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ORIGINAL ARTICLE

Regulatory Role of Chemerin in Lung Tumorigenesis and Cancer-associated Inflammation

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ABSTRACT

Objective: Interaction between the chemokine-like receptor 1 (CMKLR1) and its cognate peptide ligand, chemerin, can downregulate the inflammatory responses. This study investigates the regulatory role chemerin in lung tumorigenesis and cancer-associated inflammation.
Materials and methods: Animal experiments, cell culture, gene cloning and transfection, chemotaxis assay, Lewis lung carcinoma (LLC) tumorigenesis assays, histopathological evaluation, mixed leukocyte reactions, flow cytometry, proliferation and clonogenicity assays, reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (IHC) techniques were used.
Results: CMKLR1 was identified in mouse LLC tumors. The LLC cells were modified to secrete prochemerin at a level that cannot induce chemotactic activity. Inflammatory cytokine expression, TNF-α, IL-12 p40, but not IL-1β, was diminished in leukocytes when exposed to conditioned media obtained from the chemerin-expressing LLC cells, and in the tumors established with those cells. On the other hand, leukocyte proliferation and IFN-γ expression were not drastically impeded. Correspondingly, tumor forming capacity of the chemerin-expressing LLCs was decreased.
Conclusion: Chemerin may possess a potential to modulate the inflammatory responses in the tumor microenvironment.
Keywords: Tumor microenvironment, chemerin, chemotaxis, macrophage, CMKLR1

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INTRODUCTION

Inflammation is required for an effective anti-tumor immune response; however, some level of inflammatory activity has been characterized to promote tumorigenesis [1]. Especially, in several immune compartments with a high capacity of inflammation, such as the lung, immune responses are precisely modulated by the tumor [2]. Chemokines and local stress derived from the neoplastic formation can selectively increase the infiltration of tumor microenvironment by certain types of immune cells [2,3]. Additionally, chemotactic mediators may also contribute to the regulation of inflammatory responses [4].

The membrane glycoprotein chemokine-like receptor 1 (ChemR23, Dez in mouse) can be found on dendritic cells (DCs), macrophages and natural killer (NK) cells [5-7] where it was initially characterized as a chemotactic receptor. On the other hand, stimulation of CMKLR1 also favors the resolution of inflammation by regulating inflammatory mediators in activated macrophages directly and by increasing the clearance of neutrophils from mucosal surfaces, indirectly [8,9]. The only identified peptide ligand for CMKLR1, chemerin, is secreted as a precursor protein (prochemerin), ubiquitously found in plasma, and gains a high-affinity state upon proteolytic cleavage by the enzymes of inflammatory, fibrinolytic and coagulation cascades [5,6,10-12].

Chemerin mRNA has been identified in several adult and fetal tissues, including the lung [5,6]. In humans, one of the major sources of chemerin is visceral fat tissue where it acts as an adipogenic factor [13]. In mice, this molecule is expressed especially by epithelial cells [14]. As a molecule associated

with inflammation, increased levels of chemerin have been found in inflammatory disorders [5,15,16]. Conversely, the expression of chemerin is downregulated in squamous cancers of the skin and in adrenocortical carcinoma lesions [17,18].

Here, CMKLR1 was determined in Lewis lung tumors (especially on tumor-infiltrating macrophages). Thus, the aim of current study is to genetically modify the Lewis lung carcinoma (LLC) cells to express chemerin (as in secreted natural form, prochemerin) and to evaluate its effects on LLC-associated inflammatory responses.

MATERIALS and METHODS

Animals and Cell Culture

Six- to eight-week-old inbred female C57BL/6 mice (Kobay AŞ., Ankara, Turkey) were housed under environmentally controlled standard conditions. The Guiding Principles in the Care and Use of Laboratory Animals together with those described in the Declaration of Helsinki were strictly adhered in the conduct of all the experimental procedures described within this manuscript. This project was approved by the Institutional Ethical Committee of Hacettepe University, Ankara, Turkey (Approval Number: 2008/8-1) before its commencement.

The Lewis lung cancer cell line (LLC1) and J774A.1 macrophage cell line were kind gifts from Dr. Sven Brandau (University Duisburg-Essen, Germany) and Dr. Vedat Bulut (Gazi University, Turkey), respectively. The cells were cultured in RPMI medium containing L-glutamine (2 mM), 10% fetal bovine serum (Biological Industries, Kibbutz Beit Ha'Emek, Israel), penicillin (100 unit/mL) and streptomycin (100 µg/mL; Biochrom, Berlin, Germany). For co-culture experiments, LLC cells and freshly isolated plastic-adherent splenocytes (i.e. monocyte/macrophage) or J744A.1 macrophages were cultured at 1:1 ratio for 48 h.

