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Chemokine-Like Receptor 1 Expression and Chemerin-Directed Chemotaxis Distinguish Plasmacytoid from Myeloid Dendritic Cells in Human Blood[1]

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Plasmacytoid dendritic cells (pDCs) are versatile cells of the immune response, secreting type I IFNs and differentiating into potent immunogenic or tolerogenic APCs. pDCs can express adhesion and chemokine receptors for lymphoid tissues, but are also recruited by unknown mechanisms during tissue inflammation. We use a novel mAb specific for serpentine chemokine-like receptor 1 (CMKLR1) to evaluate its expression by circulating leukocytes in humans. We show that CMKLR1 is expressed by circulating pDCs in human blood, whereas myeloid DCs (mDCs) as well as lymphocytes, monocytes, neutrophils, and eosinophils are negative. We identify a major serum agonist activity for CMKLR1 as chemerin, a proteolytically activated attractant and the sole known ligand for CMKLR1, and we show that chemerin is activated during blood coagulation and attracts pDC but not mDC in ex vivo chemotaxis assays. We conclude that CMKLR1 expression and chemerin-mediated chemotaxis distinguish circulating pDCs from mDCs, providing a potential mechanism for their differential contribution to or regulation of immune responses at sites of bleeding or inflammatory protease activity. *The Journal of Immunology,* **2005, 174: 244–251.**

Over 40 years separate the morphological description of plasmacytoid dendritic cells (pDCs)[3] (1) and their subsequent identification as the key producers of type I IFNs (2–4), cytokines that can directly block viral replication and stimulate the adaptive immune response. Following activation by virus, unmethylated bacterial DNA (mimicked by oligonucleotide CpG) or CD40L+IL-3, pDCs mature into potent APCs, as defined by their ability to stimulate naive allogeneic $CD4^+$ T cell proliferation (2, 5, 6). When stimulated by CD40L without additional stimuli, pDCs can support the generation of immunosuppressive, regulatory CD8 T cells (7). pDCs have been characterized primarily in peripheral blood and are identified as $Lin^-HLA\text{-}DR^+CD123^+CD11c^-$ (8); they are also positive for two recently identified pDC-selective Ags, blood DC Ag-2 (BDCA2, a C-type lectin) and BDCA4 (neuropilin-1) (9). Blood pDC are relatively rare blood leukocytes, comprising <0.4% of total PBMC and declining in abundance by ~1% per year in adults (10). In the steady state, pDCs have also been reported in the thymus (11) and peritoneal lavage fluid (12).

Current evidence suggests that pDCs express receptors that can explain their migration from blood into lymphoid tissues, including L-selectin, CXCR4, CCR7, and α_4 integrins (13). In addition, they can chemotax to homeostatic chemokines, either CXC chemokine ligand (CXCL)12 when they are immature, or CCR7 ligands following in vitro maturation (14, 15). pDCs are found in lymphoid tissues, but they are also present in diverse tissue sites, often associated with inflammation and lymphocytic infiltrates, including reactive tonsils (1), inflamed nasal mucosa (16), a variety of cutaneous lesions (herpes zoster (17), skin blisters simulating syphilic infection (triggered by lipopeptide analogues of *Treponema pallidum*) (18), psoriasis vulgaris, lupus erythematosus, contact dermatitis, but not atopic dermatitis (19), and melanoma (20)), ovarian epithelial tumor (21), cerebral spinal fluid from multiple sclerosis patients or patients with Lyme neuroborreliosis (22), and synovial fluid from patients with spondyloarthropathy (23) or rheumatoid arthritis (24). The mechanisms by which pDCs traffic from the blood to extralymphoid tissue sites are not well understood. Unlike myeloid DCs (mDCs), circulating pDCs do not respond to inflammatory chemokines (14, 15). Therefore, we reasoned that additional chemoattractants might be involved, and we set out to identify novel receptors that might distinguish pDCs from mDCs in human blood.

Our studies focus on the candidate chemokine-like receptor 1, CMKLR1 (also referred to as ChemR23 or DEZ), a recently deorphaned chemoattractant receptor. CMKLR1 has been shown to be expressed by DC generated in vitro from monocytes (25), and to mediate their migration to the proteolytically regulated chemoattractant chemerin (25, 26), but its expression and function on physiologic leukocyte subsets has not been studied. We confirm that CMKLR1 is expressed by circulating DC in human blood, but we also show surprisingly that it is selectively expressed by immature pDCs, distinguishing them from mDC. We show that serum contains an active attractant for CMKLR1 identical with chemerin, and that this activity is triggered during blood coagulation, suggesting a mechanism for attraction of pDCs to sites of

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[3] Abbreviations used in this paper: pDC, plasmacytoid; DC, dendritic cell; mDC, myeloid DC; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; KLH, keyhole limpet hemocyanin; CMKLR1, chemokine-like receptor 1; TIG2, tazarotene-induced gene 2.

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tissue damage and bleeding. We propose that expression of CMKLR1 and migration to locally activated chemerin may allow the rapid and differential recruitment of pDCs in vivo.

Materials and Methods

Abs and Reagents

Anti-CD3, -CD11c, -CD14, -CD16, -CD19, -CD20, -CD56, -CD83, -CD123, -HLA-DR dye-linked mAbs, purified HLA-DR, BDCA2, BDCA4, and secondary anti-mouse allophycocyanin and anti-rat PE for immunofluorescence studies were obtained from BD Pharmingen, Miltenyi Biotec, eBioScience, Jackson ImmunoResearch Laboratories, and Caltag Laboratories. CXCL12, CC chemokine ligand (CCL)2, CCL19, CCL21, IL-4, GM-CSF were purchased from R&D Systems, LPS was purchased from Sigma-Aldrich, CMFDA (5-chloromethylfluorescein diacetate) was purchased from Molecular Probes, and phosphothioated CpG oligonucleotides (27) were purchased from Operon Technologies.

