

Leptin-mediated cytokine release and migration of eosinophils: Implications for immunopathophysiology of allergic inflammation

Chun Kwok Wong¹, Phyllis F.-Y. Cheung¹ and Christopher W. K. Lam^{1,2}

¹ Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong

² Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Taipa, Macau

Leptin is a pleiotropic adipocyte-derived cytokine used in hypothalamic regulation of body weight and modulation of immune response by stimulating T cells, macrophages and neutrophils. Leptin has been shown to be an eosinophil survival factor. We examined the immunopathological mechanisms for the activation of human eosinophils from healthy volunteers by leptin in allergic inflammation. Adhesion molecules, cytokines and cell migration were assessed by flow cytometry, ELISA and Boyden chamber assay, respectively. Intracellular signaling molecules were investigated by membrane array and Western blot. Leptin could up-regulate cell surface expression of adhesion molecule ICAM-1 and CD18 but suppress ICAM-3 and L-selectin on eosinophils. Leptin could also stimulate the chemokinesis of eosinophils, and induce the release of inflammatory cytokines IL-1 β and IL-6, and chemokines IL-8, growth-related oncogene- α and MCP-1. We found that leptin-mediated induction of adhesion molecules, release of cytokines and chemokines, and chemokinesis were differentially regulated by the activation of ERK, p38 MAPK and NF- κ B. In view of the above results and elevated production of leptin in patients with allergic diseases such as atopic asthma and atopic dermatitis, leptin could play crucial immunopathophysiological roles in allergic inflammation by activation of eosinophils *via* differential intracellular signaling cascades.

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Introduction

Leptin, an adipokine of the obesity (ob) gene [1], has been shown to regulate food intake and energy expenditure *via* a hypothalamic-mediated effect [2]. It can act as part of a feedback mechanism signaling to the brain the size of the available energy resources as a

function of the body's fat stores, and its plasma concentration correlates positively with adipose tissue mass [3, 4]. In addition, leptin has been considered to be a pleiotropic cytokine because of its diversified effects on angiogenesis, hematopoiesis, lipid and carbohydrate metabolism, and on the reproductive, cardiovascular and immune systems [5].

Leptin receptor (ObR), is a member of class I cytokine receptor superfamily and has at least six isoforms (obRa–obRf) sharing an identical extracellular ligand-binding domain [6]. One “long form” of ObR contains a long intracellular domain (obRb), whereas obRa, obRc, obRd and obRf possess short intracellular domains [7]. Leptin's functional receptor (ObRb) is expressed in the hypothalamus and all cell types of innate and adaptive immunity including T and B lymphocytes, natural killer

Correspondence: Christopher Wai-kei Lam, Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong
Fax: +852-2636-5090
e-mail: waikeilam@cuhk.edu.hk

Abbreviations: **GRO:** growth-related oncogene · **LFA-1:** leukocyte function-associated antigen-1 · **ob:** obesity · **ObR:** leptin receptor

cells, monocytes, and eosinophils [8–13]. Leptin is a link between adipose tissue and inflammation because it can induce Th1-mediated immune response, cytokine production such as IFN- γ and reverse starvation-induced immunosuppression [8, 14]. In addition, leptin can induce proliferation and differentiation of leukocytes, activate the phagocytosis of macrophages, and up-regulate the expression of adhesion molecules on endothelial cells [5, 15]. High leptin concentration may lead to an abnormally active immune response predisposing to autoimmune reaction [14].

In peripheral blood mononuclear cells (PBMC), leptin binding to its functional receptor activates intracellular Janus-activated kinases (JAK)-signal transducer and activator of transcription factor (STAT) pathways, mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3'-kinase (PI3K) pathways [16–18]. The MAPK pathway seems to mediate anti-apoptotic effects [19], whereas the PI3K pathway may be important in regulating glucose uptake in PBMC [20].