Generation of Recombinant Chemerin Gene and Transfection of LLC Cells

To maintain a natural scenario for chemerin expression by LLC cells, the mouse pro-chemerin coding sequence was used for genetic modification. The coding sequence of the gene was obtained from cDNA synthesized by reverse transcription from the total RNA isolated from LPS-stimulated (10 ng/mL, 12 h; Sigma, St. Louis, CA, USA) C57BL/6 splenocytes. To amplify the insert chemerin DNA, PCR reaction was performed by using Pfu DNA polymerase (Promega, Madison, WI, USA) using the PCR conditions, 30'' at 94°C; 45'' at 55°C; 2' at 72°C. The cloning primers (sense, 5'-GAG TGA GCT AGC GCC ATG AAG TGC TTG CTG; antisense, 5'-CTC ACT CCC GGG TTA TTT GGT TCT CAG GGC) were designed to carry NheI and XmaI restriction enzyme recognition sites (underlined). After NheI-XmaI (New England Biolabs, Ipswich, MA, USA) double digestion, chemerin insert and pIRES2-EGFP vector (Clontech, Palo Alto, CA, USA) DNA were ligated and the resulting recombinant clones were checked by sequencing on both strands (ABI Prism 310 Genetic Analyser, PE Applied Biosystems, Foster City, CA, USA).

LLC cells were transfected (Lipofectamine2000TM, Invitrogen, Carlsbad, CA, USA) with linearized recombinant chemerin construct or the control vector pIRES2-EGFP, and selected in the medium containing G418 (450 µg/mL; Promega, Madison, WI, USA). Several clones of stable transfected LLCs were analyzed for enhanced green fluorescent protein (EGFP) expression by flow cytometry. These LLCs modified with recombinant chemerin gene or pIRES2-EGFP vector were designated as Chem-LLC and IRES-LLC, respectively.

Chemotaxis Assay

Since the enzymes secreted from the activated neutrophils increase the chemotactic activity of chemerin [19], an additional conditioning step was included in the chemotaxis assay setup. Prior to chemotaxis assay, splenic polymorphonuclear cells were isolated using the Ficoll (Sigma) density gradient separation method following the hypotonic lysis of erythrocytes. Following the 3h formyl-methionyl-leucyl-phenylalanine (fmLP) stimulation, the cells were extensively washed in order to remove the fmLP. These stimulated polymorphonuclear cells (2x105 cells/mL) were incubated in the supernatants obtained from Chem-LLCs or IRES-LLCs (106 cells/mL) for 16h. The lower chamber of 24-well chemotaxis chamber (Transwell®; Corning, Lowell, MA, USA) with 5 µm pore size was filled with the polymorphonuclear cell-treated supernatants of Chem-LLCs or IRES-LLCs. Freshly isolated peritoneal macrophages (25x104 cells/100µl) were loaded in the upper chamber and allowed to transmigrate for 16h. The membranes were stained with Giemsa and counted under a light microscope.

Tumorigenesis and Histopathological Analysis

Wild type LLC, Chem-LLC, or IRES-LLC cells (5x105 cells suspended in 0.1 mL phosphate-buffered saline) were inoculated subcutaneously to the syngeneic C57BL/6 mice. The size of the tumors was measured weekly, and mice were sacrificed at the fourth week following the first appearance of palpable

tumors. The tumor samples were collected and fixed in 10% formalin and then embedded in paraffin. Histopathological evaluation of the paraffin sections was done under conventional light microscopy after hematoxylin-eosin staining. For the evaluation of EGFP expression in vivo, frozen sections of the tumors were examined using fluorescence microscopy.

Ex Vivo Assays on Mixed Leukocytes in Conditioned Media

The ex vivo analyses were performed with mixed immune cells, i.e. splenocytes. Freshly isolated splenocytes (106 cells/mL for proliferation; 2x105 cells/mL for ELISA and RT-PCR analyses) were incubated in the supernatants obtained from 24 h culturing of 2x105 cells/mL Chem-LLC or IRES-LLC in the G418-free media. Also, a set of control experiments were performed with standard media. For the evaluation of proliferation, splenocytes were initially labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes, Invitrogen, Carlsbad, CA, USA), stimulated with phytohemagglutinin (PHA, 1 μg/mL; Sigma, St. Louis, CA, USA), and following a 96 h incubation, the cells were analyzed on flow cytometry. For the ELISAs, splenocytes were incubated with or without PHA (1 ug/mL) for 24 h and the supernatants were collected and stored at -86°C. For RT-PCR analysis, splenocytes were cultured with or without LPS (0.1 μg/mL) for 3 h and at the end of incubation the cells were harvested for RNA isolation.

Flow Cytometry

LLC cells, freshly isolated splenocytes, cells isolated from tumors and co-cultures were labeled with anti-mouse CMKLR1 (clone BZ194 (194); eBioscience, San Diego, CA, USA) and F4/80 (clone BM8; eBioscience) antibodies. Cells were analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA) or a FACSAria II cell sorter (Becton Dickinson, San Jose, CA, USA). The percentage of positive cells was calculated by comparison with the appropriate isotype-matched antibody controls. EGFP reporter expression and CFSE assays were analyzed at 488 nm. Untransfected or unlabeled cells were used for autofluorescence correction.

Assessment of Proliferation and Clonogenicity

For proliferation analysis, the cells were fixed in 90% ethanol, washed, and treated with 10 μg/mL ribonuclease (Sigma) and 50 μg/mL propidium iodide (Sigma). The percentage of proliferating cells was determined according to the flow cytometric analysis of stained DNA (Multicycle software, Phoenix Flow System, San Diego, CA, USA). Proliferation rate of the cells (i.e. duplication time) was also calculated after counting the cells on a hematocytometer, periodically.