Mammalian expression vector construction and generation of stable cell lines

The coding region of human CMKLR1 was amplified from genomic DNA with an engineered N-terminal hemagglutinin tag, and cloned into pcDNA3 (Invitrogen Life Technologies). The full-length tazarotene-induced gene (TIG)2 cDNA encoding chemerin was amplified from human liver RNA (BD Clontech) and engineered to have an N-terminal 6x His tag after the signal sequence and cloned into pcDNA3. Transfectants of human CMKLR1, chemerin or empty vector were generated and stable lines selected in the murine pre-B lymphoma cell line L1.2 essentially as described (28). Transfected cells were in some cases treated with 5 mM *n*-butyric acid for 24 h before experimentation (29).

Chromatography and LC/MS/MS

A total of 1.6 L of human serum (Serologicals) was filtered and used as starting material. Heparin-Sepharose (Amersham Biosciences) and surfactant protein-Sepharose cation exchange (Amersham Biosciences) chromatography were performed using 50- and 2-ml columns and a low-pressure peristaltic pump (Masterflex; Cole-Parmer Instrument). Single bed volumes of 0.1 M stepwise increments of NaCl buffer (in 50 mM MES, pH 6.3) were used to elute proteins off the column. Columns were washed twice with 0.1 M NaCl buffer before salt increments were started. Gel filtration FPLC (Superdex75; Amersham Biosciences) was performed, and 250-μl fractions were collected. Following all chromatography steps, protein concentration was determined in each fraction by bicinchoninic acid (Pierce), protein eluants were assayed in Transwell migration with CMKLR1/L1.2 transfectants, and active fractions were pooled, diluted as appropriate, and applied to subsequent separation columns. The purified protein was separated by SDS-PAGE, and the bands were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS; Protein Chemistry Core Facility, Columbia University, New York, NY). The tryptic mass values were used in a Mascot search (⟨www.matrixscience.com/cgi/index.pl?page=/search_intro.html⟩) of public peptide databases. Acrylamide adducts (cysteine modifications) were taken into consideration when mass values were searched.

Bacterial production of recombinant chemerin

The coding region of the predicted secreted form of chemerin was amplified by RT-PCR from human liver RNA (BD Clontech) and directionally cloned into the *Eco*RI/*Hin*dIII sites of pET42a (Novagen), in-frame with upstream GST and 6x His tags. Following isopropyl β-D-thiogalactoside induction in *Escherichia coli* strain BL21, inclusion bodies were harvested, and the fusion protein was solubilized in 6 M guanidine HCl and refolded by dropwise dilution with refolding buffer (0.1 M Tris-HCl, pH 8.0, 1 mM oxidized glutathione, and 0.1 mM reduced glutathione) to final protein concentrations of 10–100 μg/ml.

RNA expression analysis

Dot blot RNA arrays were purchased from BD Clontech and hybridizations were performed according to the manufacturer's recommendation. A full-length gel-purified chemerin cDNA probe were radiolabeled with ^{32}P using RediPrime reagents (Amersham Biosciences) according to manufacturer's specifications. RT-PCR expression analysis of chemerin was performed using 500 ng total RNA (BD Clontech) as cDNA synthesis template. Full-length chemerin was amplified using intron-spanning primers, and G3PDH primers that spanned intron H were used. "No RT" controls were negative for chemerin amplicons (data not shown).

Harvesting PBMC and generating cultured monocyte-derived DC

The Institutional Review Board at Stanford University (Stanford, CA) approved all human subject protocols, and informed consent was obtained for all donations. Plasma was collected from blood samples drawn into tubes containing heparin, EDTA, or sodium citrate (BD Vacutainer). Human blood was collected and PBMC were harvested following Histopaque 1077 gradient separation. Miltenyi MACs magnetic bead CD14$^+$ separation was performed according to the manufacturer's specifications. CD14$^+$ monocytes were cultured in RPMI 1640 plus 10% FCS with additives at 2–10 million cells/ml with 100 ng/ml GM-CSF and 100 ng/ml IL-4 for 7 days to generate immature DC. In some cases, the DCs were cultured an additional 24 h with 10 ng/ml LPS to generate mature (activated) DC.

Cell sorting and Wright-Giemsa stain

Human blood leukocytes were stained as described and sorted by standard flow cytometric techniques (FACSVantage; Stanford University Digestive Disease Center Core Facility). Between 1 and 10 $\times$ 10^3 sorted cells were loaded into cytospin chambers and centrifuged onto glass slides. The slides were stained with Wright-Giemsa dye by standard automated techniques at the Veterans Affairs Hospital, Hematology Section (Palo Alto, CA) and examined by light microscopy with a $\times$40 objective.

In vitro Transwell or transendothelial chemotaxis

The 5-μm pore Transwell inserts (Costar) were used. Bare (uncoated) Transwell inserts were used for all chemotaxis experiments, except for the transendothelial migration experiment (see Fig. 6). For transendothelial migration, Transwell inserts were coated with gelatin, seeded with 10^5 HUVECs (passage $<$8), and incubated overnight, as previously described (15). Monolayers were rinsed with chemotaxis medium before use. Chemotaxis medium consisted of RPMI 1640 plus 10% FCS with additives, and 100 μl of cells were added to the top well, and test samples were added to the bottom well in a 600-μl volume. Migration was assayed for 2–5 h at 37°C, then the inserts were removed, and the cells that had migrated through the filter to the lower chamber were in some cases stained and counted by flow cytometry. An equivalent number of beads were added to each tube to allow the cell count to be normalized. A ratio was generated and percentage input migration is displayed. For in vitro cultured DC migration, 1–6 $\times$ 10^5 cells were added to the top well and migrated cells were counted using a DC cell gate based on forward and side light scatter. For human primary blood cell migration, cells were preincubated 1–3 h in medium to allow for recovery of receptor expression. A total of 1 $\times$ 10^6 PBMC were added to the top well, and migrated cells were stained (Lin FITC, HLA-DR PE, CD123 CyChrome, CD11c allophycocyanin) and analyzed by four-color flow cytometry. For some donors, incubation times resulting in optimal recovery of chemerin functional response was determined empirically and used. For transfectant migration, ~2.5 $\times$ 10^5 cells/well were used, and the number of cells counted in 30 s was used as the migration output. The results are reported as a percentage of input migration. Either a predetermined volume of chemerin conditioned medium eliciting $>$30% CMKLR1/L1.2 transfectant migration (along with an equivalent volume of empty vector (pcDNA3) L1.2 transfectant conditioned medium as a negative control), or refolded recombinant *E. coli*-expressed chemerin was used. Student's *t* test (two-tailed with unequal variance) was used to determine statistical significance.

