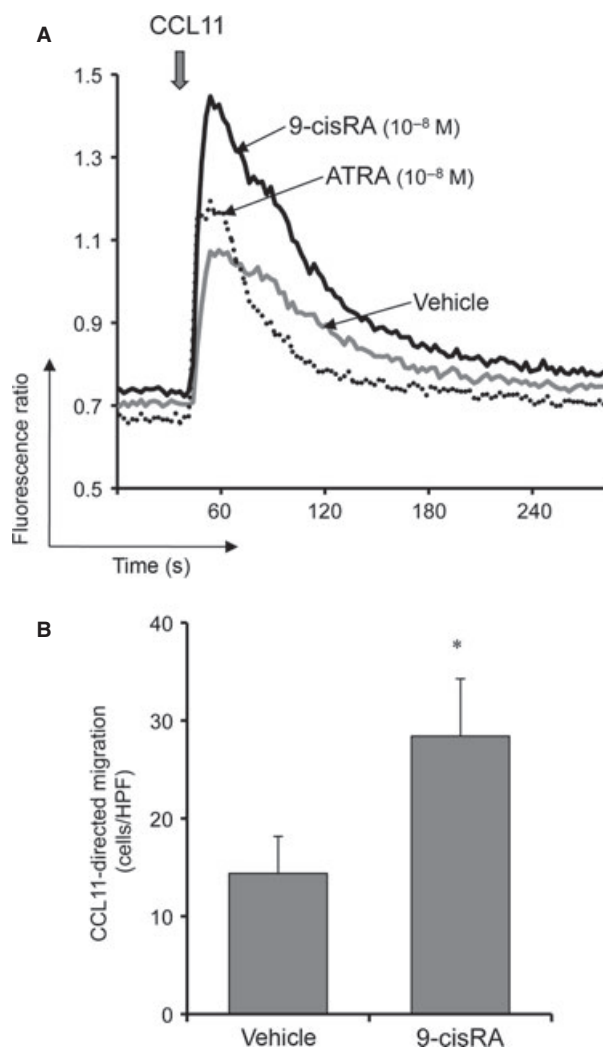


Figure 2 Retinoic acids (RA) enhanced CCL11-induced calcium mobilization and chemotaxis. (A) Purified eosinophils were incubated with vehicle or 10^{-8} M RAs for 18 h and then loaded with intracellular calcium indicator fura-2. The changes in intracellular free calcium levels induced by CCL11 (eotaxin-1; 50 nM) were detected as the increase in fluorescence intensity. The results are representative of three independent donors with similar results. (B) 9-cis RA enhanced CCL11-directed chemotaxis. Purified eosinophils were incubated with vehicle or 10^{-8} M 9-cis RA for 18 h, and then CCL11- (10 nM) induced migration assays were performed using Boyden chambers. CCL11-directed migration was assessed by subtraction of the number of cells that migrated to medium alone. *, $P < 0.05$, vs vehicle control.

presence of these cytokines, suggesting RAs maintain CCR3 expression in both normal and inflammatory conditions.

The biological actions of RAs are exerted via two families of nuclear receptors: RA receptors (RARs) and retinoid X receptors (RXRs), each of which has three major isoforms. Among them, peripheral blood eosinophils constitutively express RAR- α , - β , - γ , and RXR- α , - β (12). To determine the involvement of these receptors, synthetic agonists and antagonists were tested. Both RAR-selective agonist TTNPB and RXR-selective agonist HX630 increased the CCR3 expression, although HX630 had a less potent effect (Fig. 1E). The effect of 9-cis RA was completely blocked with RAR/RXR antagonist HX531 and RAR α antagonist Ro41-5253 (Fig. 1F). These results indicate that RAR α activation is critical for CCR3 up-regulation, although RXR can partially compensate for the effect.

The signaling capacities of RA-induced CCR3 in terms of calcium influx were probed using intracellular calcium indicator fura-2. As we expected, calcium mobilization induced by CCL11 was increased after incubation with 9-cis RA and ATRA (Fig. 2A). Additionally, 9-cis RA-treated cells showed significantly enhanced chemotactic response toward CCL11 compared with vehicle-treated cells (Fig. 2B). These results indicate that RA-induced CCR3 possess its functional capacities.

In conclusion, our current data provide novel insights into the roles of vitamin A in eosinophil functions. It is suggested

that RAs act as a significant contributor to tissue accumulation of eosinophils. RAs and their receptors may be important targets for future therapeutic strategies in allergic inflammatory diseases.

Acknowledgments

We are grateful to Masako Tanabe for outstanding technical assistance and Lisa A. Spencer for helpful discussion. This study was funded in part by Grants-in-aid for Young Scientists (17790666 and 21790934) supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure

The authors declare no competing financial interests.

Author contributions

S.U. designed and performed experiments and wrote the manuscript. J.N., Y.Y., Y.K., M.T., M.I. and Y.K. performed experiments and involved in the analysis of the data. M.T., Y.M. and W.I. contributed with scientific advice and provided technical assistance. J.C. supervised the research and edited the manuscript. All authors offered their criticisms of the manuscript and approved this version for publication.

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