The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues

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CHEMOKINES, CYTOKINES, AND INTERLEUKINS

Chemerin is a chemotactic agonist recently identified as the ligand of ChemR23, a serine receptor expressed by mononuclear phagocytes and dendritic cells (DCs). This study shows that blood CD56lowCD16+ natural killer (NK) cells selectively express functional ChemR23 and that this receptor is coexpressed with CXCR1, the CXCL8 receptor, and the KIR receptors. In vitro culturing of NK cells with IL-2 or IL-15 induced a delayed and time-dependent down-regulation of ChemR23 that was associated with the inhibition of NK cell migration to chemerin. Biopsies obtained from patients with oral lichen planus presented an infiltration of CD94+CD3−CD56+ NK cells that coexpressed ChemR23. The same biopsies were infiltrated by myeloid, DC-SIGN+ and plasmacytoid, CD123−BDCA2+ ChemR23+ dendritic cells that were occasionally associated with NK cells. In the same histologic sections, chemerin was expressed by inflamed dermal endothelium. These findings propose a role for the ChemR23/chemerin axis in the recruitment of blood NK cells and strongly implicate chemerin as a key factor for the colocalization of NK cells and DC subsets in pathologic peripheral tissues. (Blood. 2007;109: 3625-3632)

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Introduction

Natural killer (NK) cells represent a minor population (5%-20%) of peripheral blood lymphocytes that is also present in secondary lymphoid organs, such as spleen, and lymph nodes, as well as in liver, bone marrow, and maternal uterus.1-3 The function of NK cells in humans is regulated by a balance between opposite signals delivered by a set of HLA class I-specific inhibitory receptors and by a number of activating receptors and coreceptors responsible for NK cell triggering. By the combined use of these receptors, NK cells can discriminate between normal HLA class I+ cells and cells that have lost the expression of HLA class I molecules as a consequence of tumor transformation or viral infection.4,7 Most NK cells in peripheral blood express the CD56lowCD16+ phenotype, whereas the remainders are CD56highCD16− cells. It was proposed that CD56high NK cells represent a primary source of immunoregulatory cytokines, whereas the CD56lowC16+ subset represents the principal cytotoxic population.8

During inflammation, viral infection and tumor growth, NK cells are rapidly recruited from the blood into injured tissues.9-11 NK cell recruitment is governed by integrated signals, which include adhesion molecules and chemotactic factors. CD56lowCD16+ NK cells express both β1 and β2 integrins, as well as the ligands for E- and P-selectins. In addition to these molecules, CD56high NK cells also express high levels of L-selectin, a pivotal molecule for the interaction with lymph node high endothelial venules,12-14 With respect to chemokine receptors, CD56lowCD16+ NK cells express high levels of CXCR1 and CX3CR1.9,12 By contrast, CD56high NK cells express CCR7 as well as CCR5 and CXCR3.8,9,15 It is likely that the different expression profile of adhesion molecules and chemokine receptors between the 2 major blood NK cell subsets is responsible for the preferential migration of CD56lowCD16+ and CD56highCD16− NK cells into inflamed tissues and secondary lymphoid organs, respectively.16 In fact, the CD56lowCD16− NK cell subset although poorly represented in peripheral blood constitutes the only type of NK cells present in secondary lymphoid tissues.2,3

We have recently identified a novel protein, chemerin, as the natural ligand of the previously orphan receptor ChemR23.17 ChemR23 exhibits a unique expression profile among leukocyte populations being expressed preferentially by monocyte/macrophages and by immature myeloid and plasmacytoid dendritic cells (DCs).18 Chemerin, originally isolated from inflamed biologic fluids, such as ovarian cancer ascites and rheumatoid arthritis synovial fluids, is synthesized as a secreted precursor protein. Prochemerin is poorly active but can be rapidly converted into a full ChemR23 agonist by the proteolytic removal of the last 6 amino acids by neutrophil-derived proteases (elastase and cathepsin G), mast cell products (tripatase), and proteases of the coagulation cascade.19,20 Therefore, prochemerin represents a “ready to
use” chemotactic factor for the rapid recruitment of leukocytes into pathologic tissues.