Anchorage-independent growth assay was performed to determine the clonogenicity of Chem-LLC and IRES-LLC cells. Briefly, 0.6% (w/v) noble agar was mixed with an equal volume of 2x growth medium, layered on the bottom of 6-well plates, and following the gelling, 2.5x103 cells were seeded per well. After 3 weeks, colonies were stained with crystal violet (0.04% in ethanol (w/v), Sigma) and counted.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from homogenized tumor tissues or cells using QIAamp RNA Blood Mini Kit (QIAGEN, Maryland, MD, USA). The RNA samples were treated with RNase-free DNase (DNA-free kit; Ambion, Austin, TX, USA). RT-PCR controls without reverse transcriptase (RT-negative control) were performed to ensure the absence of contaminating plasmid or genomic DNA. cDNA was synthesized from 270 ng of RNA, using oligo(dT) primers and RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

For quantitation of gene expression, real-time RT-PCR analysis was used. PCR was carried out in a reaction mixture containing 1x LightCycler-DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.125 μM primer oligonucleotide and 3.5 mM MgCl2 using the cycling conditions; 30" at 95°C, 20" at 60°C, 20" at 72°C on a Rotor-Gene 6000TM cycler (Corbett Research, Sydney, Australia). Three independent assays were performed for each experiment. Each reaction was performed in duplicates or triplicates. Comparative Ct (2-ΔΔCt) method was used for the quantification of relative gene expression. Briefly, in this method, expression data of the gene of interest obtained from experimental and control samples are first internally normalized according to their β-actin and then, the experimental samples are re-normalized against the control samples [20]. Accordingly, in our study, gene expression in the experiments performed with Chem-LLC was normalized against that of the control IRES-LLC experiments. The primers for TNF-α, IL-1β, IL-12 p40, IFN-γ genes were published previously [21]. The primer sequences designed for mouse β-actin, CMKLR1, recombinant prochemerin, and EGFP amplification are listed in Table 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants of Chem-LLC or IRES-LLC (3.5x106 cells/mL) cultures were collected and used in mouse chemerin ELISAs (R&D Systems, Minneapolis, MN, USA). Supernatants derived from ex vivo experiments were used for mouse IFN-γ (Becton Dickinson) quantitation. The assays were performed according to manufacturer's instructions.

Immunohistochemistry (IHC)

Primary antibodies against CMKLR1 (clone C-14; dilution 1/25) and major histocompatibility complex (MHC) class II (clone ER-TR3; dilution 1/100) were used for immunohistochemical staining of tumor sections. Appropriate isotype control antibodies were used for control staining. Binding of the primary antibodies was detected with polyvalent secondary antibodies using a biotin/streptavidin/horseradish peroxidase detection system (Goat ImmunoCruzTM Staining System and Rat ABC Staining System) according to the manufacturer's protocol. Antibody binding was visualized with diaminobenzidine (DAB) substrate. The antibodies and reagents were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Statistical Analysis

The data presented in this manuscript were produced from three or more independent experiments performed with at least two different genetically-modified LLC series.

All values are expressed by arithmetic mean ± standard deviation (SD). Statistical difference between experimental groups was determined using Student's paired or unpaired t-test where appropriate. Differences were regarded as statistically significant when $p \leq 0.05$.

RESULTS

CMKLR1 is expressed in LLC tumor microenvironment

The presence of CMKLR1 in the LLC tumors was determined by immunohistochemistry and gene expression analyses (Figure 1A and B). LLC cells were not positive for this marker (Figure 1B). In the immunohistochemical analyses, positively stained cells were observed with histiocytic morphology. In

