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

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

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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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Polymorphonuclear neutrophils (PMNs) are well recognized as important effector cells in host defense and IgG-mediated humoral immune responses (1). Although they have been viewed primarily as a phagocytic cell type, they do synthesize and release a multitude of inflammatory mediators. These include the proinflammatory cytokines TNF-α, TGF-β, and IL-1β; the growth factor GM-CSF; and the chemokines macrophage inflammatory protein-1α and -1β, IL-8, and IFN-induced protein-10 (2). As such, PMNs have the potential to produce mediators considered to be involved in the pathogenesis of various inflammatory diseases (1–3).

Although the role of PMNs in the pathogenesis of allergic disease remains undefined, studies have reported evidence linking the presence of PMNs with airway damage and dysfunction (4–6). In particular, the number of PMNs detected in bronchoalveolar lavage (BAL) and airway mucosa has been correlated with the degree of lung dysfunction (7–9). Furthermore, it has recently become apparent that PMNs express many cytokine and chemokine receptors such as IL-4, IL-13, GM-CSF, and IL-8, as well as IgE receptors (CD23/FcεRII, galectin-3/Mac-2) (10–15), indicating the ability of these cells to respond to the stimuli considered integral to the allergic process.

IL-9 is a Th2 cytokine first described in the mouse as a T cell growth factor (16–18). Recently, a role of IL-9 in asthma and allergy has been supported by the findings that it has pleiotropic activities on cell types associated with allergic diseases including Th2 lymphocytes, mast cells, B cells, eosinophils, and airway epithelial cells (19–26). The functions of IL-9 are mediated by the IL-9 receptor (IL-9R), which is a member of the hemopoietin receptor superfamily (27). The IL-9R consists of ligand specific α-chain and γ-chain that is shared with IL-2, IL-4, IL-7, and IL-15 receptors (25). More recently, the IL-9R locus has been genetically linked to asthma and broncho-hyperresponsiveness in humans (28).

In view of these studies, and based on the evidence that complex interactions between various inflammatory cells, including the Th2 subset and PMNs, may occur during the bronchial inflammation associated with asthma, we investigated whether human PMNs express the IL-9R and respond to this inflammatory-associated cytokine. Here, we report that PMNs from asthmatic patients express high steady-state levels of IL-9Rα mRNA and protein compared with normal subjects. Furthermore, IL-9 stimulates PMNs to produce and release IL-8, an effect that is significantly reduced by anti-IL-9 neutralizing Ab.

Materials and Methods

Subjects

This study was approved by the Ethics Committee of the Montreal Chest Hospital (Montreal, Quebec). Seventeen asthmatic patients, as defined by the American Thoracic Society, were included in this study (29). Twenty-four nonatopic nonasthmatic controls, with normal spirometry were also studied. Patients had not received inhaled or systemic corticosteroids in the last 3 mo and were not receiving any medications other than β2 agonists. Subjects who had upper respiratory tract infection within the last month were excluded from the study.

Reagents and Abs

Rabbit polyclonal affinity-purified anti-IL-9 (C18) α-chain directed to C-terminal intracellular domain-specific FITC-conjugated goat anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb anti-IL-9α directed to N-terminal extracellular domain, neutralizing goat anti-human IL-9, human rGM-CSF, and rIFN-γ were purchased from R&D (Minneapolis, MN). Recombinant human IL-9 was purchased from Calbiochem (La Jolla, CA). Normal rabbit serum was obtained from Cedarlane Laboratories (Toronto, Ontario, Canada). Biotinylated horse anti-mouse IgG was obtained from Vector Laboratories (Burlingame, CA). Affinity-purified human IgG, FITC-conjugated mouse
with anti-CD16-coated microbeads for 30 min at 4°C in PBS/1% BSA, and RBCs were lysed with hypotonic saline. Granulocytes were then incubated

Table I.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8 (sense)</td>
<td>GCTGGACCTGGAGAGTTG</td>
<td>208–224</td>
</tr>
<tr>
<td>R18 (sense)</td>
<td>GCAAGATCAGTTCTGACC</td>
<td>642–662</td>
</tr>
<tr>
<td>R6 (antisense)</td>
<td>CTTTTGAGCTGGACCTTG</td>
<td>811–828</td>
</tr>
<tr>
<td>R7 (antisense)</td>
<td>TGCTTCAGGCTCCCGAG</td>
<td>925–954</td>
</tr>
</tbody>
</table>

(IgGI) anti-CD16, IgGI isotype control (clone MOPC21), FITC-conjugated mouse anti-CD16 (clone 3G8), FITC-conjugated mouse IgGI 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 phosphatase alkaline 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, 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 1% buffer, 200 μmol 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 ultraviolet light. 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 1% buffer, 200 μmol 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 ultraviolet light 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

![FIGURE 1](http://www.jimmunol.org/)  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.
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 ng/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 20°C before immunocytochemistry.