Anti-CMKLR1 mAb

The immunizing N-terminal CMKLR1 peptide was synthesized by Stanford Protein and Nucleic Acid (Stanford, CA) facility and conjugated to keyhole limpet hemocyanin (KLH) according to the manufacturer's specifications (Pierce). CFA and IFA were purchased from Sigma-Aldrich. Wistar-Furth rats were purchased from Charles River Breeding Laboratories. An ELISA-based assay (BD Pharmingen) was used to determine the isotype of our rat anti-human CMKLR1 mAb.

Results

A CMKLR1-specific mAb stains culture-derived DC

We generated a mAb designated BZ332 (IgG2aκ) to human CMKLR1 after immunizing rats with a KLH conjugate of an N-terminal CMKLR1 peptide comprised of residues 8–32 and having the sequence NH_2-TSISYGDEYPDYLDSIVVLEDLSPLC-COOH (a non-native C-terminal cysteine was added to facilitate conjugation to KLH). Hybridomas producing anti-human CMKLR1 mAbs were subcloned, and specificity was confirmed by reactivity with human but not mouse CMKLR1 transfectants, and by lack of reactivity with

L1.2 cells expressing human CCR9 and CCR10. Mouse CMKLR1 shares 80% amino acid identity and is more homologous to human CMKLR1 than any human protein, and thus represents the most probable candidate for mAb cross-reactivity, which was not observed. Reactivity with CXCR1 through CXCR6 and CCR1 through CCR10 was excluded by lack of staining of blood cell subsets or cultured human cells known to express these receptors.

We used mAb BZ332 to assess expression of CMKLR1 by DCs. Consistent with results by Wittamer et al. (25) we found that in vitro-cultured, monocyte-derived immature DCs generated with IL-4 and GM-CSF expressed CMKLR1, whereas precursor monocytes or LPS-matured DCs did not express the receptor (Fig. 1). The down-regulation of CMKLR1 observed upon LPS maturation, however, was more extensive than previously reported (25). Monocyte-derived DC have been considered to be a model of mDC observed in vivo, suggesting involvement of the receptor in mDC function. Because culture models of specialized leukocytes may fail to recapitulate the phenotypic characteristics of physiologic cell subsets in vivo (30), we asked whether CMKLR1 is expressed by circulating mDC, where it might influence their recruitment from the blood.

Expression of CMKLR1 by circulating pDC but not mDC

Blood DCs make up ~1% of circulating PBMC, and are characterized as Lin^-MHC class II^+ ($CD3^-CD14^-CD16^-CD19^-CD20^-CD56^-HLA\text{-}DR^+$) (31). Immunofluorescence staining of total PBMC revealed CMKLR1 expression limited to a small subset of lineage marker negative cells, consistent with expression by a subset of circulating DC (Fig. 2*A*). Lin^+ cells were negative for mAb reactivity, suggesting that the receptor is not expressed detectably by circulating lymphocyte subsets (Fig. 2*A*). As shown in Fig. 2*B*, this was confirmed by gating on $CD3^+$ T cells, $CD19^+$ B cells, $CD16^+$ NK cells. In addition, circulating monocytes among the Histopaque-isolated PBMC were also negative (Fig. 1*A*). Peripheral blood DCs can be further subdivided by expression of CD123: mDCs are $CD123^-$, whereas pDCs are $CD123^+$ (4, 5, 8). Surprisingly, CMKLR1 was not expressed by $Lin^-HLA\text{-}DR^+CD123^-$ mDC, whereas $CD123^+$ pDC were uniformly positive (Fig. 2*C*). Blood $CD123^+CD11c^-CMKLR1^+$ cells coexpress the pDC-specific markers BDCA2 and BDCA4 (9) as well, confirming the staining of pDCs in blood (Fig. 2*D*).

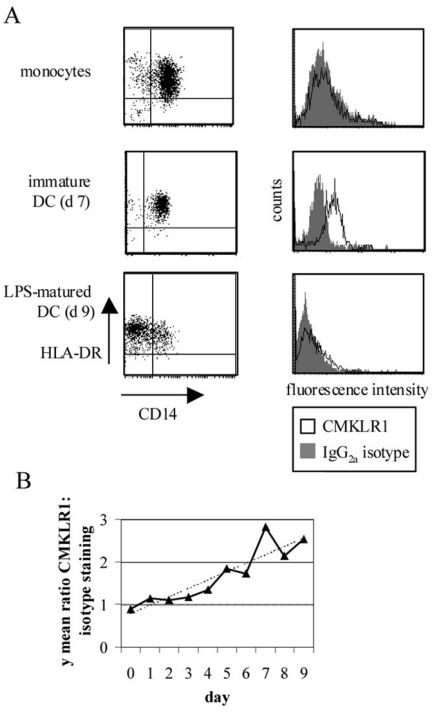


FIGURE 1. CMKLR1 is expressed on immature monocyte-derived DCs. *A*, Monocytes were cultured for 7 days with GM-CSF and IL-4 (100 ng/ml each). In vitro cultured DCs down-regulate CD14 upon maturation, while maintaining MHC class II expression throughout differentiation. Immature day 7 DCs express CMKLR1, while day 0 monocytes and day 9 LPS-matured DCs are negative by staining. *B*, CMKLR1 receptor expression increases over time as monocytes differentiated into immature DCs (no LPS added).