In this study we show that blood CD56lowCD16+ but not CD56highCD16+ NK cells, express functional ChemR23. In human biopsies of lichen planus, ChemR23 is expressed by infiltrating NK cells and by both myeloid and plasmacytoid DCs. DC subsets and NK cells colocalize in areas where chemerin is detected on the surface of inflamed endothelium. These results strongly suggest that the ChemR23/chemerin axis is involved in the trafficking of CD56lowCD16+ NK cells into inflamed peripheral tissues and in their colocalization with myeloid and plasmacytoid DCs. Furthermore, these results represent one of few reports that directly support NK-DC cell cross-talk, in vivo.21

Materials and methods

The study was conducted in accordance with a protocol approved by the Spedali Civili of Brescia institutional ethical board and informed consent was obtained from all patients.

Monoclonal antibodies and cytofluorimetric analysis

The following monoclonal antibodies (mAbs), produced in our laboratories, were used in this study: JT3A (IgG2a, anti-CD3), c227 (IgG1, anti-CD69), FS24 (IgG2b, anti-HLA-DR), BAB281 and KL247 (IgG1 and IgM, respectively, anti-CD56), Z25 and F252 (IgG1 and IgM, respectively, anti-CD30), c127 and KD1 (IgG1 and IgG2a, respectively, anti-CD16), c218 and c280 (IgG1 and IgG2a, respectively, anti-CD56), GL183 (IgG1, anti-CD158b), A2115 (IgG1, anti-CD158a), Z27 (IgG1, anti-CD158e), XA185 (IgG1, anti-CD94), Z270 (IgG1, anti-NKG2A), F278 (IgG1, anti-CD85j), and anti-ChemR23 (clone 1H2; IgG2a). Anti-CXCR1 (IgG1) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). For one- or 2-color cytofluorimetric analysis, cells were stained with the appropriate mAbs followed by PE- or FITC-conjugated isotype-specific goat anti–mouse secondary antibody (Beckman Coulter, Immuno-tech, Marseille, France).15 CD3 contamination in purified NK cells was less than 1%. Freshly purified NK cells were resuspended in RPMI 1640 (Life Technologies, Linz, Austria) and cultured as previously described22 in the presence of 5% CO2 for 4 hours. The number of migrated cells, recovered from the lower well, was expressed as the percentage of input cells. For transendothelial migration, human umbilical vein endothelial cells (HUVECs, passage < 5) were subcultured to confluent monolayers on Transwell inserts precoated with gelatin, as previously described.18 In vivo migration of NK cells was evaluated following the intraperitoneal injection of 5 μg/mouse of murine recombinant chemerin (R&D Systems, Minneapolis, MN). Cells were recovered from the peritoneal cavity 20 hours later and NK cells were stained with anti-CD3 and anti-NK1.1 mAb (PharMingen, San Diego, CA), as previously described.21

