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Non-redundant role of CCRL2 in lung dendritic cell trafficking

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Abstract

CCRL2 is a heptahelic transmembrane receptor that shows the highest degree of homology with CCR1, an inflammatory chemokine receptor. CCRL2 mRNA was rapidly (30 min) and transiently (2-4 hrs) regulated during dendritic cell (DC) maturation. Protein expression paralleled RNA regulation. *In vivo*, CCRL2 was expressed by activated DC and macrophages, but not by eosinophils and T cells. CCRL2$^{-/-}$ mice showed normal recruitment of circulating DC into the lung but a defective trafficking of antigen-loaded lung DC to mediastinal lymph nodes. This defect was associated to a reduction in lymph node cellularity and reduced priming of Th2 response. CCRL2$^{-/-}$ mice were protected in a model of OVA-induced airway inflammation with reduced leukocyte recruitment in the BAL (eosinophils and mononuclear cells) and reduced production of the Th2 cytokines IL-4 and IL-5 and chemokines CCL11 and CCL17. The central role of CCRL2 deficiency in DC was supported by the fact that adoptive transfer of CCRL2$^{-/-}$ antigen-loaded DC in wild type animals recapitulated the phenotype observed in knock out mice. These data show a nonredundant role of CCRL2 in lung DC trafficking and propose a role for this receptor in the control of excessive airway inflammatory responses.

Introduction

Dendritic cells (DC) are professional antigen presenting cells and key regulators of T cell functions [1,2]. In the airway epithelium DC form an extensive network where they

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continuously sense environmental antigens. Following antigen capture, lung DC migrate to mediastinal lymph nodes where antigens are presented to T cells. Activation of a Th2-skewed response by airway DC is responsible for allergic immune responses in the lung [3,4].

Chemokine receptors play a crucial role in the migration of maturing DC to secondary lymphoid organs [5,6] and several studies have shown that this process is a crucial event for the appropriate activation of the immune response [7-10]. DC migration to lymph nodes relies on the functional expression of CCR7 [5,6] as well as other chemotactic receptors, such as CCR8 and BLT1 [9-11]. In addition, lung DC migration is also regulated by PD1, one of the two PGD_2 receptors, and by the transcription factors PPRγ and Runx3 [12-14]. However, at difference from DC localized in other anatomical compartments, the homing of lung DC to lymph nodes is independent of the action of MRP1, the LTC_4 transporter that regulates CCR7 functions [11]. This finding indicates that DC trafficking is regulated in a tissue-specific manner.

CCRL2 (chemokine CC motif receptor-like 2), also known as L-CCR (LPS-inducible CC chemokine related gene and Eo1), is a heptahelic serpentine receptor that shares the highest homology with the chemokine receptors CCR1 and CCR5. CCRL2 is structurally characterized by the presence of a non-canonical DRYLAIV motif. CCRL2 was originally identified in the mouse macrophage cell line RAW 264.7 [15] and was recently reported to bind the chemotactic protein chemerin, though in the absence of any detectable intracellular signalling [16]. CCRL2 expression at the mRNA level has been described in murine macrophages [15], glial cells, astrocytes and microglia stimulated with LPS [17,18] and in mast cells [16]. CCRL2 was also reported to be upregulated in lung macrophages and epithelial cells following *in vivo* sensitization [19]. The human gene most closely related to CCRL2 is HCR with its two splicing variants CRAM-A and CRAM-B.

Here we describe that CCRL2 is rapidly induced during mouse DC maturation with a kinetics that precedes CCR7 induction. In order to evaluate the relevance of this receptor in DC biology, we generated CCRL2 deficient mice and used them in an established model of allergen-induced airway inflammation in which DC are known to play a crucial role [20]. The results here reported highlight a nonredundant role for CCRL2 in the migration of lung DC to regional lymph nodes and in the induction of Th2-oriented airway allergic inflammation. These results propose CCRL2 as a new potential target for therapeutic strategies aimed at controlling lung hypersensitivity.

Materials and Methods

Dendritic cells

Bone marrow-derived DC were generated from $CD34^+$ bone marrow cells and were characterized for antigen expression, pinocytic capacity and chemotaxis as described [21]. Migration of lung DC was evaluated in sensitized mice by the intratracheal (i.t.) injection of 80 μl of FITC-OVA (10 mg/ml). $CD11c^+FITC^+$ DC were FACS counted in mediastinal lymph nodes 8-24-48-72 hrs later. For adoptive transfer, bone marrow-derived DC were pulsed overnight with 100 μg/ml OVA, washed and instilled intratracheally. *In vitro* chemotaxis assays were performed using micro Boyden chambers as previously described [21].