To confirm our immunophenotyping results, we sorted $CMKLR1^+Lin^-$ blood mononuclear cells for Wright-Giemsa staining to examine the morphology of the cells. We also sorted and stained blood pDCs ($Lin^-HLA\text{-}DR^+CD123^+CD11c^-$) and mDCs ($Lin^-HLA\text{-}DR^+CD123^-CD11c^+$) for comparison. As predicted, sorted $CMKLR1^+$ cells and pDC shared similar morphology, consistent with descriptions of pDCs appearance in the literature (Fig. 2*E*). $CMKLR1^+$ cells and pDCs were round, smooth cells with generally circular nuclei and pale perinuclear regions. Sorted mDCs display a clearly different morphology, with multiple cytoplasmic projections and lobulated, protruding nuclei. Thus, both traditional morphologic and immunophenotypic analysis indicates selective expression of CMKLR1 on pDC vs mDC.

DCs alter their chemoattractant receptor expression profiles upon stimulation with Toll receptor or costimulatory receptor ligands. pDCs activated by overnight incubation with CpG oligonucleotides (or $CD40L^+$IL-3, data not shown) down-regulated CMKLR1 receptor expression (Fig. 2*F*).

The selective expression of CMKLR1 by immature pDCs in blood was surprising in light of the observed expression of CMKLR1 on monocyte-derived DCs in vitro. However, the culture-derived cells may present an atypical or specialized DC phenotype, sharing some features of both mDC (e.g., CD11c) and pDC differentiation. (In fact, these monocyte-derived DCs also express another Ag that is specific for pDCs vs mDCs in blood, BDCA4 (32)). It is known that DCs of different phenotypes can be generated based on specific combinations of cytokines present during their in vitro derivation, and it will be of interest in future studies to assess the factors responsible for regulation of CMKLR1, as well as those regulating other markers of physiologic pDC differentiation.

Identification of a potent serum CMKLR1-dependent chemoattractant as chemerin

We used CMKLR1 transfected L1.2 cell chemotaxis to detect and isolate a natural ligand for the receptor from human serum. Briefly, 1.6 L of human serum (128 g of total protein) was filtered and applied to a heparin-Sepharose column, and fractions were eluted using stepwise increments of NaCl buffer (Fig. 3*A*). The 0.7 M NaCl fraction contained the bulk of activity, and was highly enriched in chemoattractant protein, as >99.9% of serum proteins were eliminated. The separation and protein identification proceeded via cation exchange and gel filtration column chromatography, SDS-PAGE, and liquid chromatography/tandem mass spectrometry (LC/MS/MS) (data not shown). Consistent with the recent reports describing a CMKLR1 ligand from human ascites (25) or hemofiltrate (33), four mass values from a tryptic digest of the protein confirmed the identity of the active chemotactic agent in serum as the protein product of the TIG2, or chemerin (Fig. 3*B*) (25). Conditioned medium from chemerin transfected cells acted as a chemoattractant for CMKLR1 transfectants (Fig. 3*C*), and for immature monocyte-derived DCs (Fig. 3*D*) (25).

Chemerin RNA is expressed at readily detectable levels in numerous tissues and organs (Fig. 4). For example, chemerin message is found in the lymph nodes, where it may contribute to pDC