Previous studies have indicated that serum leptin concentration was elevated during allergic reactions in the airways and IgE-associated atopic dermatitis, and may play a role in the relationship between obesity and allergic inflammation [21, 22]. Weight reduction in obese patients with asthma results in decline in disease severity and symptoms [23]. In an experimental study, leptin was shown to exhibit anti-apoptotic effects on eosinophils, the prominent effector cells of allergic inflammation, via the delayed cleavage of Bax [11]. However, the exact intracellular mechanisms are not well defined for the association of obesity-related leptin and allergic asthma. To further elucidate the immunopathological mechanisms for the activation of eosinophils in obesity-related allergic inflammation, we investigated the intracellular signaling mechanisms regulating the expression profile of adhesion molecules including those of the (i) integrin family: leukocyte function-associated antigen (LFA-1) (CD11a/CD18); (ii) selectin family: L-selectin (CD62L); and (iii) Ig family: intercellular adhesion molecule (ICAM)-1 (CD54) and ICAM-3 (CD50) [24], cytokine release, and cell migration of eosinophils activated by leptin.

Results

Effect of leptin on enhancing survival of eosinophils

After culture for 24 h, the cell viability was 75.6% for leptin (50 ng/mL)-treated eosinophils compared to only 46.1% without leptin treatment (Fig. 1).

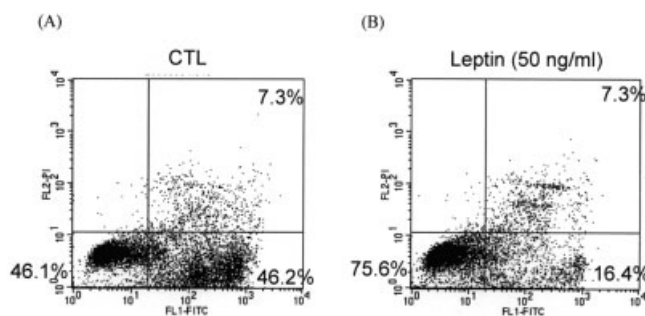


Figure 1. Effects of leptin on viability on eosinophils. Eosinophils (5×10^5 /well) were cultured with or without leptin (50 ng/mL) for 24 h in a 24-well plate. Apoptosis of eosinophils was assessed by the TACSTM Annexin V-FITC assay using flow cytometry. Representative dot plots of (A) medium control (CTL), and (B) leptin-treated eosinophils were shown from triplicate experiments.

Effect of leptin on the expression of adhesion molecules on human eosinophils

To generate the expression profile of cell surface adhesion molecules that is regulated by leptin, the effect of leptin on ICAM-1, ICAM-3, L-selectin and CD18 on eosinophils was examined. Fig. 2 shows that the dose-response of leptin inducing effects on the surface expression of ICAM-1, ICAM-3, L-selectin and CD18. We found that leptin (10–100 ng/mL) could significantly up-regulate the surface expression of ICAM-1 and CD18 (Fig. 2B and C), while down-regulating those of ICAM-3 and L-selectin (Fig. 2D and E) in a dose-dependent manner.

Effect of leptin on cell migration of eosinophils *in vitro*

To verify the stimulatory effects of leptin on the chemotactic or chemokinetic behavior of eosinophils, checkerboard analysis was performed with the combination of various concentrations of leptin and RANTES, a potent eosinophil chemoattractant, in upper and lower compartment of the Boyden chamber. As shown in Table 1A, leptin alone at upper compartment of Boyden chamber (50 and 100 μ g/mL) could induce chemokinetic eosinophil migration. However, RANTES but not leptin (50 and 100 μ g/mL) alone at lower compartment could induce eosinophil chemotaxis (Table 1B). These results indicate that the effect of leptin on eosinophil migration is chemokinetic rather than chemotactic. RANTES-induced chemotaxis in eosinophils was significantly enhanced by leptin in a dose-dependent manner (0–100 ng/mL) (all $p < 0.05$) (Table 1B).