Generation of the CCRL2 deficient mice

A SalI-HindIII and a HindIII-BamHI fragment (6.5 kb and 3 kb respectively) containing the CCRL2 gene but lacking its ORF were obtained and ligated to a PGK-neomycin resistance gene cassette. This construct was electroporated into R1 embryonic stem cells and resistant clones were isolated and analyzed by Southern blots after KpnI digestion using an external

probe. One targeted clone was injected into C57BL/6 blastocysts, and resulting chimeras were mated with C57BL/6 mice (Charles River Breeding Laboratories) to obtain an outbred line carrying the mutated CCRL2 allele that was backcrossed in the C57BL/6 background for 10 generations. Age- and gender-matched littermates were used in all studies. Procedures involving animals and their care were conformed to institutional guidelines in compliance with national (4D.L. N.116 G.U. supplement 40, 18-2-1992) and international law and policies (European Economic Community Council Directive 86/609, OJ L 358,1,12-12-1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Quantification of mRNA levels by Northern blot and Real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, San Diego, CA, USA). Northern blots were performed as described [21]. The ApaI-HincII fragment of CCRL2 cDNA was used as probe. The CCR7 probe was obtained as described [21]. For Real-time PCR analysis, total RNA samples were treated with DNAse I (Invitrogen) prior to RT. Real-time quantitative PCR reactions were performed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Carlsbad, CA, USA) using a SYBR Green PCR master mix (Applied Biosystems) and specific primers (Fig. S5). Gene expression was normalized to β-actin or 18S mRNA.

Generation of mAb to CCRL2

10^7 X-rayed CCRL2/L1.2 cells in PBS were injected i.p. every other week for 6 weeks into CCRL2-deficient mice. Three days after the last challenge, spleen cells were fused following conventional protocols. Hybridomas recognizing CCRL2/CHO-K and CCRL2/L1.2 transfectants but not parental cells were cloned and tested for specificity on CCRL2, CCRs and CXCRs transfectants. One hybridoma (hybr 4, IgG2a) was selected for these studies, purified and biotinylated.

FACS analysis

Cells were blocked with CD16/32 2.4G2, incubated with biotinylated anti-CCRL2 Ab (3 μg/ml) and then with streptavidin-FITC or -PE (BD). Directly conjugated Abs to CD3e (145-2C11), CD4 (A15.1.17), CD8α (53-6.7), CD11b (M1/70), Gr-1 (RB6-8C5), I-Ab (2G9), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD40 (3/23) from BD (Franklin Lakes, NJ, USA), CCR7, CD103 (2E7) and Siglec H (440c) from eBioscience (San Diego, CA, USA), PDCA-1 (JF05-1C2.4.1) from Miltenyi (Bergisch Gladbach, Germany) were used for analysis of cell subpopulations. Staining was analyzed by a FACScan flow cytometer (BD) using CellQuest software. Flow cytometry analysis of lung single cell suspensions was performed as described [22].

Allergen-induced airway inflammation

Mice were sensitized with OVA 0.01 mg/mouse i.p. in 0.2 ml alum on day 0 and 12, exposed daily to OVA aerosol (5%; 20 min) from day 18 to 23 and sacrificed 24 hours after the last aerosol. Airways were washed three times with 1 ml of PBS via a tracheal cannula. Differential cell counts were performed using Diff-Quik () stained cytospins. Cells from lung tissue were obtained after 0.15 mg/ml collagenase (type D; Roche, Indianapolis, IN, USA) and 25 μg/ml DNase (type I; Roche) incubation (37°C for 1 h) as described [23]. For histopathology, lungs were fixed in 10% normal buffered formalin. 4-μm paraffin-embedded sections were stained with hemotoxylin and eosin. For immunohistochemistry sections were obtained from frozen lungs from three mice/experimental group. Immunostain was performed by using anti-CD11c (BD), anti-Siglec H (rat anti-mouse, kindly provided by Prof. M. Colonna, Washington University, STL, MO, USA) and anti CD103 (eBioscience).

Primary antibodies were revealed using anti-hamster or anti-rat (Vector, Burlingame, CA, USA) biotin-conjugated secondary antibodies followed by streptavidin-HRP (for CD11c and Siglec H) or streptavidin-PA (for CD103) and DAB (all from Dako, Denmark) or Ferangi Blue (Biocare Medical, Concord, CA, USA).

Airway responsiveness was evaluated 24 hours after the last OVA challenge by measurements of lung resistance (RL) and dynamic compliance in anaesthetized and tracheostomized mice in response to inhaled methacholine in a Buxco system (Buxco). Goblet cells were counted on Periodic Acid-Schiff (PAS)-stained lung sections using an arbitrary scoring system, as previously described [24]. Data are expressed as mean ± SEM, n = 14-15 mice/group. For *in vitro* antigen restimulation of lymphocytes, mediastinal lymph nodes were excised, 5×10^5 cells in 200 μl were seeded in U-bottomed plastic plates and OVA was added at varying concentrations; plates were incubated at 37°C for 72 hrs.

Cytokine and IgE amounts evaluation

Cytokines and total IgE were measured by standard sandwich ELISA from Pharmingen (IL-4, IL-5, INF-γ and IgE) and R&D (IL-2, IL-13, CCL2, CCL5, CCL11, CCL17 and CCL22). OVA-specific IgE were detected using anti-mouse IgE mAb (BD) as capture antibody and biotinylated OVA (EZ-Link Sulfo-NHS-LC-Biotinylation Kit, Pierce) as the detection reagent.

Statistics

Statistic significance was calculated by Student T test, Mann-Whitney *U* test and one-way ANOVA as appropriate. Differences were considered significant at $p < 0.05$.

