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

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

*J Immunol* 2001; 166:2768-2774; doi: 10.4049/jimmunol.166.4.2768
http://www.jimmunol.org/content/166/4/2768

**References**
This article cites 40 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/166/4/2768.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Functional Expression of IL-9 Receptor by Human Neutrophils from Asthmatic Donors: Role in IL-8 Release

Soussi Gounni Abdelilah, Koussih Latifa, 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.

The Journal of Immunology, 2001, 166: 2768–2774.

Abbreviations used in this manuscript: PMNs, polymorphonuclear neutrophils; BAL, bronchoalveolar lavage; RT, reverse transcription.
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>
</tr>
</thead>
<tbody>
<tr>
<td>R8 (sense)</td>
<td>GCTGGACCTGGAGAGTG</td>
<td>208–224</td>
</tr>
<tr>
<td>R18 (sense)</td>
<td>GCAAGATCGATCTGGCC</td>
<td>642–662</td>
</tr>
<tr>
<td>R6 (antisense)</td>
<td>CTTTTAGCTGGACCTGT</td>
<td>811–828</td>
</tr>
<tr>
<td>R7 (antisense)</td>
<td>TGCTTCCAGGCTCCCCAGA</td>
<td>925–954</td>
</tr>
</tbody>
</table>

(rg1) 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 Fab'1, swine anti-rabbit IgG, mouse alkaline phosphatase-anti-alkaline phosphatase, Fast red, and streptavidin alkaline phosphatase were obtained from Dako (Dakopatts, 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). Taq 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 Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS, then overlaid onto a Ficoll-Paque gradient and centrifuged at 400 g for 20 min. The granulocyte-rich fraction was then mixed with dextran 70, and the RBCs were allowed to sediment for 30 min at room temperature. Supernatants were collected and centrifuged for 10 min at 400 g to recover the granulocytes, and residual RBCs were lysed with hypotonic saline. Granulocytes were then incubated with anti-CD16-coated microbeads for 30 min at 4°C in PBS/1% BSA, and washing the cells through a MACS column eluted the contaminating cells (mainly eosinophils). The differential cell count was consistently >98% PMNs as determined by staining cytospin preparations with Diff-Quick (Fisher Scientific, Ontario, Canada). The viability of the cells was >98% as determined by trypan blue exclusion.

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 1% buffer, 200 μmol of each dNTPs, 20 pmol of each oligonucleotide primer, and 0.2 U Ampli-Taq polymerase. Oligonucleotide probes 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 2× SSC, 0.05% SDS and twice at 60°C in 0.1× 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. 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.](http://www.jimmunol.org/)
min, then incubated goat-anti digoxigenin-alkaline phosphatase Fab (1: 20,000) in blocking solution for 30 min, washed twice with washing buffer at room temperature for 15 min each. After equilibration with detection buffer (100 mM Tris-HCl, 100 MM NaCl; pH 9.5) for two min, the blots were incubated 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.

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 (filled histograms) and mouse 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 cytopreparations 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 (1:60) 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 (Surgipath Canada, Manitoba, Canada).

Double immunohistochemistry

BAL was performed as described previously (32). 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 for 5 min with TBS, polyclonal anti-IL-9α (1 μg/ml) and 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 (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.

PMNs stimulation and IL-8 quantification

Freshly isolated PMNs from asthmatics (2 × 10^9/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 concentration of human rIL-9 (0.1, 1, and 10 ng/ml) or medium alone. PMNs stimulated with rIL-8 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. Immunoactive IL-8 within the supernatants was quantitated using an
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. 1, B, 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 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 normals. 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 was 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 = 34, 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).
The expression of the IL-9Rα protein on human PMNs was widely heterogeneous according to individual donors, and there was a significant difference between the percentage of IL-9Rα-positive PMNs in asthmatics compared with normal controls analyzed by FACS and immunohistochemistry analysis (p < 0.02). This suggests that the expression of IL-9Rα 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-9Rα 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-9Rα in PMNs (S. G. Abdelilah, unpublished observation).

Although >50% of PMNs from normal controls do not express the IL-9Rα 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-9Rα 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 these cytokine receptors may have a negative regulatory effect on IL-9Rα 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-9Rα 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.

Acknowledgments

We thank Dr. Z. Zhu and Dr. J. Elias for IL-8 quantification. We also acknowledge Elsa Schotman for technical assistance.

References

18. Renauld, J. C., A. Goethals, F. Houssiau, H. Merz, E. Van Roost, and 
14. Truong, M. J., V. Gruart, J. P. Kusnierz, J. P. Papin, S. Loiseau, A. Capron, and 
5. Verheyen, D., C. N. Nicolaides, K. J. Holroyd, and R. C. Levitt. 1995. Genetic susceptibility to asthma bronchial hyperplasia, and bronchial hyperresponsiveness linked to the XY long arm pseudou-
3. Fiskoff, S. A. 1988. Graded increase probability of eosinophilic differentiation of 