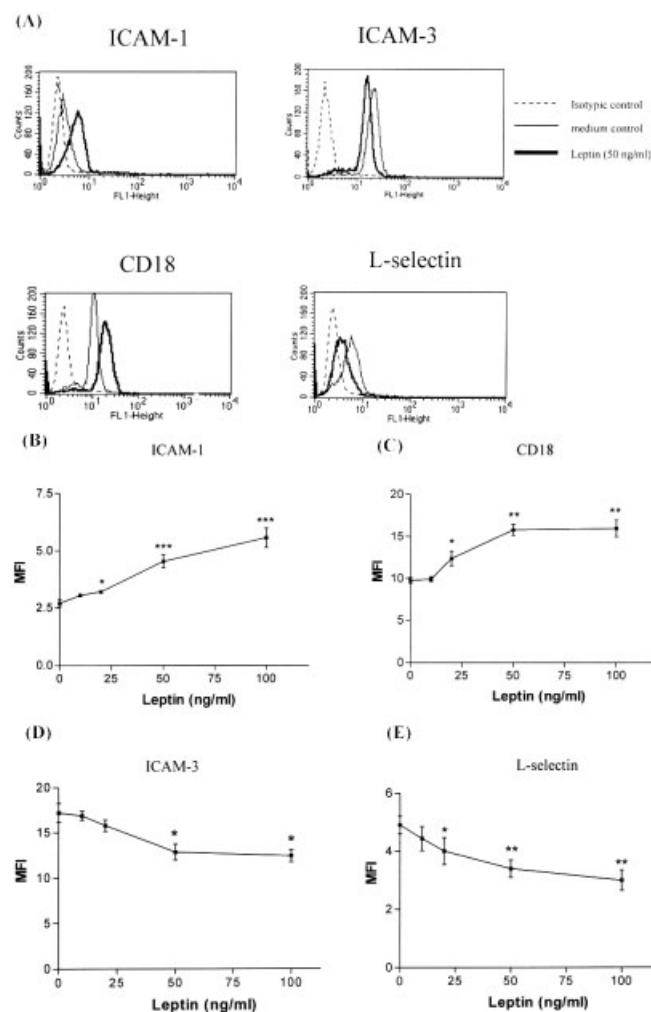


Figure 2. Effects of leptin on cell surface expression of ICAM-1, CD18, ICAM-3 and L-selectin on eosinophils. Eosinophils (5×10^5 /well) were cultured with leptin (0–100 ng/mL) for 16 h in a 24-well plate. Surface expression of adhesion molecules per 10 000 viable cells was analyzed by flow cytometry as MFI; (A) representative histograms of the changes in the expression of adhesion molecules. Results of (B) ICAM-1, (C) CD18, (D) ICAM-3 and (E) L-selectin have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean $\pm$ SD of five independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control.

Effect of leptin on the release of chemokines and IL-6 from eosinophils

We firstly assessed the cytokine expression profile using an antibody-based human cytokine protein membrane array. We found that leptin (50 ng/mL) could activate eosinophils to markedly induce the release of inflammatory cytokines IL-1 β and IL-6, and chemokines IL-8, monocyte chemoattractant protein (MCP)-1 and growth-related oncogene (GRO) among the 79 different cytokines being screened after 24-h incubation com-

pared to that of control (data not shown). As shown in Fig. 3, leptin could activate eosinophils to release IL-1 β , IL-6, IL-8, GRO- α and MCP-1 dose dependently (0–100 ng/mL) at 12 and 24 h. Moreover, the release at 12-h incubation time points was significantly lower than that at the 24-h time points (all $p < 0.05$) (Fig. 3).

Effects of neutralization antibody against leptin on its biological activities on eosinophils

As shown in Fig. 4, neutralization antibody against human leptin (0.1–10 μ g/mL) could abolish the leptin-mediated survival, cell surface expression of adhesion molecules, chemokinesis and induction of cytokines and chemokines, thereby indicating that the observed biological activities were due to leptin effects but not due to impurities of the leptin preparation.