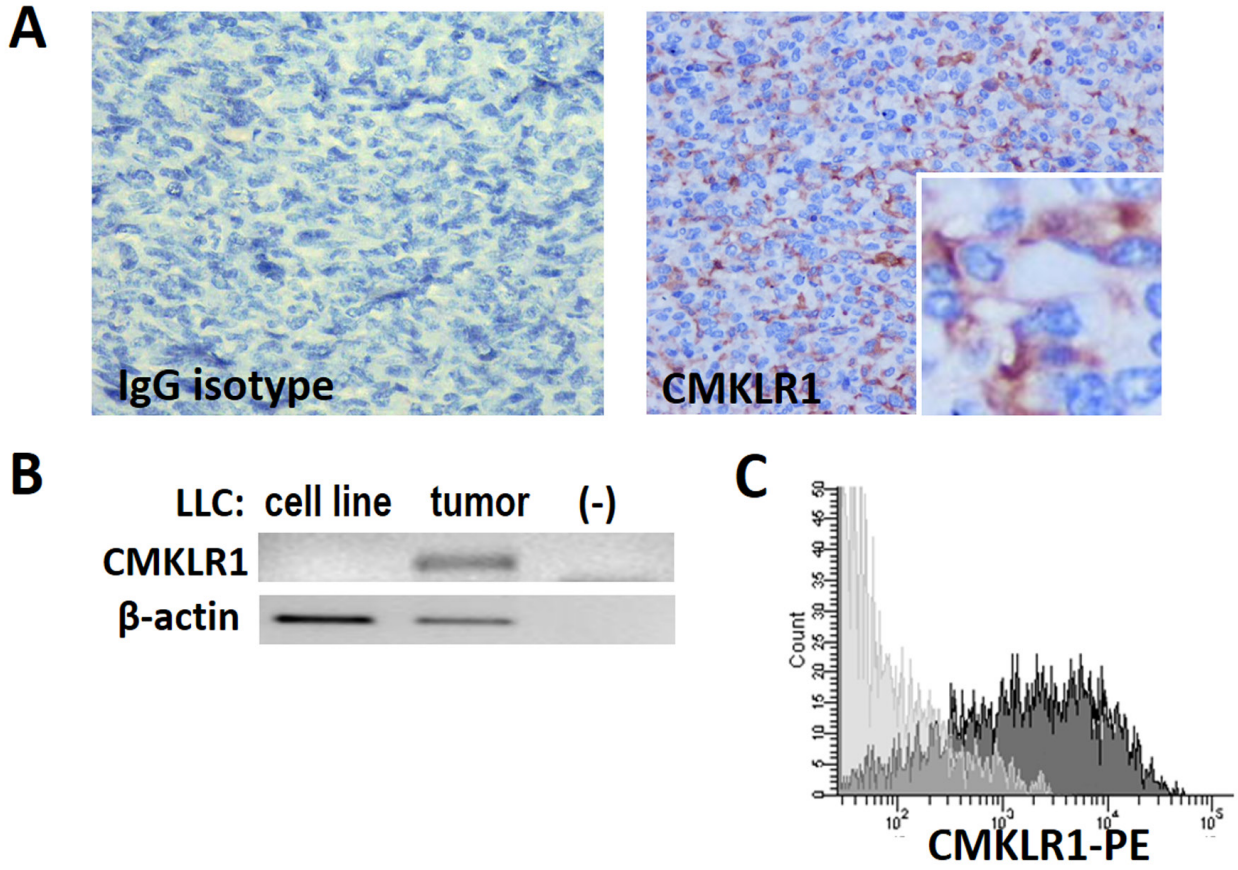

Figure 1 Presence of CMKLR1 in the tumor.

(A) The presence of CMKLR1 in the wild-type LLC tumors was confirmed by IHC. Staining with isotype IgG (left panel, x200) or anti-CMKLR1 antibody (right panel, x200) are shown. Higher magnification (x800) is displayed on the lower-right corner. (B) CMKLR1 gene expression in LLC cell line and wild-type LLC tumors were studied with RT-PCR. (-), PCR negative control. (C) Expression of CMKLR1 on macrophages was analyzed by flow cytometry. Grey histogram, isotype control staining; dark grey histogram, specific staining with anti-CMKLR1 antibody.

support of this, when the cell suspensions obtained from the tumors were examined, the majority of macrophages carrying specific marker F4/80 was determined to express CMKLR1 (n=4, 84.4±5.7%), (Figure 1C).

Chemotaxis and infiltration capacity of CMKLR1+ cells were not affected by low-level expression of recombinant prochemerin in LLC cells

Several experiments were performed with the genetically-modified LLC cells carrying the recombinant prochemerin gene (Chem-LLC cells) or the control vector (IRES-LLC cells). De novo expression of chemerin was detected only in Chem-LLCs whereas reporter EGFP expression was also present in IRES-LLC control cells (Figure 2A and B).

Chemerin can affect inflammatory responses at low concentrations (and higher levels are required for the induction of chemotaxis) [22,8]. As our aim was to determine the inflammatory effects of chemerin (without interference by leukocyte recruitment into the tumor mass), we preferred to use Chem-LLCs expressing low levels of chemerin. The amount of chemerin secreted into culture supernatants was 71.3±5.4 ng/mL (Figure 2C). Besides, Chem-LLCs and IRES-LLCs that were employed in the further stages of the study, were identified with similar proportion of proliferating cells (57.2±2.2% vs. 50.8±1.2%; n=3, P>0.05) and clonogenic properties (39±5 vs. 35±2 colonies; n=3, P>0.05). In vivo stability of the genetic