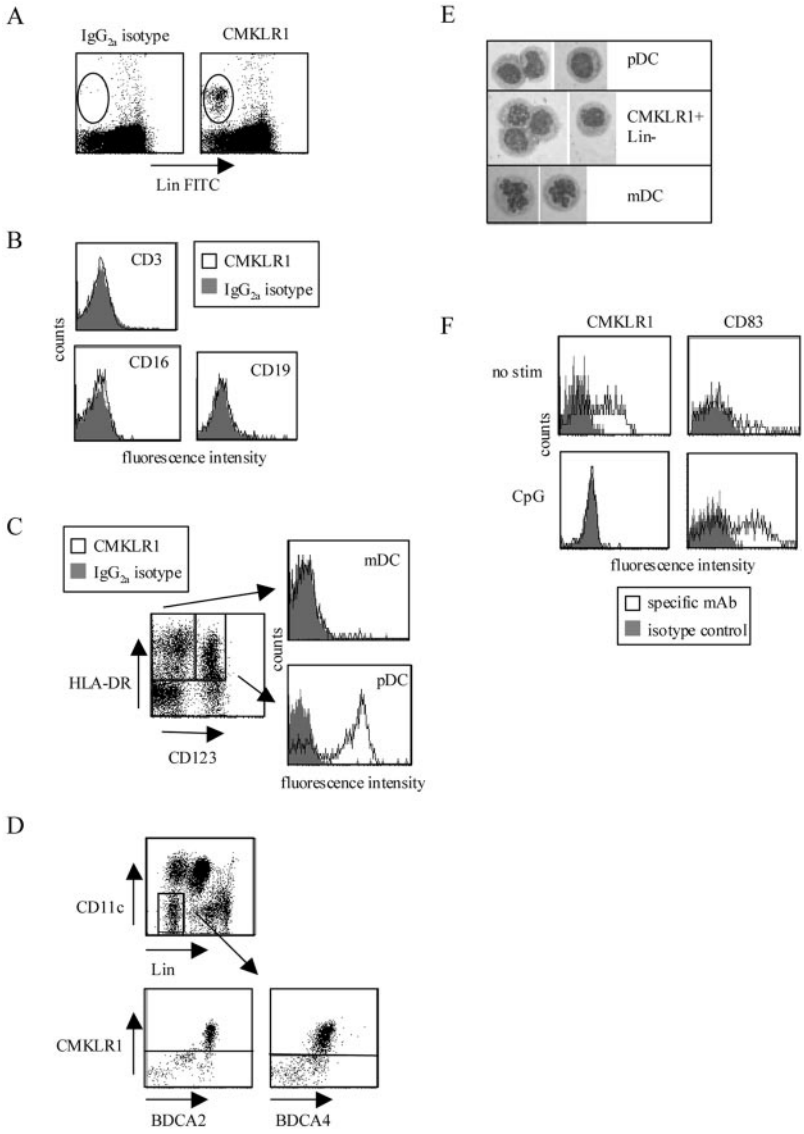


FIGURE 2. CMKLR1 is selectively expressed on blood pDCs and down-regulated upon activation. *A*, A small subset of freshly isolated Lin^- (negative for CD3, CD14, CD16, CD19, CD20, CD56) PBMC express CMKLR1 (circled population is ~0.5% total PBMC). *B*, Circulating $CD3^+$ T cells, $CD16^+$ NK cells, and $CD19^+$ B cells are negative for CMKLR1 expression. *C*, Blood PBMC were stained with Lin FITC, rat anti-CMKLR1 (2° anti-rat PE), CD123 CyChrome, mouse anti-HLA-DR (2° anti-mouse allophycocyanin). $Lin^-HLA\text{-}DR^+$ blood dendritic cells were subdivided by CD123 expression. $Lin^-HLA\text{-}DR^+CD123^+$ pDCs stained for CMKLR1, whereas $Lin^-HLA\text{-}DR^+CD123^-$ mDCs were negative for the receptor ($n = 13$ different blood donors). *D*, Blood PBMC were stained with Lin FITC, CD11c allophycocyanin, BDCA2 or BDCA4 (2° anti-mouse CyChrome). $Lin^-CD11c^-BDCA2^+$ and $Lin^-CD11c^-BDCA4^+$ pDC were $CMKLR1^+$ (horizontal line indicates isotype control staining levels, $n = 3$). *E*, $CMKLR1^+Lin^-$ cells, pDCs, and mDCs ($Lin^-HLA\text{-}DR^+CD123^+CD11c^-$ and $CD123^-CD11c^+$, respectively), were sorted, harvested by cytospin, and stained by Wright-Giemsa. Cells were examined by light microscope using a ×40 objective. *F*, PBMC were cultured overnight with either no stimulation or CpG oligonucleotides, and the pDCs were examined for CMKLR1 expression (in separate experiments, CD83 up-regulation was used to demonstrate pDC activation by CpG, $n = 3$).

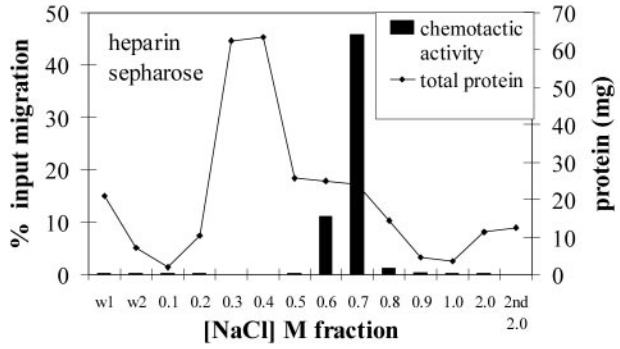
homing to secondary lymphoid tissues. Although most tissues, except components of the nervous system, seem to express significant levels, the most abundant sources of chemerin mRNA appear to be liver, pancreas, and adrenal gland. High-level expression by the liver may be responsible for the high levels of chemerin in the serum. Chemerin message is also abundant in the skin (Fig. 4*C*).

In our experiments leading to the identification of chemerin as a dominant serum chemoattractant for CMKLR1, we observed that, in contrast to serum, human plasma displayed very little attractant activity. We hypothesized that factors activated upon blood clotting were responsible for the increased chemerin activity, and because coagulation and fibrinolysis are enzymatic and thus time dependent processes, we compared chemerin activity in either serum with plasma from normal or anticoagulated blood from the same donor over time. We found that serum displays significantly more chemoattractant activity than plasma over matched time intervals (Fig. 5). To determine whether the increase in chemerin activity was dependent on the presence of blood cells, we collected cell-free fluid from blood centrifuged immediately after blood draw (from the same donor) and assayed the samples for chemerin activity. Chemerin activity in "cell-free serum" also increased over time, although with somewhat delayed kinetics (Fig. 5). We conclude that chemerin circulates in a less or inactive proform in blood and that factors associated with or induced by the clotting or fibrinolytic cascades can activate chemerin in plasma. Furthermore, our results are consistent with previous studies demonstrating a role for cellular factors in the proteolytic activation of chemerin (Ref. 25 and our personal observations) because the presence of blood cells accelerates chemerin activation during blood coagulation.

Chemerin attracts blood pDCs but not mDCs

We evaluated the ability of the ligand to attract blood DCs in assays of cell migration across monolayers of human umbilical vein endothelium, an assay shown by de la Rosa et al. (15) to provide a more efficient system than bare Transwells for pDC migration, pDC migrated significantly to recombinant attractant in a dose-dependent manner (in transendothelial migration assays, mean 11.1 ± 2.5% (±SE) input migration to 4.0 nM recombinant bacterial-expressed chemerin vs 0.3 ± 0.1% background migration ($n = 3$ different donors; $p < 0.05$), Fig. 6). The full-length recombinant chemerin was highly active in these assays, likely reflecting spontaneous processing to the active form by the endothelial cells or blood leukocytes in the assay system. pDCs also migrated well to the general leukocyte attractant CXCL12 (10.3 ± 1.6%), and

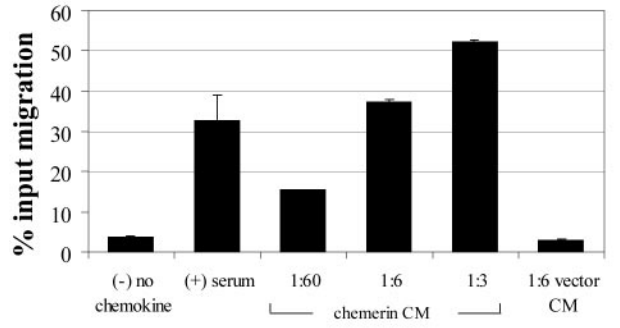
A



B

Residue position	Sequence	Predicted MW (+propionamide cys)	LC/MS data
114-125	LVHCPIETQVLR*	1477.81	1477.77
126-137	EAEEHQETQCLR*	1542.67	1542.73
105-113	GSEDKVLGR*	959.53	959.50
141-155	AGEDPHSFVPGQFA	1668.66	1668.72