Tissues and reagents

Mucosal specimens from 9 patients with active oral lichen planus (OLP) were analyzed. Each specimen was snap frozen in isopentane precooled to liquid nitrogen temperature and stored at −80°C. Thin (5-μm) cryostat sections were air-dried overnight at room temperature and fixed in acetone for 10 minutes before staining. Tissue expression of ChemR23, CD56, CD94, CD123, and BDCA-2 was evaluated by immunohistochemistry using anti-ChemR23 mAbs (4C7 and 1H7; mouse, IgG2b and IgG2a, respectively; dilution 1:15),15 anti-CD56 mAb (clone 2B8, mouse, IgG2a), anti-CD94 mAb (clone 39 BIO-CL1, mouse, IgG1, dilution 1:10, kindly provided by M. Colonna (Washington University, St Louis, MO), anti-CD123 mAb (clone 7G3, mouse, IgG2a, dilution 1:40, PharMingen), and anti-BDCA-2 mAb (clone AC144, mouse, IgG1, dilution 1:5, Miltenyi Biotec, Auburn, CA). Chemerin expression was evaluated by immunohistochemistry as previously described.13 After treatment with goat serum for 5 minutes to inhibit nonspecific binding, tissue sections were incubated with primary antibodies at room temperature for 1 hour, followed by biotinylated goat-polyvalent immunoglobulin, the streptavidin-peroxidase amplification system (StreptAViGen Multilink Kit, Biogenex, San Ramon, CA) and visualized by applying 3-aminop-9-ethylcarbazole (LabVision, Fremont, CA). Nuclei were counterstained with hematoxylin. MxA immunostaining was performed on paraffin sections (3 μm) using anti-MxA (clone M143, mouse, IgG2a, dilution 1:500, provided by Otto Haller, University of Freiburg, Germany) and an indirect immunoperoxidase technique (Dako LSAB + Kit peroxidase; Dako, Carpinteria, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. Double immunofluorescence was performed using a combination of primary antibodies, including anti-ChemR23 (mouse, IgG2b), anti-CD56 (mouse, IgG2a), anti-CD94 (mouse, IgG1), anti-CD123 (mouse, IgG2a), anti-BDCA-2 (mouse, IgG1), anti-DC-sign/CD209 (clone DCN46, mouse, IgG2b, dilution 1:200, PharMingen), anti-Langerin/CD207 (mouse, IgG2b, dilution 1:500, kindly provided by Dr G. Trinchieri, National Cancer Institute, Bethesda, MD), anti-CD69 (mouse, IgG1, dilution 1:50, Dako), anti-CD3 (polyclonal rabbit, IgG, dilution 1:100, Dako). Appropriate Texas red- and FITC-conjugated isotype-specific secondary antibodies were used to reveal the primary antibodies, as previously described.15,18 Immunofluorescence was analyzed and photographed using an Olympus BX60 fluorescence microscope, equipped with the Olympus DP-70 digital camera. Pictures were analyzed by Analysis 3.2 soft imaging system GMBH and Adobe Photoshop 5.0, limited edition. Lenses used were: Olympus: 20×/0.70, ×0.17, and 40×/0.85, ×0/11-0.23.

Results

Expression of ChemR23 in human peripheral blood NK cell subsets

In a previous study, we reported that ChemR23 is mostly expressed by MHC class II+ cells, including monocytes, myeloid and plasmacytoid DCs, and by a minor subset of CD3+ and CD20+ cells.18 Here, the expression of ChemR23 was further investigated in unfractionated PBMCs and highly purified NK cells. This analysis revealed that the majority of CD56+ cells present in PBMCs (Figure 1A) and in purified NK cells (Figure 1B) express ChemR23. Remarkably, ChemR23 expression was confined to CD56low cells, whereas it was absent from the CD56high subset.
CD56low NK cells are known to express high levels of CXCR1, the CXCL8 receptor, and ChemR23 expression in these cells was characterized by the NKG2Abright NKp46bright phenotype, a result that is consistent with previous reports showing that CD56high NK cells express high levels of both NKG2A and NKp46.

ChemR23+ NK cells were further characterized for the expression of inhibitory and activating receptors. ChemR23+ cells expressed various members of the KIR family including KIR2DL2/2DL3/2DS2 (CD158b) and KIR3DL1/3DS1 (CD158e). They also expressed LIR1/ILT2 (CD85j), NKG2A, and the activating receptors CD16 and NKp46. A subset of ChemR23− NK cells was characterized by the NKG2Ahigh NKp46bright phenotype, a result that is consistent with previous reports showing that CD56high (ChemR23−) cells express high levels of both NKG2A and NKp46.

Migration of NK cells in response to chemerin

We next evaluated the ability of chemerin, the ChemR23 ligand, to induce NK cell migration using a Transwell-based chemotaxis assay. Chemerin induced a bell-shaped dose-dependent migration of NK cells, with a peak of activity observed at 300 pM chemerin and an EC50 of 0.05 nM. In the same experimental conditions, CXCL8 induced NK cell migration with a maximal response observed at the concentration of 10 nM (EC50 = 1 nM). Therefore, chemerin is characterized by a 20-fold higher potency and by a slightly reduced efficacy (percentage of migrated cells) as compared to CXCL8 (Figure 3A). Similar results were obtained when chemerin was tested in a transendothelial migration assay (Figure 3B). The response of NK cells to chemerin was chemotaxis (ie, directional migration) rather than chemokinesis (ie, activated random migration), since chemerin induced NK cell migration only when added to the lower compartment of the chemotactic chamber, with no activity in the presence of a negative gradient (chemerin in the upper well) or in the absence of chemotactic gradient (chemerin in both lower and upper wells; Figure 3C). As expected on the basis of previous results, NK cell migration to chemerin was completely blocked by the action of Bordetella pertussis toxin (Figure 3D) and in the presence of an anti-ChemR23 mAb (data not shown). These results provide evidence that the activation of ChemR23 is responsible for chemerin-induced NK cell migration. Finally, the ability of chemerin to induce NK cell recruitment in vivo was investigated in vivo following intraperitoneal administration in mice. After 20 hours, the number of NK1.1+/CD3− cells recovered from the peritoneal cavity was strongly increased in chemerin-injected animal compared to the control group (0.89 × 10⁶ and 0.38 × 10⁶ cells, respectively; n = 3).