Results

Regulation of CCRL2 expression in mouse DC

In a preliminary screening of orphan/uncharacterized putative chemotactic receptors (unpublished), CCRL2 was selected for its unique ability to be upregulated during DC maturation. CCRL2 mRNA was barely detectable in resting bone marrow-derived myeloid DC. LPS, a prototypic maturation factor for DC, rapidly induced CCRL2 expression, starting at 30 min and reaching a peak at 2 hrs, and decreasing thereafter (Fig. 1A). As expected [21], CCR7 transcripts levels were also increased by LPS, but with delayed kinetics, starting after 4 hrs and reaching peak levels at 24 hrs (Fig. 1A). Regulation at the mRNA level was paralleled by protein expression, with peak levels detected at 12 hrs that returned to nearly basal levels at 40 hrs of stimulation. The evaluation of CCR7 membrane expression confirmed the delayed kinetics observed at the mRNA level (Fig. 1B). Other inflammatory mediators, such as TNFα and poly(I:C), known to induce DC maturation, also upregulated CCRL2 expression (Fig. 1C). No constitutive CCRL2 membrane expression was detected in $CD11c^+$ DC purified from lymph nodes, spleen, thymus, bone marrow and lung (data not shown). However, CCRL2 was readily induced in spleen $CD11c^+$ DC 6 hrs following LPS i.v. administration (Fig. 1D), a timeframe compatible with the *in vivo* redistribution of activated DC from the marginal zone to the T cell areas [25].

Evaluation of WT and CCRL2$^{-/-}$ mice in a model of OVA-induced airway hyper responsiveness

To directly evaluate CCRL2 biological relevance we generated CCRL2 deficient mice ($CCRL2^{-/-}$) by homologous recombination techniques as detailed in Materials and Methods and Figure 2A and B. After electroporation of R1 embryonic stem cells, neomycin-resistant clones were analyzed by Southern blots (Fig. 2C). Mice were routinely genotyped by PCR with a set of three primers that detected the WT and the targeted alleles (Fig. 2D). CCRL2

deficiency was assessed by Real-Time RT-PCR in bone marrow-derived DC and tissues, using specific primers (Fig. 2E and data not shown). CCRL2$^{-/-}$ mice developed normally to term and were fertile. No significant alterations were found after histological and flow cytometric analysis of lymphoid organs and blood, when compared with age- and gender-matched WT control mice (not shown). CCRL2$^{-/-}$ mice had a normal lifespan and did not show an overt phenotype under steady-state conditions.

Lung DC play a crucial role in the transport of antigens to mediastinal lymph nodes and in the induction of airway hypersensitivity [11,26]. The role of CCRL2 was therefore investigated in a model of airway hypersensitivity. OVA-immunized CCRL2$^{-/-}$ mice, challenged by aerosol for six consecutive days, showed a dramatic reduction in the total number of leukocytes and in particular of eosinophils and lymphocyte/mononuclear cells, with respect to WT animals (Fig. 3A). The decrease in T cells was evident for three main cell subsets, namely CD4^{+}, CD8^{+} and ST2^{+}CD3^{+}/Th2 cells (Fig. 3B). However, a possible direct role for CCRL2 in the recruitment of eosinophils and T cells into the airways was ruled out by the lack of expression of CCRL2 in these two cell types; conversely BAL MHCII^{+} cells (macrophages and DC) expressed CCRL2 (Fig. 3C).

In parallel, the BAL of CCRL2$^{-/-}$ mice contained lower levels of the Th2 cytokines IL-4 and IL-5 and similar levels of IL-13 and the Th1 cytokine IFNγ (Fig. 3D). Of note, although Th2 cells and eosinophils represent an important source for Th2 cytokines, IL-13 can also be secreted by lung smooth muscle cells [27]. This may explain the different regulation of IL-13 versus IL-4 and IL-5 here observed. The alterations observed in the BAL were not paralleled by changes in the lung parenchyma where normal degrees of infiltrating leukocytes (Fig. 1S, panels A and B) and cytokine levels were detected (Fig. 1S, panel C). To further examine the mechanisms associated with the defective leukocyte recruitment observed in CCRL2$^{-/-}$ mice, the levels of some relevant chemokines were evaluated. The eosinophil-attracting chemokine CCL11/eotaxin and the Th2-attracting chemokine CCL17/TARC levels were significantly decreased in BAL (Fig. 3D) but not in lung tissue (Fig. S1 panel C). This finding is consistent with the decreased number of eosinophils and Th2 cells observed in the airway lumen. Conversely, no difference was observed in the expression of CCL22 (BAL and lung) and CCL11 and CCL17 in the lung. CCL2 and CCL5 levels were decreased in lung tissues of OVA-sensitized CCRL2$^{-/-}$ (Fig. S1 panel C) but not in the BAL (Fig. 3D). In addition, OVA-sensitized CCRL2$^{-/-}$ mice showed normal levels of circulating total and OVA-specific IgE (Fig. 3E). This finding is consistent with the ability of CCRL2$^{-/-}$ mice to mount a normal primary and secondary antibody response following i.p. immunization with OVA/Alum or OVA/complete Freund's adjuvant (unpublished results). Finally, the typical features of asthma, namely airway hyperresponsiveness (AHR) and mucus hypersecretion were assessed in OVA-sensitized CCRL2$^{-/-}$ and WT mice. Both strains showed a significant increase in methacholine responsiveness evaluated as lung resistance (Fig. 3E) and dynamic compliance (data not shown), that was however similar in the two mouse strains (Fig. 3E). Similarly, OVA-sensitized CCRL2$^{-/-}$ and WT mice displayed a comparable number of goblet cells (Fig. 3E).