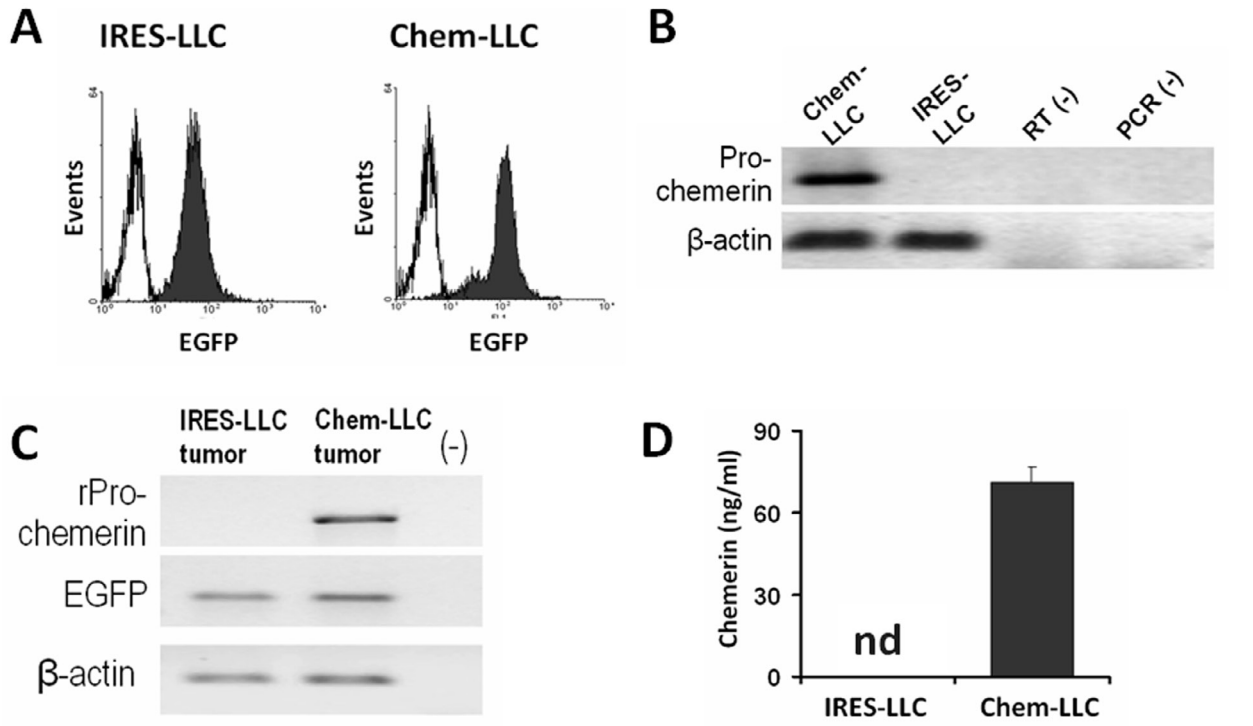

Figure 2 De novo expression of prochemerin in LLC cells.

(A) Representative flow cytometry histograms of EGFP reporter expression in the LLC cells genetically-modified with the recombinant prochemerin plasmid or with the control vector. The expression of prochemerin was also confirmed (B) in vitro and (C) in vivo, respectively by RT-PCR, and (D) by ELISA. RT (-), RT-PCR-negative control; PCR (-) or (-), PCR negative control; nd, not detected.

modification in LLC cells was confirmed with EGFP reporter expression in the tumor tissues on fluorescence microscopy (data not shown) and by the presence of recombinant prochemerin mRNA (Figure 2D).

CMKLR1+ peritoneal macrophages were employed in the chemotaxis assays. The migration of macrophages into the Chem-LLC conditioned media and the conditioned media obtained from the control IRES-LLC cells were similar (Figure 3A). In addition, the tumors established with Chem-LLCs or IRES-LLCs showed similar amounts of CMKLR1+ or MHC class II+ cells (Figure 3B).

CMKLR1 is upregulated in the presence of LLC cells

Upon culturing with LLC cells, the expression of CMKLR1 was significantly increased both on freshly isolated plastic-adherent splenic monocytes/macrophages (control, 9.8±2.7%; co-cultured, 19.3±3%; n=3, P<0.01) or on J744A.1 macrophage cell line (control, 29.2±10.6%; co-cultured, 47.4±5.6%; n=3, P<0.05) (Figure 4A and B).

The effect of prochemerin-expressing LLCs on proinflammatory gene expression ex vivo and in vivo

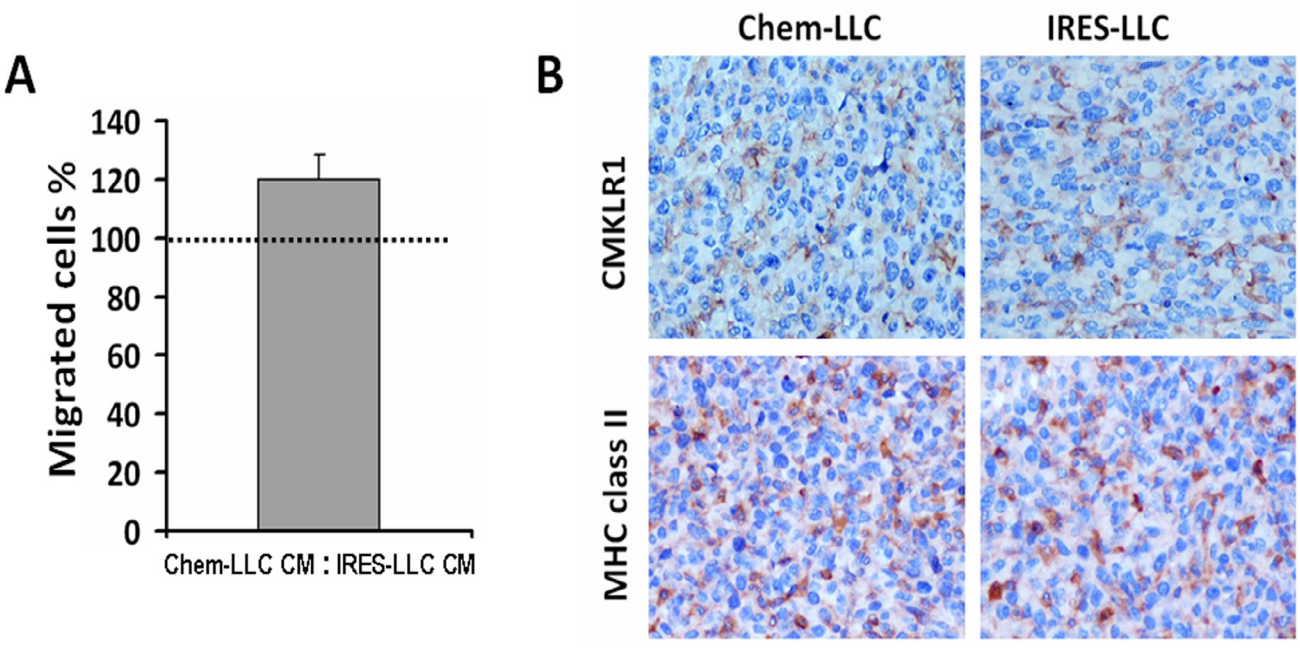

Figure 3 Migration of CMKLR1+ cells towards LLC-derived recombinant chemerin gradient.