Regulation of ChemR23 expression and function in NK cells

The results so far presented were generated using freshly purified blood NK cells. When cultured in vitro in the presence of cytokines, such as IL-2, NK cells acquire an activated phenotype in terms of cytokine production and cytotoxic ability and modify their repertoire of chemokine receptors. To analyze the regulation of ChemR23 expression, purified NK cells were cultured in the presence of either IL-2 or of IL-15 and analyzed for ChemR23 expression at different days of culture. As shown in Figure 1B (upper panel), after 1 day of culture in the presence of IL-2, ChemR23 expression was virtually unchanged as compared to freshly purified NK cells although at this stage CD69 was already expressed by most of the cells and both CD56 and NKp46 were up-regulated (data not shown). However, a sharp down-regulation of ChemR23 expression was detected after 4 days with a complete down-regulation observed at day 6 of culture. Similar results were obtained when NK cells were cultured with IL-15 instead of IL-2 (data not shown). It is interesting to note that CXCR1 was down-regulated in parallel to ChemR23 with a similar kinetics (Figure 1A bottom panels). The down-regulation of ChemR23 membrane expression was paralleled by a decrease of ChemR23 mRNA and by the inhibition of cell migration in response to chemerin (Figure 1B-C). At longer time of IL-2 activation, such as after 2 to 4 weeks, NK cells were virtually ChemR23−. These results were likely due to receptor down-modulation rather than to the in vitro selection of ChemR23− cells. In fact, consistent with their derivation from CD56low NK cells, the IL-2–cultured population was mostly CD16+ and expressed KIR2DL2/3 and KIR3DL1 (36% and 22%, respectively). These data suggest that NK cells express ChemR23 only during the early phases of their recruitment to the site of infection.
Role of chemerin in the colocalization of NK cells and DC in lichen planus lesions

Lichen planus is a mucocutaneous inflammatory disease histologically characterized by basal keratinocyte damage and lymphocyte reaction. Analysis of the T-lymphocyte infiltrate suggests that activated cytotoxic T cells may trigger apoptosis of epithelial cells.\(^{29-31}\) Figure 5 shows that in OLP, NK cells also infiltrate skin lesions as documented by the presence of numerous CD56\(^+\) and CD94\(^+\) cells. Of note, only a minor fraction (\(\leq 2\%)\) of CD3\(^+\) cells coexpressed the CD94 marker, indicating their true NK cell nature (Figure S1, available online at the Blood website; see the Supplemental Figure link at the top of the online article). NK cells were mostly located in the dermis with fewer sparse cells in the epidermis; this distribution mirrors the distribution of ChemR23\(^+\) cells (Figure 5). Therefore, ChemR23 expression by OLP-associated NK cells was evaluated by double immunofluorescence; most (\(\geq 70\%)\) of the CD56\(^+\) and CD94\(^+\) cells also expressed ChemR23. A minor fraction (approximately 40%) of the CD56\(^+\) cells were also CD69\(^+\) suggesting that ChemR23 was mostly expressed by freshly recruited NK cells (Figure 5). Conversely, CD3\(^+\) cells were mostly negative (\(\geq 97\%)\) for ChemR23 expression (Figure 1S).

