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Functional Expression of IL-9 Receptor by Human Neutrophils from Asthmatic Donors: Role in IL-8 Release

Soussi Gounni Abdelilah,‡ Koussih Latifa,Λ Nutku Esra,‡ Lisa Cameron,* Lamkhioued Bouchaib,† Nicholas C. Nicolaides,‡ Roy C. Levitt,§ and Qutayba Hamid*‡

Human polymorphonuclear neutrophils (PMNs) express surface receptors for various inflammatory mediators, including IgE and IL-4. Recently, the IL-9R locus has been genetically linked to asthma and bronchial hyperresponsiveness in humans. In this study, we evaluated expression of the IL-9R and the effect of IL-9 on human PMNs. RT-PCR analysis showed the presence of IL-9Rα-chain mRNA in PMN RNA preparations from asthmatic patients. Using FACS analysis, surface expression of IL-9Rα was detected on PMNs freshly isolated from asthmatics, and to a lesser extent on normal controls. In addition, protein expression of IL-9Rα was also detected in peripheral blood and bronchoalveolar lavage PMNs. Furthermore, functional studies showed that IL-9 stimulation of PMNs results in the release of IL-8 in a concentration-dependent manner. The anti-IL-9 neutralizing Ab suppressed this effect, but had no effect on GM-CSF-induced IL-8 release from PMNs. Taken together, these findings suggest a novel role for PMNs in allergic disease through the expression and activation of the IL-9R.

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§ Abbreviations used in this manuscript: PMNs, polymorphonuclear neutrophils; BAL, bronchoalveolar lavage; RT, reverse transcription.

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(IgG1) anti-CD16, IgG1 isotype control (clone MOPC21), FITC-conjugated mouse anti-CD16 (clone 3G8), FITC-conjugated mouse IgG1 isotype control, and goat IgG were obtained from Sigma (Oakville, Ontario, Canada). FITC- and biotin-conjugated F(ab’)2 swine anti-rabbit IgG, mouse alkaline phosphatase anti-alkaline phosphatase, Fast red, and streptavidin alkaline phosphatase anti-alkaline phosphatase, Fast red, and streptavidin alkaline phosphatase were obtained from Dako (Dakopatt, Denmark). Anti-CD16 immunomagnetic beads were obtained from Miltenyi (Auburn, CA). FBS with low endotoxin (<25 endotoxin U/ml by Limulus amebocyte lysate Gel Clot) was obtained from HyClone (Logan, UT). RPMI 1640, antibiotics (penicillin, streptomycin), dNTP, and Superscript reverse transcriptase were obtained from (Life Technologies, Grand Island, NY). Ficoll-Paque gradients, dextran 70, and polymerase were obtained from Pharmacia Biotech (Uppsala, Sweden). Anti-digoxigenin-alkaline phosphatase Fab, CPD-Star, digoxigenin dideoxy-UTP, terminal transferase, and blocking reagent were obtained from Roche (Laval, Quebec, Canada).

**Isolation and purification of human peripheral blood PMNs**

PMNs were purified as previously described (30). In brief, human blood was diluted with Ca2+/Mg2+-free PBS, then overlaid onto a Ficoll-Paque gradient and centrifuged at 4°C for 30 min. The blots were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% v/v Tween 20) for 5 min, washed 3 times at 0.1 mg/ml poly(A), 0.02% SDS, and 2% blocking reagent. Hybridization was performed with digoxigenin-labeled oligonucleotide probe for 18 h at 60°C (primers R7-R8) or 62°C (primers R6-R8); β actin, 55°C for 2 min), and extension 72°C (90 s). The initial denaturation period was 5 min, the final extension was 10 min. β actin was amplified as an internal control. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, visualized by ethidium bromide staining under UV illumination and blotted on Hybond N membrane (Amersham, Arlington Heights, IL) using standard methods (31). Oligonucleotide probes (R6 and R18) were labeled with digoxigenin-11-dideoxy-UTP using terminal transferase (31). The blots were prehybridized for 2 h at 42°C in hybridization solution (50% formamide, 5% SSC, 0.1% sodium laurylsarcosine, 0.1 mg/ml poly(A), 0.02% SDS, and 2% blocking reagent). Hybridization was performed with digoxigenin-labeled oligonucleotide probe for 18 h at 42°C. The blots were washed at high stringency conditions: three times at room temperature in 2X SSC, 0.05% SDS and twice at 60°C in 0.1X SSC, 0.05% SDS for 30 min each. The blots were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% v/v Tween 20) for 5 min.