Defective trafficking of pulmonary CCRL2$^{-/-}$ DC to regional lymph nodes

The migration of lung DC from periphery to regional lymph nodes is a key step for the induction of immune response and tolerance [5,6,28]. Therefore, lung DC trafficking was investigated in OVA-challenged CCRL2$^{-/-}$ mice. In agreement with previous reports [29], Figure 4A shows that the influx of DC into the lung was already detectable after 2 hrs of OVA challenge and reached peak levels at 24 hrs. No significant difference in the absolute number of DC (Fig. 4A), neither in their activation phenotype (i.e. CD80, CD86, CD40 and MHCII expression) (data not shown) was found between CCRL2$^{-/-}$ and WT mice at any of the time points investigated. Similarly, no difference was observed in the relative proportion

of the three major lung DC subsets, namely, myeloid $CD103^+$ and $CD11b^+$ DC and plasmacytoid DC (Fig. 4B). Furthermore, the *in vivo* distribution of DC subsets was investigated by immunohistochemistry of lung frozen tissue sections. $CD103^+$ cells were mainly identified in the peri-bronchiolar space lining the conducting airways. Siglec H^+ and $CD11c^+$ cells were localized in the peri-bronchiolar space admixed to other immune cells as well as in the interstitial space of the lung parenchyma. CD11c stain was also observed in large cells located in the alveolar space likely reflecting expression by alveolar macrophages. However, no difference was observed between WT and $CCRL2^{-/-}$ mice in the distribution of these DC subsets in either resting or OVA-stimulated conditions (Fig. S2). In order to study the migration of airway DC, OVA-sensitized animals were instilled i.t. with a single administration of FITC-OVA and $CD11c^+FITC^+$ DC were enumerated in mediastinal draining lymph nodes [4,23]. Figures 4C and D report that the migration of $FITC^+$ DC was already detectable after 8 hr stimulation and reached a peak at 24 hrs, as previously reported [23]. $CCRL2^{-/-}$ mice showed a statistical significant reduction in $FITC^+$ DC trafficking to lymph nodes at 48 and 72 hrs after antigen administration; of note, only $FITC^+$ DC expressed CCRL2 (Fig. 4E). These results highlight a crucial role of CCRL2 in directing pulmonary DC migration to draining lymph nodes. In order to investigate the molecular basis for the defective migration of lung DC, experiments were performed using bone marrow derived DC. *In vitro*, DC generated from $CCRL2^{-/-}$ and WT bone marrow progenitors were similar in terms of cell yield (not shown), expression of membrane markers and costimulatory molecules, and in their ability to take up antigens (Fig. S3, panels A and B). These results postulate that CCRL2 deficiency does not interfere with DC development and maturation *in vitro*. DC from $CCRL2^{-/-}$ DC were also tested for the expression and function of chemotactic receptors. When evaluated at the mRNA level, $CCRL2^{-/-}$ DC showed a normal expression of the chemokine receptors CCR1, CCR2, CCR5, CXCR4 and CXCR6 and CCR7 (Fig. S3, panel C). Expression of CCR-3, -4, -6, -8, -9, CXCR-2, -3, -5, -6, -7, CX3CR1 and XCR1 was low in both WT and $CCRL2^{-/-}$ DC (less than 500 mRNA molecules per 10^6 β-actin molecules) and was not modulated during maturation (data not shown). When tested in chemotaxis assays, immature $CCRL2^{-/-}$ DC showed a normal *in vitro* migration to CCL5 and chemerin, and a slightly reduced response to CCL3 and CXCL12. Mature $CCRL2^{-/-}$ DC were competent in migrating to CCL19, a CCR7 ligand (Fig. 4 panel F). Finally, skin painting experiments did not reveal any alteration in the *in vivo* migration ability of endogenous $CCRL2^{-/-}$ DC and no alteration was seen in the migration to lymph nodes of in vitro generated CFSE-labelled DC (from WT and CCRL2 mice), when injected in the footpad of WT mice, (data not shown). Altogether, these results exclude the possibility that CCRL2 deletion may be associated to a major alteration in the expression and function of DC chemotactic receptors.

Defective lymphocyte priming in mediastinal lymph nodes of $CCRL2^{-/-}$ mice

The anti-OVA specific immune response was evaluated using cells collected from lung draining lymph nodes. For this purpose, lymph nodes were collected 24 hrs after the last aerosol challenge, homogenized, and cell suspensions analyzed. Consistently with the reduced migration of antigen-loaded DC [30], mediastinal lymph nodes from $CCRL2^{-/-}$ mice showed a 2-fold reduction in total cellularity and a significant reduction in the percentage of total $CD11c^+$ cells (Fig. 5A). Lymph node cell suspensions were then exposed to OVA and Th1/Th2 cytokine levels assessed in culture supernatants. As shown in Figure 5B, at the end of 4-day culture, WT cells secreted IL-5 and IL-13 at levels that were significantly higher than those detected using cells from $CCRL2^{-/-}$ lymph nodes. In the same experiments, IL-4 and IFNγ levels were below the assay detection limit.