Effect of leptin on the MAPK and NF- κ B activity of eosinophils

As shown in Fig. 5, the results of phosphorylation profile of MAPK and other serine/threonine kinases demonstrated that leptin (50 ng/mL) could induce the phosphorylation of ERK1 and p38 δ of eosinophils at 15-min incubation. Fig. 6 shows that leptin (50 ng/mL) could activate eosinophils by rapidly inducing the phosphorylation of ERK, p38 MAPK, JAK2 and I κ B- α within 30 min. These results confirmed the activation of ERK1, p38 δ , JAK and NF- κ B activity in eosinophils by leptin.

Effects of signaling molecule inhibitors on viability and apoptosis of leptin-treated eosinophils

Based on the results of viability test on eosinophils, we chose the following selective signaling molecule inhibitors AG490 (10 μ M), BAY11-7082 (1 μ M), PD98059 (10 μ M), LY294002 (5 μ M), SB203580 (7.5 μ M) and SP600125 (3 μ M) for the subsequent inhibition experiments. Fig. 7 shows that pre-treatment of eosinophils with JAK inhibitor AG490, I κ B kinase inhibitor BAY11-7082 and p38 MAPK inhibitor SB203580 for 1 h could significantly suppress the leptin-enhanced survival of eosinophils (all $p < 0.05$).

Effects of signaling molecule inhibitors on leptin-regulated activities of eosinophils

Fig. 8 shows that pre-treatment of eosinophils with I κ B kinase inhibitor BAY11-7082, ERK inhibitor PD98059 and p38 MAPK inhibitor SB203580 for 1 h could significantly suppress the leptin-induced up-regulation of ICAM-1 and CD18 and restore the leptin-induced

Table 1. Checkerboard analysis of the effect of leptin and RANTES on the migration of eosinophils^{a)}

Lower compartment:		Upper compartment: Leptin (ng/mL)			
		0	10	50	100
(A) Leptin (ng/mL)	0	16.7 ± 3.1	26.7 ± 7.6	46.0 ± 7.2**	66.7 ± 6.1**
	10	12.0 ± 3.5	40.7 ± 10.3	44.0 ± 10.6*	77.3 ± 16.0*
	50	28.0 ± 10.6	46.0 ± 12.2	62.7 ± 14.7*	74.7 ± 11.0*
	100	13.3 ± 2.3	50.0 ± 8.0*	82.0 ± 9.2**	117.3 ± 25.3*
(B) RANTES (ng/mL)	0	16.7 ± 3.1	26.7 ± 7.6	46.0 ± 7.2**	66.7 ± 6.1**
	10	16.0 ± 7.6	49.0 ± 15.4*	52.0 ± 9.6*	124.0 ± 4.6***
	50	44.0 ± 8.7*	73.0 ± 1.7***	161.0 ± 23.3**	167.7 ± 15.7**
	100	43.0 ± 6.2*	120.0 ± 23.4*	150.0 ± 16.7**	178.0 ± 24.4**

^{a)} Eosinophils (5×10^4 cells) were treated with leptin (0–100 ng/mL) for 16 h, and then incubated in a 48-well microchamber in the upper chambers, while the RPMI medium with (A) leptin (0–100 ng/mL) and (B) RANTES (0–100 ng/mL) was placed in the lower chambers. Migration was carried out for 6 h. After the incubation, the number of migrated cells was counted. Results are expressed as arithmetic mean ± SD from triplicate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with medium control.

down-regulation of ICAM-3 and L-selectin (all $p < 0.05$). As shown in Fig. 9, BAY11–7082, PD98059 and SB203580 could also significantly abolish the leptin-

induced chemokinetic migration of eosinophils (all $p < 0.05$).