**RT-PCR and Southern blot analysis**

Total cellular RNA was extracted from highly purified peripheral blood PMNs isolated from asthmatics and normal controls, or HL-60 cell line using TRIzol method (Life Technologies, Gaithersburg, MD). Reverse transcription (RT) was performed by using 2 μg of total RNA in a first-strand cDNA synthesis reaction with SuperScript reverse transcriptase as recommended by the supplier. PCR was performed by adding 1 μl of the RT product into 50 μl of total volume reaction containing 1X buffer, 200 μM of each dNTPs, 20 pmol of each oligonucleotide primer, and 0.2 U Ampli-Taq polymerase. Oligonucleotides specific for IL-9Rα sequences were used in the PCR. Oligonucleotide primers were synthesized on the basis of the entire coding region of the human IL-9Rα (GenBank accession no. M84747) as described in Table I. The PCR (IL-9Rα, 35 cycles; β actin, 25 cycles) was conducted in a thermal cycler (PTC100; MJ Research, Watertown, MA). Each cycle including denaturation (94°C, 1 min), annealing (IL-9Rα-chain, 60°C (primers R7-R8) or 62°C (primers R6-R8); β actin, 55°C for 2 min), and extension 72°C (90 s). The initial denaturation period was 5 min, the final extension was 10 min. β actin was amplified as an internal control. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, visualized by ethidium bromide staining under UV illumination and blotted on Hybond N membrane (Amersham, Arlington Heights, IL) using standard methods (31). Oligonucleotide probes (R6 and R18) were labeled with digoxigenin-11-dideoxy-UTP using terminal transferase (31). The blots were prehybridized for 2 h at 42°C in hybridization solution (50% formamide, 5% SSC, 0.1% sodium laurylsarcosine, 0.1 mg/ml poly(A), 0.02% SDS, and 2% blocking reagent). Hybridization was performed with digoxigenin-labeled oligonucleotide probe for 18 h at 42°C. The blots were washed at high stringency conditions: three times at room temperature in 2X SSC, 0.05% SDS and twice at 60°C in 0.1X SSC, 0.05% SDS for 30 min each. The blots were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% v/v Tween 20) for 5 min.

**Figure 1.** Detection of IL-9Rα-chain transcripts in human PMN preparations. A, Schematic presentation of IL-9Rα-chain cDNA and primers position used for PCR. B and C, IL-9Rα-specific amplified fragments were detected in peripheral blood human PMN RNA preparations from all asthmatics (lanes 1–7) in three of seven normal controls tested (lanes 8–14). Peripheral blood mononuclear cells and undifferentiated HL-60 cell line was used as positive and negative controls, respectively (lanes 15 and 16). Lane 17 corresponds to a PCR performed without adding cDNA. D, β actin was used as control. PMN RNA was isolated, and first-strand cDNA synthesis was performed followed by PCR amplification using specific primers to IL-9α (R7-R18), or (R6-R8) as described in Materials and Methods.

**Table I.** IL-9Rα-chain primers used for RT-PCR and Southern blot analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
<th>Position</th>
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<tbody>
<tr>
<td>R8 (sense)</td>
<td>GCTGGACCTGGAGAGTG</td>
<td>208–224</td>
</tr>
<tr>
<td>R18 (sense)</td>
<td>GCAAGATCGTTCTGCCC</td>
<td>642–662</td>
</tr>
<tr>
<td>R6 (antisense)</td>
<td>CTTTGGACCTGGACCTG</td>
<td>811–828</td>
</tr>
<tr>
<td>R7 (antisense)</td>
<td>TGCTCCAGCTCCCCAGA</td>
<td>925–954</td>
</tr>
</tbody>
</table>
FIGURE 2. Cell surface expression of IL-9Rα-chain on peripheral blood PMNs. Highly purified peripheral blood human PMNs from asthmatics (A and B) and normal controls (C and D) were analyzed with mouse anti-human IL-9Rα-chain mAb (filled histograms) and mouse IgG1 isotype-matched control Ab (dashed lines). The bold histograms represent the CD16 expression by PMNs; FITC-labeled mouse IgG1 isotype control is represented by a thin line.

**FACS analysis**

Samples of 10^5 PMN in 100 μl of 1× PBS/5% FBS were incubated for 30 min on ice with the primary Abs (mAb anti-IL-9R (5 μg/ml) or IgG1 control) in the presence of 1 mg/ml of human IgG Ig to block nonspecific binding. The cells were washed twice with PBS/2%FBS and incubated with FITC-conjugated goat anti-mouse IgG (1:200) in the dark for 30 min on ice. The cells were washed again with PBS/2%FBS, resuspended in 0.3 ml of PBS, and analyzed on FACScan. PMNs preparations were analyzed for the expression of FcyRIII using fluorescein-conjugated CD16 mAb (IgG1, dilution 1:20) or isotype-matched control used at the same concentration. The results are presented as percentage of positive cells using CellQuest software (Becton Dickinson, Mountain View, CA).