Previous work documented a high degree of accumulation of both myeloid and plasmacytoid DCs in OLP lesions.\(^{24}\) Since the number of ChemR23\(^+\) cells regularly outnumbered CD56\(^+\) and CD94\(^+\) cells, the expression of ChemR23 by DCs was also investigated. Figure 6 shows that OLP lesions presented an elevated number of CD123\(^+\), BDCA2\(^+\) plasmacytoid DCs, and DC-SIGN\(^+\) myeloid DCs. A proportion of DC-SIGN\(^+\) mononuclear cells, thus corresponding to stromal DCs, as well as plasmacytoid DCs (CD123\(^+\)) coexpressed ChemR23 (Figure 6 central panels), whereas Langerhans cells were negative (data not shown). Therefore, the majority of the ChemR23\(^+\) cells coexpressed a DC phenotype, whereas approximately 25% of them coexpressed CD56 or CD94. It is interesting to note that NK cells and DCs not only are colocalized in OLP sections but also that close contacts can occasionally be observed between CD56\(^+\) cells and BDCA2\(^+\) or DC-SIGN\(^+\) cells (Figure 6 bottom left panels; see arrowheads). To investigate the possible consequences of this NK cell/DC colocalization, the secretion of type I interferon, the major cytokine released by plasmacytoid DCs,\(^{32}\) was investigated. For this purpose it was evaluated by immunohistochemistry the expression of MxA, an IFN-α-inducible protein.\(^{33}\) MxA was strongly expressed on epithelial and inflammatory cells in all OLP lesions (Figure 6). In particular,
epithelial MxA expression was significantly enhanced when compared with contiguous unaffected mucosa. Conversely, no MxA expression was noticed in oral mucosal biopsies from 3 patients showing reactive acanthosis.

To gain further information about the possible role of ChemR23 in the colocalization of DC subsets and NK cells, the expression of chemerin, the ChemR23 ligand, was examined by immunohistochemistry. Chemerin reactivity was observed on the endothelial cells lining stromal vessels in 7 of the 9 cases of OLP investigated (Figure 6). In addition, granular cytoplasmic positivity for chemerin was observed in rare mononuclear cells with epithelial morphology located in the epithelium. Altogether, these data strongly support the hypothesis that chemerin may orchestrate the colocalization of DCs and NK cells in OLP lesions.

Discussion

This study shows that ChemR23, a chemotactic receptor mainly expressed by antigen-presenting cells, such as monocytes/macrophages and DCs, is also a functional receptor for human NK cells. ChemR23 expression marks the major CD56lowCD16+ peripheral blood NK cell subset, with no expression by CD56high NK cells. Consistent with their CD56low phenotype, ChemR23+ cells express KIRs as well as CD16 molecules. Chemerin, the ChemR23 ligand, promotes the migration of NK cells at picomolar concentrations in a dose-dependent manner, and this migration is dependent on ChemR23 as well as for DCs that are recruited to skin lesions in lupus erythematosus. The distribution and regulation of ChemR23 in peripheral blood NK cells closely parallel the expression of CXCR1 and the 2 receptors were found to be coexpressed by circulating NK cells. This finding strongly suggests a role for the ChemR23/chemerin axis in the recruitment of CD56low NK cells into inflamed peripheral pathologic tissues.

Chemerin belongs to the cathelicidin/cystatin family of proteins, which includes precursors of bactericidal peptides (cathelicidins), precursors of mediators active on leukocytes through G protein-coupled receptors (prokininogen, cathelicidin precursors), as well as cysteine protease inhibitors (cystatins). Elevated chemerin production was found in ovarian cancer ascites and in the synovial fluids of patients with rheumatoid arthritis. Furthermore, prochemerin was found expressed in skin biopsies obtained from nonlesional psoriatic skin and in patients with systemic lupus erythematosus. Therefore, it is likely that chemerin represents an important signal involved in leukocyte recruitment during inflammation and autoimmune diseases and at tumor sites. Chemerin was shown to serve as a chemoattractant for plasmacytoid and myeloid DCs that populate secondary lymphoid compartments as well as for DCs that are recruited to skin lesions in lupus...
erythematous. Thus, chemerin local production may promote the colocalization of DCs and CD56low NK cells in pathologic tissues.