To directly investigate the role of CCRL2 in the elicitation of the Th2 response *in vivo*, WT mice were sensitized with OVA at days 0 and 11, and at days 18 and 20 received the i.t.

injection of OVA-pulsed DC generated from either WT or CCRL2$^{-/-}$ mice; 48 hrs after the last DC instillation, mediastinal lymph node cells were collected and cultured *in vitro* in the presence of OVA. As shown in Figure 5C, cells obtained from mice that had received WT DC showed a strong Th2 response in terms of IL-5 and IL-13 production that was not further stimulated by the *in vitro* addition of OVA. The lack of response to OVA *in vitro* is possibly due to the high concentration of the antigen used to load DC before adoptive transfer. On the contrary, lymph nodes cells from mice that had received CCRL2$^{-/-}$ DC showed very modest response, that could be elicited in a dose-dependent manner by the *in vitro* addition of OVA (Fig. 5C). Cells from lymph nodes of mice instilled with unpulsed WT or CCRL2$^{-/-}$ DC did not produce IL-5 or IL-13 both under basal conditions and in response to OVA (not shown). The defective ability of CCRL2$^{-/-}$ DC to prime a Th2 response could not be ascribed to an intrinsic defect of CCRL2$^{-/-}$ DC since they were fully able to induce T cell proliferation in allostimulatory mixed leukocyte reactions (MLR). Indeed, Figure 4S, panel A shows similar levels of CFSE-labelled CD4^{+} cell proliferation when cultured with either allogenic WT or CCRL2$^{-/-}$ DC. Similarly, CCRL2$^{-/-}$ DC induced a normal degree of OT-II OVA-specific TCR transgenic CD4^{+} T cell proliferation, *in vitro* (Figure S4 panel B). In agreement with these results, similar levels of cytokines (IL-2, IL-4 and IFN-γ) were detected in the supernatants of T cell cultures performed with WT and CCRL2$^{-/-}$ DC (Fig. S4).

Discussion

The migration of lung DC from periphery to mediastinal lymph nodes is a key step in the development of airway allergy and inflammation, including asthma [3,4,11,31]. In this study we report that the expression of the poorly characterized receptor CCRL2 is rapidly induced during DC maturation both *in vitro* and *in vivo* with kinetics that precedes CCR7 expression, the master lymph node homing chemokine receptor [5,6]. To investigate the potential role of CCRL2 in DC biology, we have generated CCRL2-deficient mice and used them in an established Th2 model of OVA-induced airway inflammation in which DC migration was shown to play a prominent role [3,6]. In this experimental model antigen-loaded DC revealed an impaired capacity to traffic to regional lymph nodes and this defect was associated with reduced T cell priming. CCL11 and CCL17 were also significantly reduced in the airways of CCRL2 deficient mice. CCL11 has been shown to be an important chemoattractant for eosinophils and Th2 cells whereas CCL17 is a chemoattractant for Th2 cells [32]. Therefore the decreased production of these two cytokines likely accounts for the reduced number of these two cell types in the airway lumen of CCRL2$^{-/-}$ mice. Indeed, lung eosinophils and T cells do not express CCRL2, ruling out a direct involvement of this receptor in their recruitment. In the lung, CCL11 and CCL17 are produced by epithelial and endothelial cells, whereas smooth muscle cells and alveolar macrophages are responsible for the production of other chemokines, such as CCL2, CCL12 and CCL22 [31,33]. CCRL2 is expressed by bronchial epithelial cells and lung macrophages following OVA challenge [19]. Therefore it is possible that CCRL2 deficiency in these additional cell types might also contribute to the reduced airway inflammation observed in CCRL2$^{-/-}$ mice. On the contrary, the degree of leukocyte infiltration and the levels of CCL11 and CCL17 in lung tissue were not altered.

The levels of the Th2 cytokines IL-4 and IL-5 were reduced in both the airway lumen and the lung interstitium, highlighting a defect of CCRL2$^{-/-}$ mice in generating Th2 responses. Consistently with these results, T cell priming in mediastinal lymph nodes was almost abolished in CCRL2$^{-/-}$ mice. Since lung antigen-specific T cell response is initiated in the lymph nodes, these results suggest a critical role for CCRL2 in the generation of local primary immune responses.

Indeed, adoptive transfer experiments of antigen-loaded CCRL2$^{-/-}$ DC into WT animals support the concept that the defective Th2 priming in mediastinal lymph nodes was strictly associated to a defect in DC trafficking. On the other hand, the recruitment of circulating DC to the lung of CCRL2$^{-/-}$ mice exposed to OVA was not different from that of WT animals. This result suggests that this receptor is not involved in the tissue recruitment of peripheral DC. Bone marrow derived DC generated from CCRL2$^{-/-}$ mice were indistinguishable from WT mice in terms of antigen uptake, membrane phenotype and antigen presentation capacity. Furthermore, CCRL2$^{-/-}$ DC expressed normal mRNA levels of all the chemokine receptors investigated and normal *in vitro* and *in vivo* migration properties, excluding the possibility that CCRL2 deletion might be associated to a more general alteration of the chemotactic receptor functions. Finally, it should be noted that *in vitro*, DC obtained from CCRL2$^{-/-}$ mice were fully competent in promoting the proliferation of allogeneic or syngeneic OT-II cells and in inducing Th2 cytokines production. Taking together, these results exclude an intrinsic defect of CCRL2$^{-/-}$ DC to promote T cell activation and support a role for CCRL2 in lung DC migration and function. In spite of the reduced cell recruitment in BAL and of the reduced Th2 priming in lymph nodes, lung function, assessed as methacholine responsiveness, was only slightly decreased. These findings suggest that, in this model, lung function correlates better with the leukocyte content of lung parenchyma rather than the airway lumen. Indeed, previous studies have shown that blocking CCL12 or CCL22 reduces leukocyte recruitment to the lung interstitium but not to the airway lumen; this defect was associated to a decrease in airway hyperresponsiveness and mucus production [34,35]. Of note, CCRL2$^{-/-}$ mice did not show any alteration when tested in a Th1 model of OVA/LPS-induced lung hypersensitivity [36] (data not shown). This result highlights a peculiar role of CCRL2 in Th2 skewed responses.