Single immunohistochemistry

The cytospreparations of purified blood PMNs were washed with TBS, and saturated for 30 min with TBS/10% human normal serum/5% normal goat serum. After washing for 5 min with CPD-Star diluted in detection buffer (1:100). For detection of chemiluminescent signal, the blots were exposed to x-ray film for 20 min at room temperature.

PMNs stimulation and IL-8 quantification

Freshly isolated 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 (100 U/ml penicillin and 100 μg/ml streptomycin). Differentiation toward neutrophils was performed as described previously (33). Briefly, cells were grown in RPMI 1640, 10% FBS in the presence of DMSO (1.25%V/V) for 7 days to generate neutrophils.

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 (33). Briefly, cells were grown in RPMI 1640, 10% FBS in the presence of DMSO (1.25%V/V) for 7 days to generate neutrophils.

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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% ($n = 17$, mean value, 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-9Rα 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 immunostaining 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 immunostaining 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-stimulated 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).

FIGURE 6. Effect of IL-9 on the release of IL-8 by PMNs. A. Concentration-dependent effect of rIL-9 on the release of IL-8 by PMNs. Assays were performed in duplicate on (2 × 10⁶) PMNs from the same donor. These results are representative of three independent experiments performed under the same conditions (*, p < 0.05). B. IL-8 release from PMNs stimulated with either rIL-9, rIFNγ, or rGM-CSF. Data are expressed as mean ± SD of four individual experiments performed in duplicate. Student’s t test was performed within the experimental group of PMNs incubated with medium alone and the experimental groups of PMNs incubated with 10 ng/ml of rIL-9 or rGM-CSF (*, p < 0.05).
The expression of the IL-9Ra protein on human PMNs was widely heterogeneous according to individual donors, and there was a significant difference between the percentage of IL-9Ra-positive PMNs in asthmatics compared with normal controls analyzed by FACS and immunohistochemistry analysis (p < 0.02). This suggests that the expression of IL-9Ra is under regulatory control. A number of potential explanations for this are under investigation. Th2 cytokines highly expressed in allergic diseases may up-regulate the IL-9Ra expression on PMNs from asthmatics. This is in line with previous report indicating that the Th-2 cytokines, in particular GM-CSF and IL-4, exert several actions on PMNs, including stimulation or changes in expression of many surface receptors (8, 10–11, 39). Further support for this possibility comes from studies showing that both IL-4 and GM-CSF up-regulate the expression of IL-9Ra in PMNs (S. G. Abdelilah, unpublished observation).

Although >50% of PMNs from normal controls do not express the IL-9Ra as described recently (40), an interesting feature of our study was that PMNs from some normal controls have an intracellular store but no surface expression. This suggests that in this particular subpopulation of PMNs the IL-9Ra protein may be associated with a yet to be defined structure(s) that precludes its surface expression. Furthermore, the γ-chain is shared by IL-2, IL-4, and IL-15 receptors (25), which have been shown to be expressed on the surface of human PMNs (11, 41, 42). One can speculate that the surface expression of those cytokine receptors may have a negative regulatory effect on IL-9Ra surface expression by competing for γ-chain during IL-9R assembly. This situation was recently reported for FcεRI in mast cells (43). In contrast, the coupling mechanisms leading to active association of IL-9R subunits are deficient or are themselves subject to distinct regulatory signals in PMNs from some normal controls. Furthermore, PMNs from some normal controls showed IL-9Ra surface expression that suggest that this population of PMNs are stimulated in vivo by factors other than Th-2 cytokines. Further studies are in progress to clarify these and other possible mechanism with regard to the regulation of IL-9R in PMNs.

In the context of local allergic inflammation, the release of IL-8 by human PMNs after IL-9 stimulation may be involved in the recruitment of inflammatory cells (23–24, 34). In line with these suggestions, IL-8 has been previously shown to be a chemotactic factor for activated T lymphocytes, eosinophils, and basophils and to enhance the expression of integrins on monocytes as well as their adherence to endothelial cells (35). Collectively, the results of this study provide a novel mechanism by which PMNs may contribute to the inflammatory reaction via an IL-9-dependent mechanism.

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References