C



D

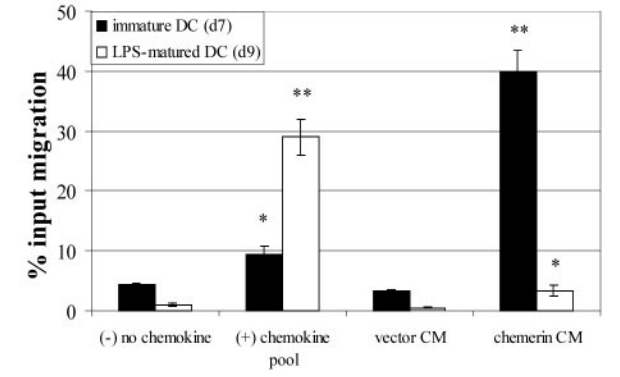


FIGURE 3. Identification of serum chemerin as chemoattractant ligand for CMKLR1. *A*, A total of 1.6 L of human serum was prefiltered and applied to a 50-ml heparin-Sepharose column, and bound protein was eluted using a NaCl gradient. The 0.7 M NaCl fraction was enriched for chemotactic activity as assayed by Transwell CMKLR1/L1.2 migration. Total protein was determined by bicinchoninic acid. *B*, Four mass values from the tryptic digest of the isolated chemotactic protein matched four peptides in public databases corresponding to the polypeptide encoded by TIG2, or chemerin (search parameters included 1 missed trypsin cleavage and cysteines modified by acrylamide adducts). The peptides marked (*) were microsequenced by MS/MS fragmentation, and the results were consistent with the predicted peptide sequence. *C*, Chemerin/L1.2 transfectants were generated and varying dilutions (1/60, 1/6, 1/3) of conditioned medium (CM) were tested for chemotactic activity. Conditioned medium was generated by culturing L1.2 transfectants in low-serum Optimem, harvesting the exhausted medium, filtering and concentrating it. A 1/6 dilution of conditioned medium from empty vector transfectants (vector CM) was tested as a negative control. Empty vector L1.2 control transfectants did not respond to chemerin conditioned medium (data not shown). Medium alone (−) as shown, and serum (+) was a 1/6 dilution of purified human serum. The mean from duplicate wells of a representative experiment with range is presented ($n > 3$). *D*, Immature DCs show a robust migratory response to chemerin conditioned medium. Chemotaxis was investigated using bare Transwell inserts. The chemokine pool (+) was 10 nM each CXCL12 (SDFI), CCL19 (ELC), CCL21 (SLC), and CCL2 (MCP-1), and no chemokine (−) as shown. Each bar represents the mean (±SEM) percentage input migration from three experiments (three different blood donors) performed with duplicate wells. *, $p < 0.05$, **, $p < 0.005$ by *t* test comparing (−) vs (+) or vector conditioned medium (vector CM) vs chemerin conditioned medium (chemerin CM).

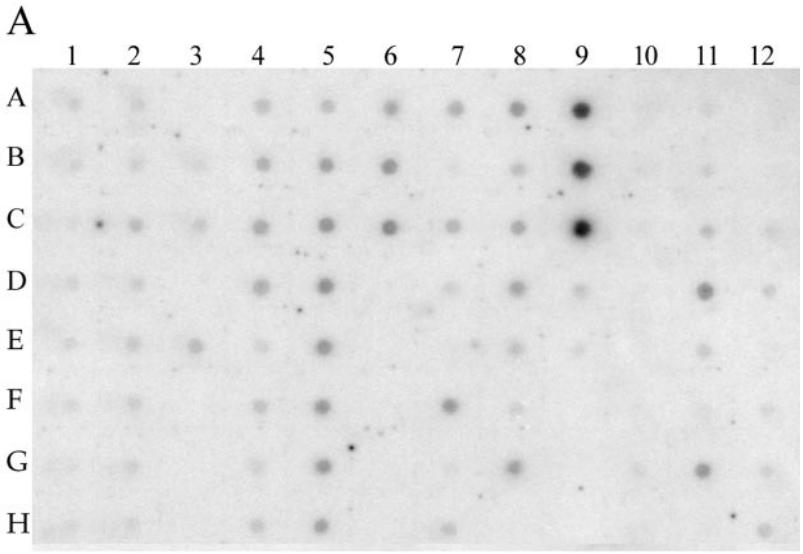
significantly but less well to the CCR7 ligands CCL19 (EBV-induced molecule-1 ligand CC chemokine, ELC) and CCL21 (secondary lymphoid-tissue CC chemokine, SLC) (2.0 ± 0.5%, $p <$ 0.05 vs medium control). In contrast mDCs did not migrate above background to chemerin at any concentration tested (0.9 ± 0.2% for 4.0 nM chemerin vs 1.1 ± 0.4% background migration). Although pDC migration across bare Transwell membranes is less efficient overall (15), chemerin in conditioned medium also attracted pDC in standard Transwell chemotaxis assays (mean 6.0 ± 1.0% (±SE) input migration to conditioned medium from chemerin-transfected L1.2 cells vs 1.6 ± 0.3% input migration to control vector transfected medium ($n = 13$ different donors; $p < 0.0005$)). Thus chemerin is a potent attractant for circulating pDCs but not for blood mDCs, correlating with the differential expression of its receptor.