In the past few years, it has been proposed that CCL2, CCL5, CCL7 and CCL8 could bind and activate CCRL2 [37]. However, we (data not shown) and others [16] have not been able to confirm these data. Of note, we have previously reported that maturation of mouse DC *in vitro* causes a marked decrease in the chemotactic response towards CCL2 and CCL5 [21], whereas this study reports that CCRL2 is induced during maturation. CCRL2 presents a non-canonical DRYLAIV motif and the ability of this receptor to signal is still a matter of debate. It was recently shown that CCRL2 binds and presents chemerin in the absence of receptor internalization and signalling [16]. Chemerin is a chemotactic protein that we and others have recently purified and characterized as the ChemR23 ligand [38,39]. This study reports that chemerin induces the *in vitro* migration of WT and CCRL2$^{-/-}$ DC and does not modify the migration to CCR7 ligands (unpublished data). Chemerin expression in mediastinal lymph nodes was not modulated by OVA immunization and challenge in WT and CCRL2$^{-/-}$ mice (data not shown), therefore the role of chemerin in the trafficking of CCRL2^{+} lung DC is presently uncertain [38].

The migration of DC from peripheral tissues to draining lymph nodes relies on the functional expression of CCR7 and CCR8 [5,6,26]. However, CCR7 proper functioning requires accessory signals. In particular, it was reported that cysteinyl leukotrienes and their transporter protein MRP1 were required for CCR7 activity [40]. We have recently reported that also LTB_4, through the activation of its high affinity receptor BLT1, promotes CCR7 expression and function in migrating DC [9]. Recent work has proposed that DC trafficking from peripheral tissues is controlled in a tissue-specific manner, with lung DC egression being independent of the expression of MRP1 [11]. In this paper we show that lung DC migration is impaired in CCRL2 deficient mice. However, we did not observe any defect in the migration of skin DC in a model of FITC-skin painting and using CCRL2$^{-/-}$ bone marrow-derived DC injected in the footpad of WT mice (data not shown). Therefore, we propose that MRP1-independent lung DC trafficking to lymph nodes may require a still unknown accessory signal provided by CCRL2.

In summary, this study reports that CCRL2 plays a nonredundant role in lung DC migration to peripheral lymph nodes and that this defective DC migration is responsible for the reduced Th2 response observed in CCRL2$^{-/-}$ mice. Therefore, CCRL2 may represent a new target for the control of lung inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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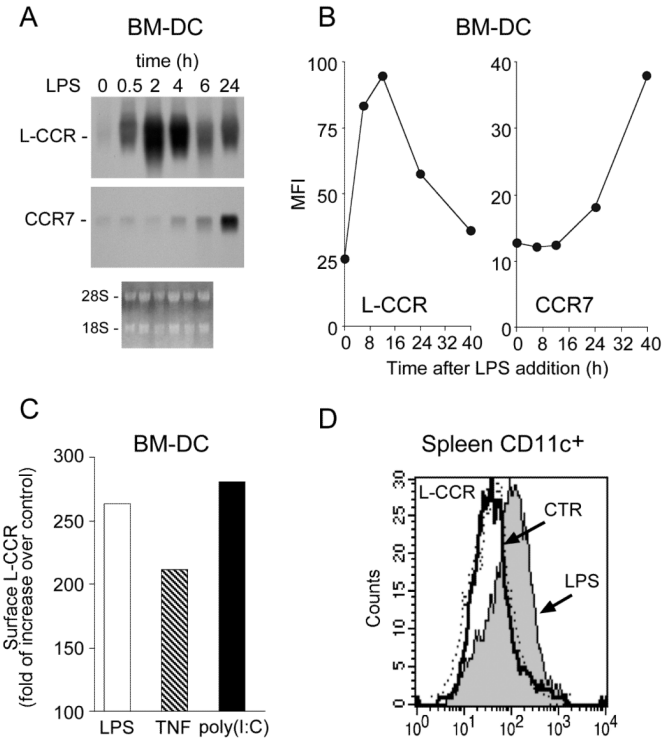

Figure 1. Induction of CCRL2 in maturing DC

DC were generated *in vitro* from $CD34^+$ bone marrow precursors. (A) Total RNA was purified from immature DC (0 hrs) or DC treated for different times with 100 ng/ml LPS and analyzed by Northern blot. Ethidium bromide staining is shown. (B) Kinetics of CCRL2 and CCR7 membrane expression in DC stimulated with 100 ng/ml LPS. The graphs show the mean fluorescence intensity (MFI) of cells labelled with CCRL2 and CCR7 mAbs. (C) FACS analysis of DC treated with 100 ng/ml LPS, 20 ng/ml TNFα or 25 μg/ml poly (I:C) for 12 hrs. Data are expressed as folds of increase of CCRL2 labelling over vehicle-treated DC. (D) CCRL2 expression in spleen $CD11c^+$ DC isolated 6 hrs after i.v. injection of 25 μg/mouse LPS. Histograms represent CCRL2 expression in WT mice (dotted line) and $CCRL2^{-/-}$ mice (solid lines). Data are representative of 2-4 separate experiments.