**Cytospin preparations**

Cytospin slides were prepared from BAL or peripheral blood PMNs, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed with 0.05 M Tris-HCl-buffered isotonic saline, pH 7.6 (TBS). After drying, the slides were stored at 4°C.

**Single immunohistochemistry**

The cytospin preparations of purified blood PMNs were washed with TBS, and saturated for 30 min with TBS/10% of human normal serum/5% of normal goat serum. Cells were incubated with monoclonal anti-IL-9Rα or isotype-matched control each at 5 μg/ml overnight at 4°C. After washing, rabbit anti-mouse Ig (1:600) was added for 30 min at room temperature followed by alkaline phosphatase anti-alkaline phosphatase (1:60) for 30 min at room temperature. After wash with TBS, the slides were developed using Fast red and counterstained with Mayer’s hematoxylin.

**Double immunohistochemistry**

BAL was performed as described previously (3). BAL slides were first hydrated with TBS for 5 min at room temperature followed by a blocking step with TBS/10% human normal serum/5% normal goat serum for 30 min at room temperature. After washing 5 min with TBS, polyclonal anti-IL-9Rα (1 μg/ml) or anti-neutrophil elastase mAb (1:100) in TBS/10% human normal serum were applied for 2 h at room temperature. Then preparations were washed with TBS three times for 5 min each and incubated with biotinylated horse anti-mouse IgG (1:100) in TBS/10% human normal serum for 1 h at room temperature. The cytopreparations were incubated with FITC-conjugated (Fab)2 swine anti-rabbit (1:200) and streptavidin-conjugated alkaline phosphatase (1:200) for 45 min at 37°C in dark. After the revelation step with Fast red, slides were washed and counterstained with Mayer’s hematoxylin.

**Cell line and culture conditions**

The human cell line HL-60 was provided from American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in humidified 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Differentiation toward neutrophils was performed as described previously (32). Briefly, cells were grown in RPMI 1640, 10% FBS in the presence of DMSO (1.25% V/V) for 7 days to generate neutrophils.

**PMNs stimulation and IL-8 quantification**

PMNs from asthmatics (2 × 10^6/ml) were incubated at 37°C in humidified 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics for 18 h in the presence of graded concentrations of human rIL-9 (0.1, 1, and 10 ng/ml) or medium alone. PMNs stimulated with rIFN-γ or rGM-CSF both at 10 ng/ml was performed under the same culture condition. The concentration of neutralizing goat polyclonal anti-IL-9 was chosen from preliminary concentration-response experiments. PMNs were preincubated for 1 h with either goat polyclonal anti-IL-9 or goat IgG both at 1 μg/ml in complete RPMI 1640 at 37°C in humidified 5% CO2. Cells were then stimulated or not with either rIL-9 or rGM-CSF both at 10 ng/ml. After culture, supernatants were removed, clarified by centrifugation, and stored at −80°C until analysis.

Immunoreactive IL-8 within the supernatants was quantitated using an ELISA kit.

**FIGURE 3.** Comparison between PMNs from additional asthmatic (n = 17) and normal control subjects (n = 18). The values expressed on the y-axis indicate the percentage of IL-9Rα-positive cells using CellQuest software (Becton Dickinson). Mann Whitney U test was performed within the experimental group of PMNs from asthmatics and the experimental groups of PMNs from normal controls (*, p < 0.001).
ELISA kit obtained from R&D Systems (Minneapolis, MN) according to the manufacturer’s protocol. The sensitivity limit of these kits is 10 pg/ml.

Statistics

Data are presented as mean ± SD. Statistical significance was determined using a Mann Whitney U test and paired Student’s t test. Values of p < 0.05 were considered statistically significant. Statistical analyses were performed with the use of a standard computer package (Systat version 7.0; Systat, Evanston, IL).