(A) Percentage of migrated CMKLR1+ peritoneal macrophages into Chem-LLC conditioned media (CM) are shown in proportion to that of migrated into control IRES-LLC CM. (B) Infiltration of Chem-LLC or IRES-LLC tumors by CMKLR1 or MHC class II expressing cells. Micrographs (x200) of representative immunohistochemical stainings are shown.

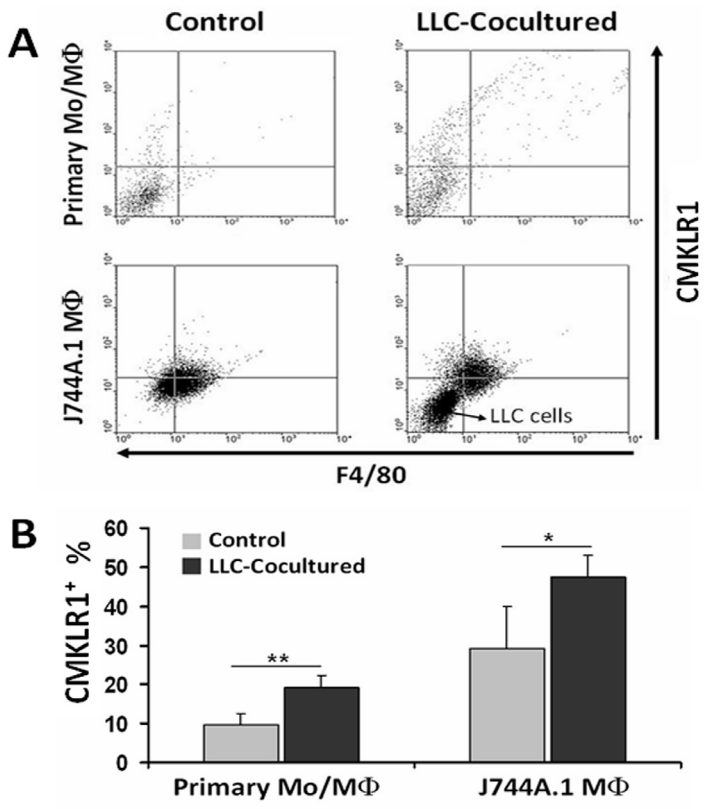

Figure 4 Effect of LLCs on CMKLR1 expression.

(A) The results of CMKLR1 flow cytometry analysis on plastic-adherent primary splenic monocytes/macrophages or on J744A.1 macrophage cell line cultured alone or co-cultured with LLC cells for 48h is shown. Prior to staining for flow cytometric analysis, the loosely adherent LLC cells could be mechanically separated from strongly adherent primary splenic monocytes/macrophages however this was not possible with the J744A.1 cells. (B) Mean percentage of CMKLR1+ cells in ex vivo and in vitro experiments. Because of similar adherence properties of LLC and J744A.1 cells, the percentage of CMKLR1+ J744A.1 cells was calculated after gating on F4/80+ cells (n=3, *P<0.05, **P<0.01). Mo, monocyte; MΦ, macrophage.

Ex vivo experiments were performed on mixed splenic leukocytes incubated in Chem-LLC or IRES-LLC culture supernatants with or without LPS for 3 hours. In comparison to the assays performed with the conditioned media from IRES-LLC, the conditioned media from Chem-LLC resulted in decreased levels of TNF-α and IL-12 p40 gene expression whereas IL-1β was slightly increased. In accordance with the previous report [8], only with the conditioned media from the Chem-LLC, decline in the expression of these proinflammatory