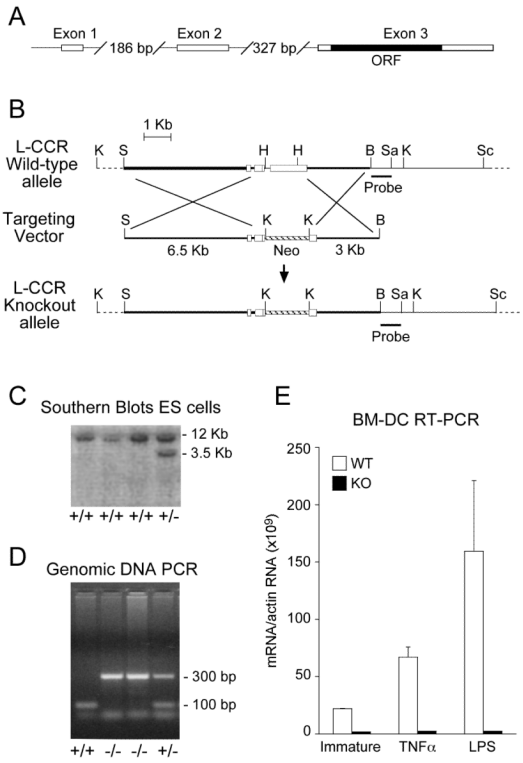

Figure 2. Generation of CCRL2 deficient mice

(A) Exon/intron structure of the CCRL2 gene. (B) Schematic representation of WT and KO alleles of the CCRL2 gene and targeting vector. Restriction sites: K, KpnI; S, SalI; H, HindIII; B, BamHI; Sa, SacI; Sc, ScaI. KpnI-digested genomic DNA fragments were detected by “Probe”. (C) Southern blot analysis of KpnI-digested genomic DNA from transfected ES cells hybridized with the CCRL2 probe generated a 12 kb WT restriction fragment and a 3.5 kb homologous recombinant restriction fragment. (D) PCR analysis of genomic DNA of $CCRL2^{+/+}$ (lane 1), $CCRL2^{-/-}$ (lanes 2,3) and $CCRL2^{+/-}$ (lane 4) mice. (E) Total RNA was isolated from immature as well as TNF-α and LPS stimulated (24 hrs) bone marrow DC.

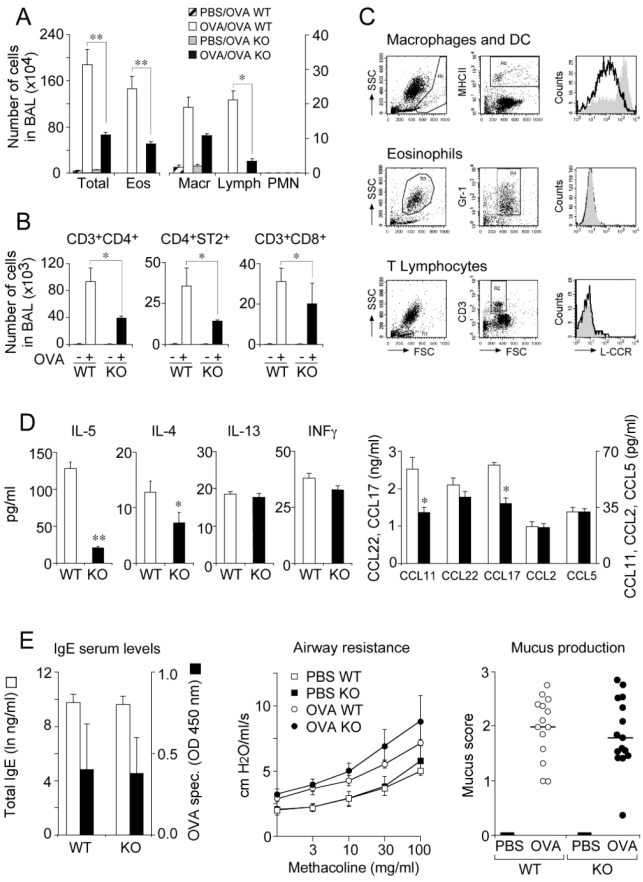

Figure 3. Role of CCRL2 in OVA-induced airway inflammation

WT and CCRL2$^{-/-}$ mice were sensitized and challenged with OVA by aerosol. BAL was collected twenty-four hours after the last aerosol. (A) Differential cell counts in BAL. (B) Evaluation of T cell subsets in BAL. (C) CCRL2 membrane expression by MHCII^{+} cells, eosinophils and T lymphocytes by FACS analysis. Histograms represent CCRL2 mAb staining of cells from WT (open curves) and CCRL2$^{-/-}$ mice (filled curves). (D) Cytokine and chemokine levels in BAL. (E) Total and OVA-specific IgE in serum (left panel), and bronchial hyperreactivity (AHR) to methacholine (middle panel) and mucus production (right panel). Data are mean ± SEM of five (A) or three (B-E) representative experiments with 6 to 12 mice per group; * P <0.05; ** P <0.01.