Effects of different signaling molecule inhibitors on leptin-induced cytokine/chemokine release from eosinophils

Fig. 10 shows that leptin-induced release of IL-1 β and IL-6, and IL-8, GRO- α and MCP-1 was suppressed by BAY11–7082 and SB203580, and BAY11–7082, PD98059 and SB203580, respectively (all $p < 0.05$).

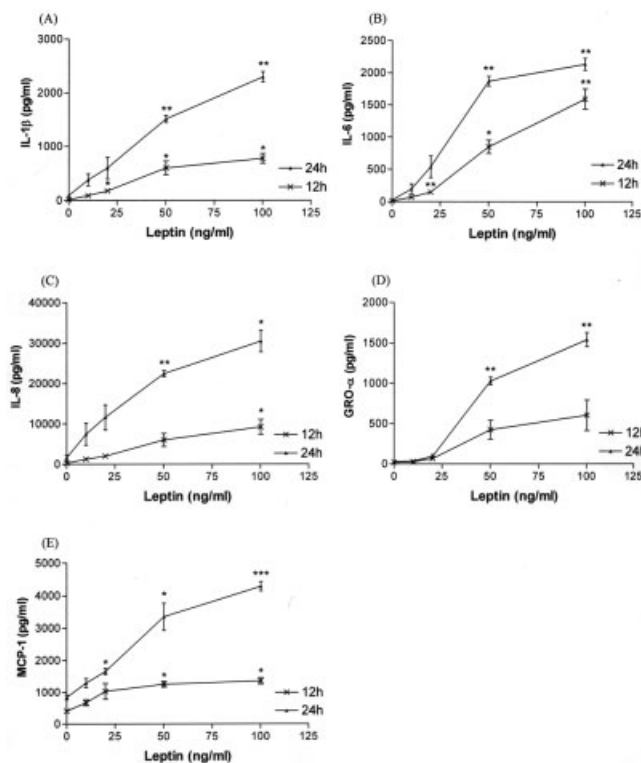


Figure 3. Leptin-induced release of (A) IL-1 β , (B) IL-6, (C) IL-8, (D) GRO- α , and (E) MCP-1 from eosinophils. Eosinophils (5×10^5 /well) were cultured with leptin (0–100 ng/mL) for 12 and 24 h in a 24-well plate. Chemokines and cytokines released into the culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control.

Discussion

Previous studies have implicated leptin in the regulation of the immune system by the stimulation of Th1 cells in Th1 immunity [8], neutrophils and monocytes in innate immunity [19, 25], and delaying the apoptosis of eosinophils [11]. However, the intracellular regulatory mechanisms of leptin on the activation of biological functions, including the release of cytokines and chemokines, and cell migration of eosinophils, are not well understood.

In the present study, we found that leptin could significantly up-regulate the cell surface expression of ICAM-1 and CD18, but suppress those of ICAM-3 and L-selectin dose dependently (Fig. 1). However, this cytokine did not induce any significant change in surface expression of CCR3 receptor for eotaxin, an eosinophil-specific chemokine (data not shown), which may be due to the differential regulation of intracellular signal transduction pathways responsible for different adhesion molecules and chemokine receptors. ICAM-1 has