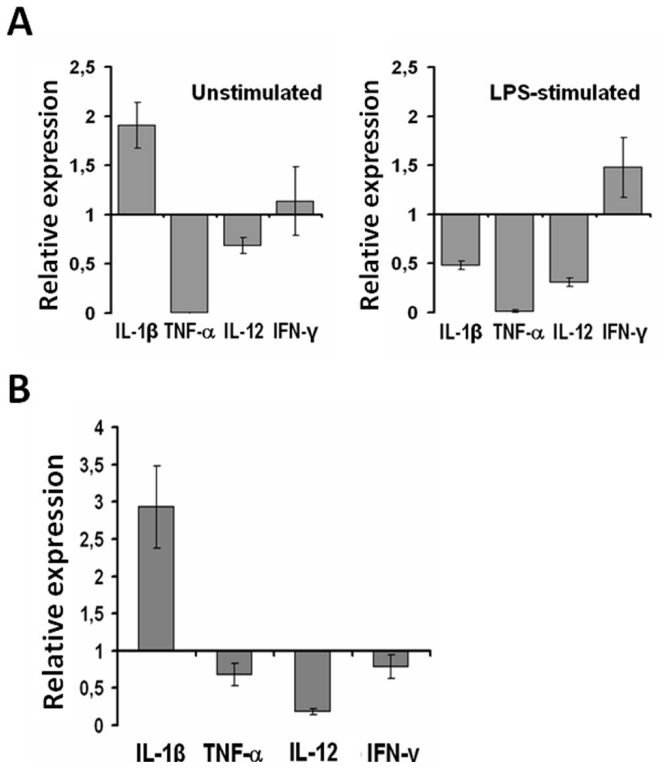

Figure 5 Modulation of inflammatory response by recombinant prochemerin-expressing LLC cells, ex vivo and in vivo.

(A) The expression of IL-1β, TNF-α, IL-12 p40, and IFN-γ genes were determined in freshly isolated splenocytes incubated with Chem-LLC or IRES-LLC supernatants without (left panel) or with LPS (right panel) stimulation for 3 hours (n=3). (B) Expression of the proinflammatory genes were also evaluated in the tumor tissues (n=3). The data obtained from the experiments with Chem-LLCs are shown comparative to control IRES-LLCs.

genes became more prominent upon LPS stimulation. On the other hand, IFN-γ showed a minor increase with the Chem-LLC supernatants, moreover stimulation with LPS enhanced its expression to some extent (Figure 5A).

In vivo, the expression of proinflammatory genes IL-1β, TNF-α, IL-12 p40 and IFN-γ was assessed in the Chem-LLC tumor tissues in comparison with the control IRES-LLC tumors (Figure 5B). Except IL-1β which was increased, the expression of other cytokines was diminished as observed in the ex vivo experiments on unstimulated leukocytes (Figure 5A and B). IL-12 p40 was the most suppressed cytokine while a very slight decrease in IFN-γ expression was observed.

Tumor forming capacity of prochemerin-expressing LLC cells was reduced

Growth of Chem-LLCs or the IRES-LLCs was recorded after subcutaneous grafting to syngenic C57BL/6 mice. In the control IRES-LLC group, all mice were bearing tumors by the fourth week of follow-up. The incidence of tumors was decreased in the mice grafted with prochemerin expressing cells (Figure 6A). Interestingly, the size of the Chem-LLC tumors that were able to develop did not significantly differ from that of the IRES-LLC tumors (Figure 6B).

To evaluate the impact of anti-tumor immune responses on the initiation of tumor formation, immune proliferation (for 96 hours PHA-stimulation period) and IFN-γ secretion (for 24 hours PHA-stimulation period) were assessed (Figure 6C and D). In the presence of immune stimulator, PHA, IFN-γ