Discussion

We have found that the chemoattractant receptor CMKLR1 is expressed by pDCs in blood, distinguishing pDCs from naive and memory lymphocytes, monocytes, granulocytes and even blood mDCs. CMKLR1 confers on circulating pDCs the ability to respond to a unique chemoattractant, chemerin. Chemerin is widely expressed at the RNA level, and the translated protein is found in abundance in blood. Our data suggest that the less or inactive proform of chemerin is present in plasma, and it is converted into a potent pDC chemoattractant following blood coagulation. These results support a potential mechanism for the recruitment of pDCs to sites of bleeding, and for bridging hemostasis with the innate and adaptive immune responses following tissue damage.

The chemerin-encoding gene TIG2 was initially cloned by differential display as being up-regulated in in vitro cultured human skin rafts treated with the anti-inflammatory retinoid tazarotene (34). It was also shown to be up-regulated in a hormone-treated, osteoclastogenic mouse bone marrow stromal cell line ST2 (35). Indeed, we found that conditioned medium from the ST2 cell line treated with 1,25-dihydroxyvitamin D_3 and dexamethasone was chemotactic for CMKLR1 transfectants (data not shown). These results, together with our RNA expression data, suggest that chemerin message is constitutively expressed in a number of tissues, and that it can be regulated as well, particularly in response to retinoid and steroid receptor stimulation.

It is clear, however, that posttranslational modification of chemerin in the form of proteolytic processing also regulates its chemoattractant activity (25, 26). Recombinant full-length chemerin effects CMKLR1 signaling only when presented at high



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebel left		heart	esoph	colon, trans	kidney	lung	liver	leuk, HL-60	fet brain	yeast tot RNA
B	cereb cortex	cerebel right	accum nucleus	aorta	stomach	colon, descen	skel muscle	placen	pancre	HeLa, S3	fet heart	yeast tRNA
C	frontal lobe	corpus colles	thalam	atrium left	duod	rectum	spleen	bladd	adren gland	leuk, K-562	fet kidne	*E.coli* RNA
D	pariet lobe	caudate nucleus		atrium right	jejun		thymus	uterus	thyroid gland	leuk, MOLT4	fet liver	*E.coli* DNA
E	occip lobe	amygd	spinal cord	vent left	ileum		PBL	prost	saliv gland	Burkitt lymph Raji	fet splen	Poly r(A)
F	temp lobe	hippo-campus		vent right	ileo-cecum		lymph node	testis		Burkitt lymph Daudi	fet thym	human Cot-1 DNA
G	pg cereb cortex	medul oblong		inter-vent septum	append		bone marrow	ovary		colorec adeno SW480	fet lung	human DNA 100 ng
H	pons	putamen		apex of heart	colon, ascend		trachea			lung carcin A549		human DNA 500 ng

C

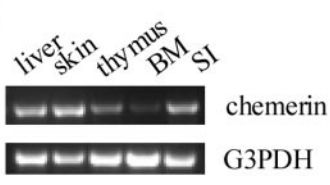


FIGURE 4. Chemerin RNA expression. *A*, A human RNA array was probed with chemerin cDNA. Chemerin is widely expressed, with highest levels in the adrenal gland, liver, and pancreas, and strong signals in many tissues. Notable exclusions include components of the CNS, bone marrow (BM), peripheral blood leukocytes (PBL), and thymus. *B*, RNA spot identifier. *C*, RT-PCR using intron-spanning primers shows chemerin expression in skin, and confirms high levels of expression in the liver (SI, small intestine). Weak expression in the bone marrow is consistent with the dot blot data. G3PDH demonstrates equivalent RNA template in each sample. "No RT" controls showed no amplicons, indicating that the PCR bands reflect RNA expression (data not shown).

concentrations compared with fully active forms (Refs. 25, 26 and our personal observations). Structural analyses show that the attractant is activated by proteolysis and release of short carboxyl-terminal peptides (25, 26, 33). Interestingly, chemerin is spontaneously activated by coculture with cells, and by factors in supernatants of cultured cells (Ref. 25 and for example the migration of CMKLR1 transfectants to conditioned medium from chemerin-expressing cells in Fig. 3*C*). This explains the potent activity of recombinant chemerin in our studies of transendothelial cell pDC chemotaxis because in this setting the endothelial cells, or the migrating cells themselves, can spontaneously activate the recombinant attractant.

Wittamer et al. (25) isolated active chemerin from patient ascites fluid, and have detected it in inflamed synovial fluid. Meder et al. (33) isolated chemerin from hemofiltrate from patients undergoing dialysis for renal failure. These conditions are associated with tissue inflammatory reactions and/or immune cell activation, and the authors hypothesized that inflammatory proteases may be responsible for cleavage and activation of tissue chemerin (25). Our results suggest that proteases activated during the coagulation or fibrinolytic cascades may also, directly or indirectly, lead to carboxyl-terminal cleavage and subsequent chemerin activation. Of course, inflammation is also associated with the activation of coagulation/fibrinolytic enzymes, as shown in allergic contact dermatitis and delayed-type hypersensitivity lesions (36), and synovial fluid from spondyloarthropathic or rheumatic joints (37). Thus, the hemostatic systems that trigger chemerin activation

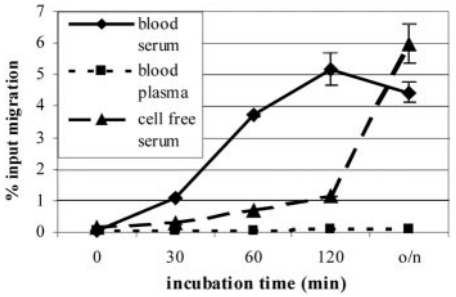


FIGURE 5. Serum but not plasma attracts CMKLR1 transfectants. For the "blood plasma" and "blood serum" samples, normal or anticoagulated blood was collected from the same donor and incubated at room temperature. At the indicated time points, plasma and serum were clarified by centrifugation, placed on ice, and tested for attractant activity with CMKLR1 transfectants at 1/17 dilution. In comparing attractant activity, an equivalent amount of anticoagulant was added to each serum sample before testing to control for the anticoagulant present in the plasma samples. For the "cell-free serum" sample, normal blood was collected from the same donor, immediately centrifuged, and the fluid phase was collected and incubated at room temperature. At the indicated time points, heparin was added to arrest coagulation, and the samples were placed on ice and then tested in chemotaxis as mentioned. The mean from duplicate wells of a representative experiment is presented with range ($n > 5$ donors). o/n, Overnight.