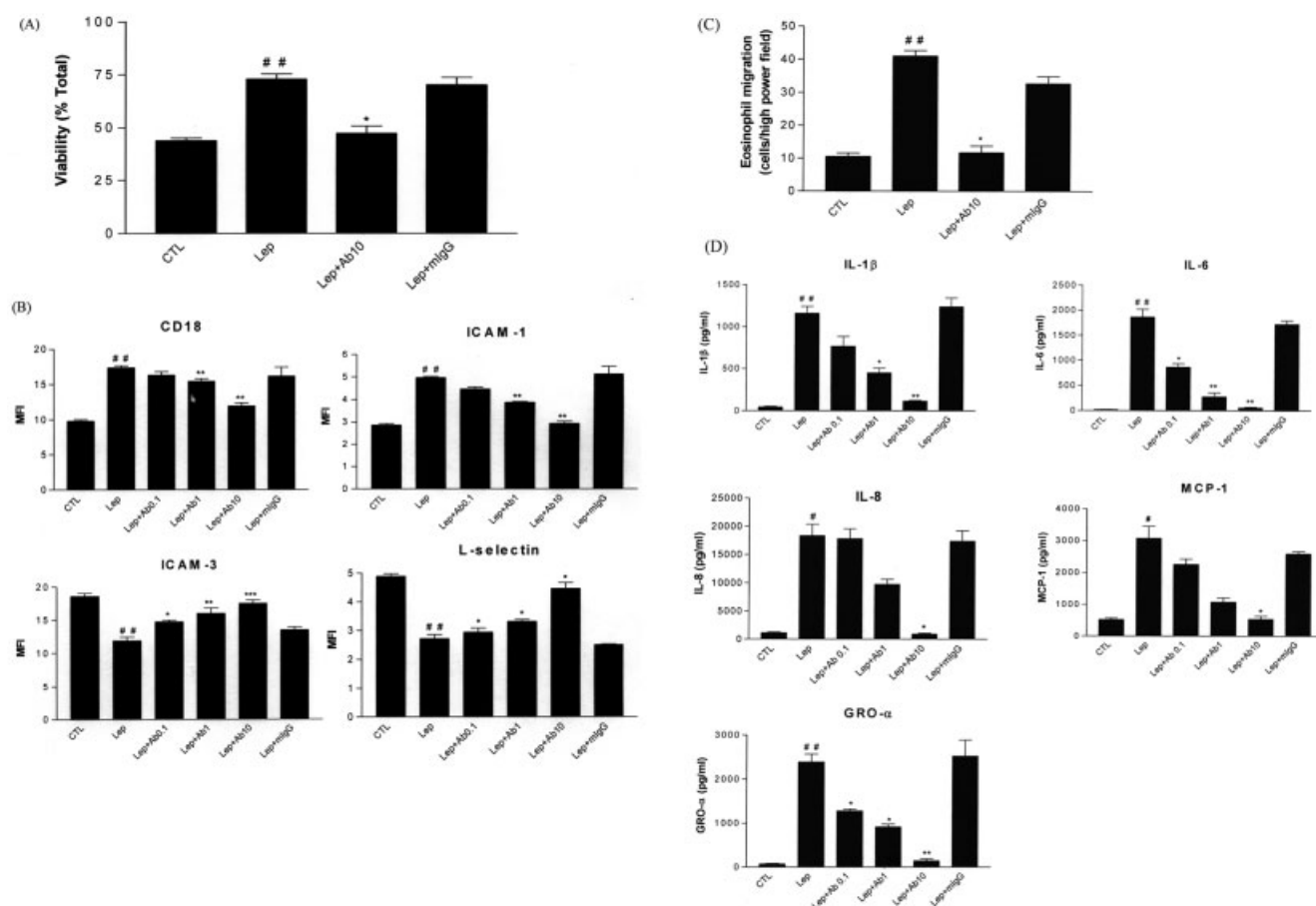


Figure 4. Effects of neutralization antibody against leptin on the biological activities of leptin on eosinophils. Leptin was incubated with or without neutralization antibody (0.1–10 μ g/mL) for 15 min and then incubated with eosinophils (5×10^5 /well) for the assay of (A) apoptosis, (B) cell surface of adhesion molecules, (C) chemokinesis and (D) induction of cytokines. Results were expressed as arithmetic mean plus SD from triplicate experiments. Mouse IgG1 was used as a nonspecific antibody for isotypic antibody control. # $p < 0.05$; ## $p < 0.01$ when compared with medium control (CTL); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with leptin control (lep). Ab0.1: neutralization antibody (0.1 μ g/mL); Ab1: neutralization antibody (1 μ g/mL); Ab10: neutralization antibody (10 μ g/mL); mIgG: mouse IgG1 (10 μ g/mL).