Recent studies have highlighted the role of NK cells during the early phases of innate immune responses. In particular, it was suggested that NK cells can interact with myeloid DCs in inflamed peripheral tissues, where a potent cross-talk would occur between these 2 cell types. During this interaction, CD56low CD16+ NK cells acquire the ability to kill immature DCs and to release high levels of cytokines including IFN-γ and TNF-α, which promote DC maturation. These functions may be facilitated by the simultaneous activation of both NK cells and DCs by pattern recognition receptors. Indeed, CD56low NK cells, in the presence of IL-12 and of the concomitant activation of Toll-like receptors, become rapidly activated and acquire cytolytic activity against tumor target cells as well as against immature DCs. Thus, the recruitment of the CD56low NK cell subset in response to chemerin, will regulate the number and the maturation process of DCs before their migration to lymph nodes, whereas the CD56high subset, recruited to lymph nodes via chemerin-independent mechanisms, would produce IFN-γ necessary for DC-dependent priming of Th1 responses.

Despite the large body of evidence about the role of NK cell/DC interactions, little evidence supports the concept that this interaction indeed occurs in peripheral tissues, in vivo. In an effort to provide evidence for this model, we have investigated the expression of ChemR23 by NK cells in a pathologic condition known to be characterized by elevated tissue accumulation of DCs. Biopsies obtained from patients with OLP showed, in addition to numerous CD123+/BDCA-2+/DC-SIGN+ DCs, the presence of CD3+CD56+CD94+ NK cells. Both DC subsets and NK cells expressed ChemR23 with NK cells accounting for about 25% of the total ChemR23+ cells present in each lesion. Only a minority of ChemR23+ NK cells expressed CD69, an early activation marker, suggesting that ChemR23 was mostly expressed by recently recruited NK cell.

Because NK cells were found in close proximity with both plasmacytoid and myeloid DCs, it is likely that this interaction will result in reciprocal cell activation. Recent work has described the effect of NK/plasmacytoid DC interaction in vitro. OLP sections characterized by plasmacytoid DC and NK cell accumulation strongly expressed MxA, an IFN-α-inducible protein. Furthermore, it is worth considering that
plasmacytoid DCs present in OLP lesions were found to produce high levels of type I IFN and that CD69 is known to be induced by this cytokine. In normal conditions, chemerin is absent in nonlymphoid peripheral tissues, including dermis and epidermis; on the other hand, in OLP lesions, endothelial cells lining blood vessels express chemerin. This finding further supports the role of chemerin in the recruitment and colocalization of NK cells and DC subsets in OLP lesions.

In summary, this study shows that CD56+ circulating NK cells express functional ChemR23 and that chemerin is expressed by inflamed endothelial cells in lichen planus lesions, a pathologic condition characterized by the accumulation of NK cells and DCs. These results strongly support the idea that the ChemR23/chemerin axis may play a crucial role in the colocalization of these 2 cell subsets in pathologic peripheral tissues and provide one of the few pieces of direct evidence that this interaction might indeed occur in vivo.

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Authorship

Contributions: S.P. performed research and analyzed data; A.S. performed research and analyzed data; E.M. performed research and analyzed data; W.L. performed research; L.M. performed research; F.F. analyzed new reagents; M.P. contributed new reagents; A. Majorana contributed tissues; M.S. performed research; G.T. performed research; A. Moretta analyzed data and wrote the paper; and S.S. planned the experiments, analyzed the data, and wrote the paper.

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References

46. Nishimura et al. Thrombin-activatable procarboxypeptidase B regulates activated complement C5a in vivo, which appeared in the March 1, 2007, issue of Blood (Volume 109:1992-1997), there were errors in the byline, the abstract, and the text. The third author’s name should have been Adrian M. Piliponsky. Also, the abstract should have begun, “Plasma procarboxypeptidase B (proCPB) is activated by the endothelial thrombin-thrombomodulin complex. Activated proCPB (CPB) functions as . . . .” Finally, the last sentence of “Discussion” should have read, “Selective activation of thrombin, enhancing its antithrombotic and anti-inflammatory properties combined activation of PC and proCPB, while minimizing its prothrombotic properties, may represent another avenue of developing new antithrombotic agents.” The byline error was corrected online in departure from print.
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