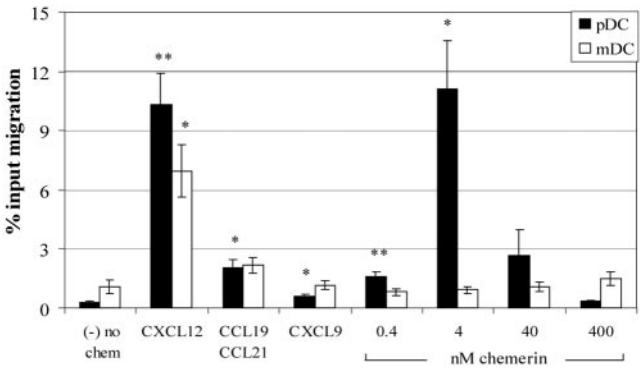


FIGURE 6. Chemerin is a potent chemoattractant for human blood pDCs. Transendothelial migration was investigated using Transwell inserts coated with HUVEC monolayers. Total PBMC were tested, and the migrated cells were collected and stained for HLA-DR, CD123, CD11c, and Lin markers. CXCL12 (20 nM) was used as a positive control, the CCR7 ligands CCL19 (100 nM) and CCL21 (10 nM) were assayed, and the proinflammatory chemokine CXCL9 (100 nM) was also tested. A range of concentrations of recombinant bacterial-expressed chemerin was assayed ($n = 3$ donors, mean percentage ($\pm$SE) migration of pDCs or mDCs is displayed. *, $p < 0.05$, **, $p < 0.005$ in pairwise comparisons with background migration ($-$).

during blood clotting may also participate, along with other inflammatory protease cascades, in regulating pDC recruitment to sites of inflammation. It will be of interest to determine the specific protease or proteases involved in chemerin processing in vivo.

Our data suggest a self-standing mechanism to explain pDC recruitment to extralymphoid sites associated with coagulation or tissue damage, but other chemoattractant receptors and ligands could also participate in this process. Although pDCs do not respond to common inflammatory chemokines such as CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IFN-γ-inducible protein, IP-10), CXCL11 (IFN-inducible T cell chemoattractant, I-TAC), or CXCL9 (monokine-induced IFN-γ, Mig) (14, 15), a recent report indicates that pDCs express adenosine receptor A1 and migrate to high concentrations of adenosine (38). The authors postulate that adenosine released by damaged cells in inflammatory lesions may recruit pDC (38, 39). Another report indicates that pDC (and not mDC) express the IL-18R and migrate to IL18 (40), which has also been described as a chemoattractant for T cells (41). Although we have not observed robust chemotaxis of pDC to adenosine (and were unable to reproduce migration to IL-18 in our Transwell models, B. A. Zabel and E. C. Butcher, unpublished observations), the effects of these receptors could be sensitive to experimental or environmental conditions, and they may play complementary roles with CMKLR1 in vivo. Interestingly, although pDC do not migrate to CXCR3 ligands (14, 15) they do express the receptor as indicated by cell surface staining; and two groups have suggested that CXCR3 ligands can exert a synergistic effect with CXCL12 (stromal cell-derived factor 1, SDF1, a CXCR4 ligand) to regulate pDC migration. In one report, pDCs activated with CpG migrated better to CXCL11 (a CXCR3 ligand) plus CXCL12 than to CXCL12 alone (42). In another report, unstimulated pDC displayed robust migration to a suboptimal concentration of CXCL12 in the presence of inducible CXCR3 ligands CXCL11, CXCL9, or CXCL10 (43). Whether such a "chemokine synergy" model alone or in conjunction with chemerin constitutes a general mechanism for pDC tissue recruitment remains to be explored.

In addition to its role as a chemoattractant receptor, CMKLR1 is a demonstrated coreceptor for primary HIV-1 strain 92UG024 (44). In this context, our finding of selective expression of CMKLR1 by pDC suggests a potential explanation for recent data that pDCs can be infected more easily than mDCs by certain HIV-1 strains (45). pDCs may be efficient targets for HIV infection because they express CD4, they are present in blood and secondary lymphoid organs, and they express coreceptors such as CXCR4, CCR5 (46) and now CMKLR1. IFN-α is known to interfere with productive HIV infection (47) and because pDCs are the primary IFN-α-producing cell in the body, targeting and eliminating pDCs may be important for productive and stable HIV-1 infection. Multiple coreceptor blockade, including agents directed against CMKLR1, may be a useful therapeutic approach to controlling HIV-1 in infected patients.

In conclusion, our findings suggest that CMKLR1 may be a key mediator of pDC recruitment from the blood into tissue sites enriched in active chemerin. Although the regulation of chemerin activation in vivo remains to be clarified, its enhanced activity in response to blood clotting and to cellular protease activators may render it uniquely suited to recruit pDCs to sites of bleeding, tissue damage, and inflammation. pDCs, through α-IFN production and Ag processing, are thought to be important in bridging the innate and adaptive immune responses: rapid recruitment to sites of inflammatory protease activation may be critical to this role. The down-regulation of CMKLR1 upon DC activation and maturation, along with enhanced responsiveness to CCR7 ligands, is consistent with the extensive reprogramming of DC migration during the immune response to pathogens, and may help permit their migration as APC to lymph nodes via lymphatics. The significance of selective CMKLR1 expression to the unique sensitivity of pDC to HIV-1 infection is now amenable to study as well. The identification of this pDC selective chemoattractant and receptor may offer opportunities to regulate the migration of these versatile and potent cells, either to enhance antiviral responses or to modulate immune activity.

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