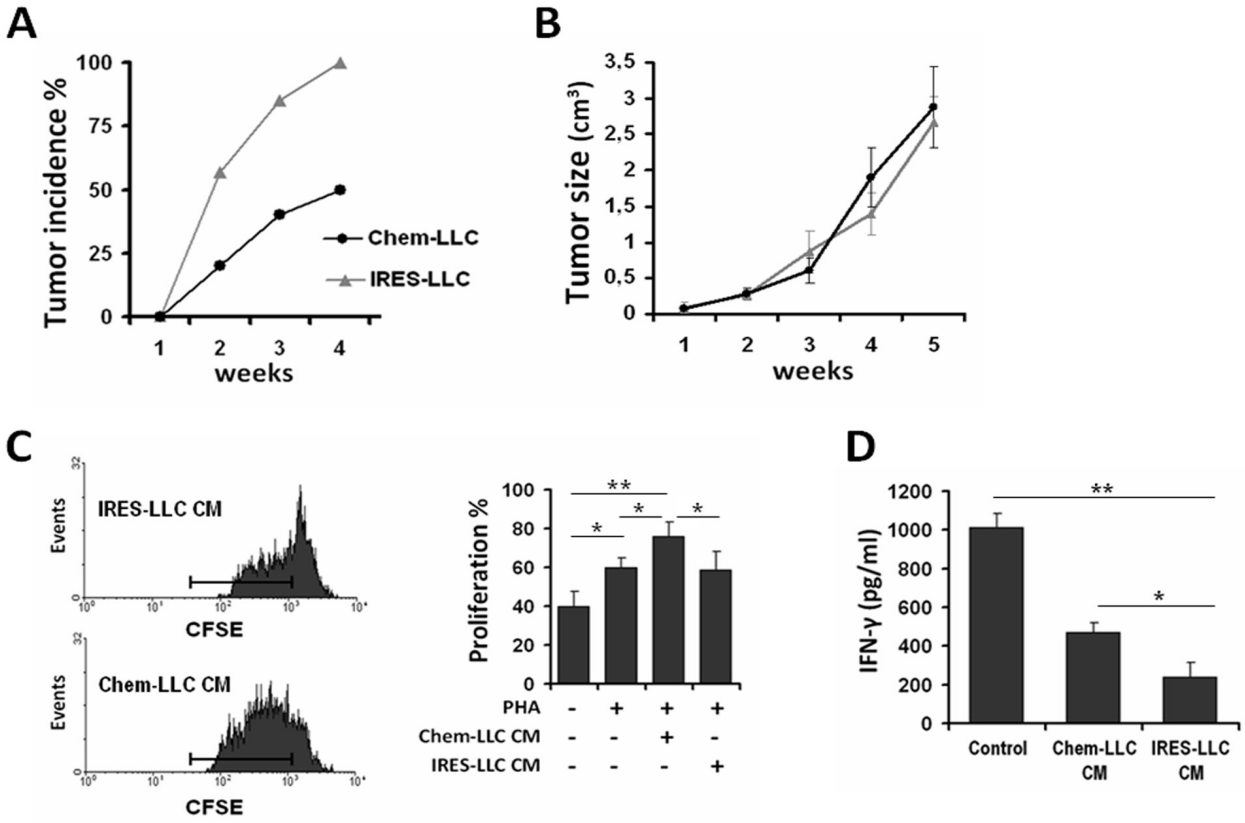

Figure 6 The tumor formation capacity of prochemerin-expressing LLC cells.

(A) The incidence of tumor formation in mice subcutaneously inoculated with IRES-LLC (n=7) or Chem-LLC (n=10), and (B) the size of the tumors calculated from two independent experiments performed with two different genetically-modified LLC series are shown. (C) Leukocyte proliferation in the presence of Chem-LLC or IRES-LLC conditioned media was assessed by CFSE assay. Upper- and lower-left histograms represent proliferation of PHA-stimulated cells incubated with IRES-LLC or Chem-LLC supernatants, respectively. (D) Upon 24 hours of PHA-stimulation, IFN-γ secretion by splenocytes incubated with Chem-LLC or IRES-LLC conditioned media (CM) or standard RPMI media (control) were tested by ELISA. (n=3, *p<0.05, **p<0.01).

was significantly higher with the Chem-LLC conditioned media compared to in that of with the IRES-LLC (Figure 6D). Besides, proliferation capacity of the leukocytes was also enhanced with the Chem-LLC supernatants (Figure 6C).

DISCUSSION

For a pro-growth environment, tumor cells modulate the intercellular signaling networks, i.e. chemokines, cytokines and growth factors, and as a result, immune responses divert to promote the cancer development [23]. Immune-associated receptors, such as CMKLR1, having both anti- and pro-inflammatory functions are candidates to be employed in this process. Here, we report the expression of CMKLR1 in the tumor microenvironment, where it may be capable of regulating the inflammation and tumorigenicity of prochemerin-expressing LLC cells.

Tumor-associated macrophages (TAMs) are good examples for the M2 subgroup of macrophages which contribute to survival, growth and metastasis of neoplastic cells [24]. Our results demonstrated that in the LLC tumors, majority of the TAMs are CMKLR1+ which may be upregulated as a result of exposure to the tumor-cell-derived factors. Accordingly, transforming growth factor-β (TGF-β) which is an abundant factor found in the tumor microenvironment, and secreted by LLC cells (unpublished data), has been previously reported to increase the macrophage CMKLR1 expression [25].

Prochemerin is expressed by a wide variety of cells, especially mouse epithelial cells including the lung epithelium [14]. However, the expression of prochemerin was not detected in several murine cancer cell lines (unpublished data), and here, in wild-type LLC cells. In humans, chemerin is downregulated in squamous cancers of the skin and in adrenocortical carcinoma lesions [17,18]. Activation

of CMKLR1 on LPS-activated macrophages reduces the expression of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, RANTES and IL-12 but increases IL-10 and TGF-β [10]. Accordingly, in our experimental setting, the expression of recombinant prochemerin decreased the proinflammatory signals ex vivo and in LLC tumors, in vivo. Thus, our results may indicate that; following the upregulation of CMKLR1 in tumor microenvironment (e.g. on TAMs), the interaction with chemerin might constitute a regulatory mechanism to control inflammation.

Initiation of tumor formation and metastasis (especially, of the LLC cells) develop in relation with inflammatory stimuli [24,26]. In our study, the inflammatory microenvironment may have become altered to not to favor tumor formation since de novo expression of prochemerin in LLCs decreased the critical inflammatory mediators, such as TNF-α, IL-12. Alternatively, the decline in tumor formation may be a result of enhanced anti-tumor immune responses, because the leukocyte proliferation and the IFN-γ secretion were higher with the prochemerin-expressing LLC cells, as simulated in the ex vivo experiments.

In the prochemerin-expressing tumors that once were able to establish, the expression of inflammatory cytokines was similar to the pattern that was evident from the splenocytes incubated with the Chem-LLC conditioned media without LPS stimulation. Only after LPS-stimulation ex vivo, IL-1β gene expression was reduced. Upon administration of chemerin-derived peptides, decreased levels of IL-1β was previously reported in LPS-activated macrophages and in bronchoalveolar lavage fluids of LPS-challenged mice [8,14]. Intriguingly, factors secreted from LLCs, e.g. versicane, can also affect the regulation of IL-1β expression. Versicane is a toll-like receptor (TLR) 2 ligand which can increase IL-1β production in macrophages [26]. Here, the data on the regulation of IL-1β gene expression may be influenced both by chemerin and by LLC-derived factors.

In conclusion, CMKLR1 was identified in the tumor microenvironment and the CMKLR1-chemerin pathway may possess the capacity to modulate the inflammatory balance and hamper the tumor formation.

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