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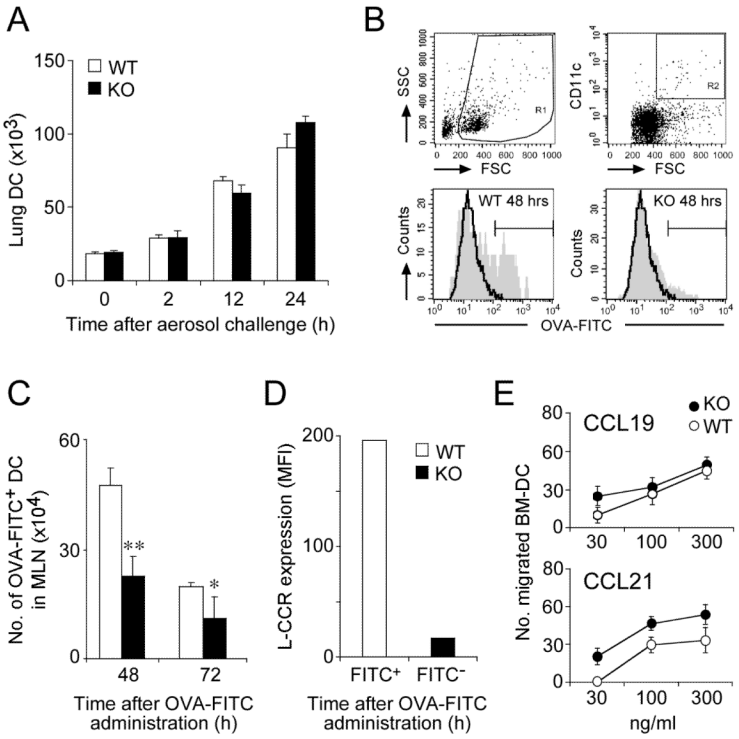

Figure 4. Role of CCRL2 in lung DC trafficking

(A) Lung tissue and trachea were isolated from sensitized mice after one OVA-aerosol challenge; DC were quantified by FACS analysis; results are expressed as total number of DC per mouse (mean ± SEM, n=3; 6 mice/group). (B) The percentage of $CD11c^{+}$/low autofluorescent DC in total lung $CD11c^{+}$ cells and DC subsets ($CD103^{+}$, $CD11b^{+}$, and Siglec H^{+}/$PDCA1^{+}$ pDC) are presented. (C and D) OVA-sensitized mice received i.t. 800 μg OVA-FITC. Panel C, representative analysis of WT and $CCRL2^{-/-}$ mediastinal lymph node cells obtained 48 hrs after FITC-OVA administration. Panel D, kinetics of $FITC^{+}$ DC in lymph nodes. Data are mean ± SEM, n=3; 12-15 mice/group. * P <0.05. (E) Surface expression of CCRL2 in lymph node $FITC^{+}$ and $FITC^{-}$ WT DC after OVA-FITC administration (n=2; 4 mice/group). (F) Chemotaxis of bone marrow-derived DC. One experiment representative of six, each one performed with independent DC cultures. Results are at the net of basal migration (10±3 and 8±2 cells for WT and KO cells, respectively; * P <0.05).

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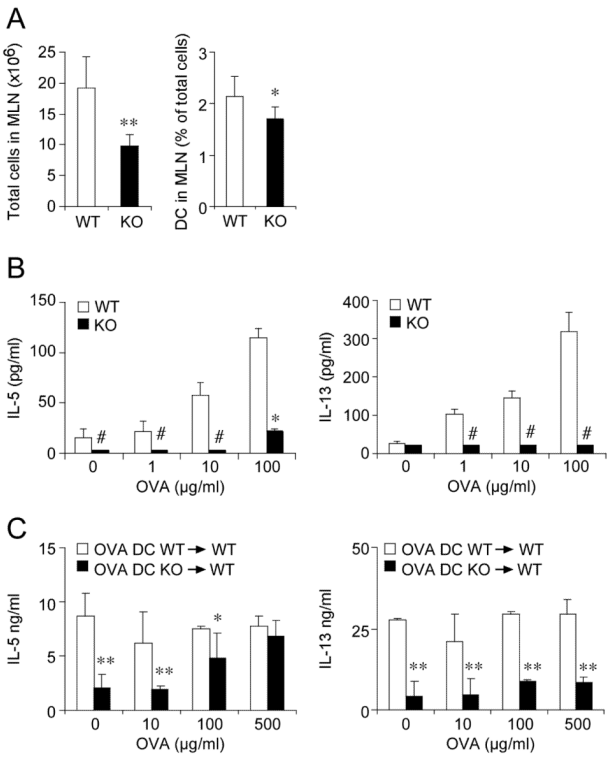

Figure 5. Impaired production of Th2 cytokines in mediastinal lymph nodes of CCRL2$^{-/-}$ mice

Lymph nodes single cell suspensions were obtained 24 hrs after the last aerosol administration. (A) Total cells/mouse and percentage of DC in gated CD11c^{+}MHCII^{+} cells. (B) Lymph node cells were cultured (0.5×10^{6} cells/0.2ml/well) with OVA for 96 hrs. (C) WT or CCRL2$^{-/-}$ OVA-pulsed bone marrow DC (10^{6} cells/mouse) were injected i.t. to WT mice at days 18 and 20. 48 hrs later, lymph nodes were excised and cytokine production determined after ex-vivo stimulation with OVA. Data are mean ± SEM of 10 to 20 mice/group from >2 separate experiments. *, P <0.05; **, P <0.01 in comparison to WT mice. #: undetectable levels.

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