Results

Detection of mRNA encoding IL-9 receptor

Previous studies have shown that IL-9Rα is expressed in many hemopoietic cells associated with allergic diseases including T cells, mast cells, and macrophages (34). In this study, we first determine whether freshly isolated peripheral blood PMNs express steady-state IL-9Rα mRNA levels. RNA preparation from highly purified PMNs was first analyzed by RT-PCR. As shown in Fig. 1B, mRNA of IL-9Rα was detected in all RNA preparations from asthmatic patients (lanes 1–7) and also in three of seven PMN RNA preparations purified from normal controls (lane 8–14). The presence of IL-9Rα mRNA in human PMNs from asthmatics and normal controls was confirmed using RT-PCR with other IL-9Rα-specific primers (Fig. 1C). Furthermore, IL-9Rα-chain was detected in PBMCs used as positive control but not in undifferentiated HL-60 cell line (negative cell line), respectively (Fig. 1, B and C, lanes 15 and 16). β actin-specific amplification products were of similar intensity between all samples suggesting equality of the RNA preparations (Fig. 1D).

Cell surface expression of the IL-9Rα in human PMNs

To investigate whether the IL-9Rα is expressed on the cell surface of human PMNs, purified cells from separate donors were analyzed by cytofluorography using a mouse anti-IL-9Rα mAb. Analysis of these samples showed that the IL-9Rα-chain was detected on the surface of PMNs from asthmatic and to lesser extent in normal controls. As shown in Fig. 2, A and B, CD16-positive PMNs from two asthmatics expressed on their surface the IL-9Rα-chain with mean percentage of positivity 88 and 49%, respectively. Cell surface IL-9R expression was subsequently confirmed in additional asthmatics. In every case, the IL-9Rα-chain expression was readily detectable with mean percentage of positive cells of 44.7 ± 6.6% (n = 17, Fig. 3). In addition, although the majority of PMNs from >50% of normal did not showed IL-9Rα surface expression (n = 10 of 18) (Figs. 2D and 3), PMNs from eight normal controls showed IL-9Rα surface expression (Fig. 2C) with a mean positivity of 22.1 ± 11.6% (n = 8). Comparison between both groups showed a statistical difference (p < 0.001, Fig. 3). Taken together, these results demonstrated that human
PMNs express the IL-9Rα with high level in asthmatics compared with controls.

**Detection of IL-9Rα protein in peripheral blood and BAL PMNs by immunohistochemistry**

To further investigate the protein expression of the IL-9Rα by human PMNs, immunocytochemistry was first performed with monoclonal anti-IL-9R α Ab on human peripheral blood PMNs. A specific staining within the cytoplasm and the membrane was observed in PMNs from an asthmatic donor (Fig. 4A). In contrast, PMNs in some normal controls showed a specific staining either within the cytoplasm or on the surface (arrows in Fig. 4, C and E). Substitution of the first Ab with an isotype control eliminated the immuno-staining of the positive cells, demonstrating the specificity of the analysis (Fig. 4, B, D, and F).

We assessed the steady state of IL-9Rα protein levels within the cytoplasm of peripheral blood PMNs isolated from asthmatics and normal controls. Interestingly, while IL-9Rα was expressed in the cytoplasm of PMNs from all asthmatic donors with a variable level, little or no expression was observed in the majority of PMNs from normal controls (n = 11 of 18), and there was a significant difference in the level of IL-9Rα expression in PMNs between asthmatics and normal controls (p = 0.001, Fig. 5).

We then evaluated the expression of IL-9Rα in BAL cells from asthmatics. Positive immunoreactivity for the polyclonal IL-9Rα-chain Ab is observed in a subset of BAL cells exhibiting PMNs morphology (Fig. 4G). Similar result was observed with monoclonal anti-IL-9Rα Ab (data not shown).

To determine whether positive cells were PMNs, we performed double immunocytochemistry using mouse anti-elastase mAb, as marker of PMNs, and the polyclonal anti-IL-9Rα-chain Ab. Here we show the coexpression of IL-9Rα-chain and the elastase within the same cell (Fig. 4, I and K, respectively). However, no staining was observed on BAL cells with the isotype-matched Abs (Fig. 4, J and L). Furthermore, a positive immuno-staining was also detected in PMNs within the airways of asthmatic patients (data not shown). This result confirmed that PMNs express the IL-9Rα-chain.

**IL-9 and IL-9R mediated IL-8 release from human PMNs**

To verify that the IL-9R expressed in human PMNs was functional, we examined whether stimulating human PMNs with IL-9 could induce the release of IL-8, because IL-8 production is a common feature in inflammation process (35). Peripheral blood PMNs from asthmatics were stimulated with or without rIL-9 and evaluated for IL-8 release in culture medium after 18 h. As shown in Fig. 6, rIL-9 induced the synthesis and release of IL-8 in a dose-dependent manner. The minimal effective dose of rIL-9 was 1 ng/ml (Fig. 6A). Indeed, the levels of IL-8 released in the external milieu of rIL-9-stimulated PMNs was almost 5-fold increased when compared with control cells (192 ± 40 pg/ml vs 35 ± 18 pg/ml, respectively, p < 0.01, n = 4), and high levels of IL-8 were detected in rGM-CSF-stimulated PMNs (1117 ± 115 pg/ml, n = 4, Fig. 6B). However, a slight decrease of IL-8 release was observed when the same preparations of PMNs were incubated with rIFN-γ (27 ± 16 pg/ml, Fig. 6B) (36).

We then used anti-IL-9 neutralizing Ab to confirm that the IL-8 release is specifically mediated by IL-9 on human PMNs. PMNs from three asthmatic donors were pretreated with neutralizing goat anti-IL-9 or goat IgG at 1 μg/ml for 1 h and then stimulated either with rIL-9 or rGM-CSF. Pretreatment with anti-IL-9 Ab significantly blocked rIL-9-induced IL-8 release from PMNs (78.5% inhibition) (Fig. 7A), but had no effect on rGM-CSF-induced IL-8 (Fig. 7B). In contrast, goat IgG had no significant effect on IL-8 release from PMNs incubated with rIL-9, rGM-CSF, or medium (Fig. 7, A and B).

Taken together, these results demonstrated that IL-9R expressed by PMNs is indeed functional and that IL-9 has a direct biological role in regulating PMNs function(s).

**Discussion**

Neutrophils have long been thought to be short lived, terminally differentiated cells incapable of synthesizing significant levels of protein, with their primary function being phagocytosis and the release of cytotoxic compounds (1). More recently, it has been demonstrated that PMNs cannot only produce a number of functionally diverse substances, including cytokines, but also express receptors that have been implicated in different inflammatory reactions including allergy (2–4, 37).
Anti-IL-9 neutralizing Abs suppressed the IL-9-induced IL-8 release from human PMNs. A. The goat polyclonal anti-IL-9 Ab, but not isotype-matched control, significantly inhibited IL-8 release from PMNs after stimulation with rIL-9. B. rGM-CSF-induced IL-8 from PMNs is not affected by pretreatment with neutralizing anti-IL-9 Ab. PMNs were pretreated with goat polyclonal anti-IL-9 Ab for 1 h at 37°C, then stimulated or not with rIL-9 or rGM-CSF as described in Materials and Methods. Data are expressed as mean ± SD of three individual experiments performed in duplicate.

In this study, we investigated the expression of the IL-9R as well as the ability of IL-9 to induce functional activation of human PMNs. The expression of the IL-9Rα was established by FACS, immunohistochemistry, and RT-PCR analysis of freshly isolated human peripheral blood PMNs. Furthermore, IL-9R expression was also detected in BAL-derived PMNs from asthmatic patients. These data demonstrate that PMNs express the IL-9 cell surface receptor. We also showed that IL-9 can induce the production and the release of IL-8 by human PMNs, an effect that was blocked by anti-IL-9 neutralizing Ab. Because IL-9 has been reported to have chemotactic activity for various inflammatory cells involved in allergic diseases (35), this result provides a possible mechanism by which PMNs may contribute to the amplification of the inflammatory response. Recently, Dong et al. have reported that IL-9 also has direct activity on airway epithelial cells to produce C-C chemokines such as eotaxin (26). Together these data suggest an orchestrated activity by which IL-9 may induce chemokines in the airway, which result in a robust infiltration of inflammatory cells.

The recruitment of leukocytes to inflammatory sites is mediated by the production of a number of chemotactic mediators (37). More recently, IL-9 has been shown to induce massive airway inflammation in vivo (23–24). Our results show that in asthmatics the expression of the IL-9R in PMNs is not isotype-matched control, significantly inhibited IL-8 release from human PMNs. Furthermore, IL-9R expression was also detected in BAL-derived PMNs from asthmatic patients. These data demonstrate that PMNs express the IL-9 cell surface receptor. We also showed that IL-9 can induce the production and the release of IL-8 by human PMNs, an effect that was blocked by anti-IL-9 neutralizing Ab. Because IL-9 has been reported to have chemotactic activity for various inflammatory cells involved in allergic diseases (35), this result provides a possible mechanism by which PMNs may contribute to the amplification of the inflammatory response. Recently, Dong et al. have reported that IL-9 also has direct activity on airway epithelial cells to produce C-C chemokines such as eotaxin (26). Together these data suggest an orchestrated activity by which IL-9 may induce chemokines in the airway, which result in a robust infiltration of inflammatory cells.

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