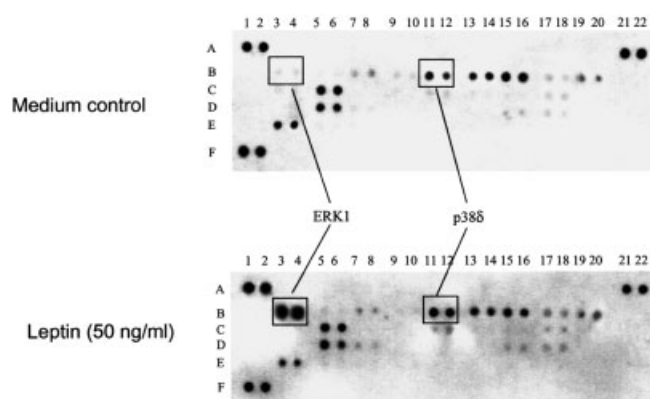


Figure 5. Representative expression profiles of intracellular phosphorylation of MAPK and other serine/threonine kinases of eosinophils upon activation by leptin. Eosinophils (1×10^7 /well) were treated with or without leptin (50 ng/mL) for 15 min. Eosinophils were then washed and lysed. Twenty-one different intracellular phosphorylated MAPK and other serine/threonine kinases in total cellular lysate were assessed semi-quantitatively using an antibody based Human Phospho-MAPK Array Kit. Positive and negative controls were designated at (A1, A2, A21, A22, F1, F2) and (E3–E14), respectively. Triplicate experiments were performed with essentially identical results. The format of the antibodies on the array are detailed in Table 2.

been well demonstrated to potentiate inflammatory process in childhood asthma [26]. The interaction of ICAM-1 and integrins has been shown to be essential for the recruitment and transendothelial migration of eosinophils [27]. The up-regulation of both ICAM-1

and CD18 expression is therefore essential for the recruitment and transmigration of eosinophils into inflammatory sites [24, 28]. ICAM-1 can bind to fibronectin, which is a broad range cell adhesion molecule [29]. Our previous study showed that up-

Table 2. The format of antibodies on the Human Phospho-MAPK membrane array

Coordinate	Target/control	Alternate nomenclature	Phosphorylation site detected
A1, A2	Positive control	Control (+)	-
A21, A22	Positive control	Control (+)	-
B3, B4	ERK1	MAPK3, p44 MAPK	T202/Y204
B5, B6	JNK1	MAPK8, SAPK1 γ	T183/Y185
B7, B8	JNK pan	-	T183/Y185, T221/Y223
B9, B10	p38 γ	MAPK12, SAPK3, ERK6	T183/Y185
B11, B12	p38 δ	MAPK13, SAPK4	T180/Y182
B13, B14	RSK1	MAPKAPK1 α , RPS6KA1	S380
B15, B16	GSK-3 α/β	GSK3A/GSK3B	S21/S9
B17, B18	Akt1	PKB α , RAC α	S473
B19, B20	Akt2	PKB β , RAC β	S474
C3, C4	ERK2	MAPK1, p42MAPK	T185/Y187
C5, C6	JNK2	MAPK9, SAPK1 α	T183/Y185
C9, C10	p38 α	MAPK14, SAPK2A, CSBP1	T180/Y182
C11, C12	P38 β	MAPK11, SAPK2B, p38-2	T180/Y182
C13, C14	RSK2	ISPK-1, RPS6KA3	S386
C15, C16	GSK-3 β	GSK3B	S9
C17, C18	Akt3	PKB γ , RAC γ	S472
C19, C20	Akt pan	-	S473, S474, S472
D5, D6	JNK3	MAPK10, SAPK1 β	T221/Y223
D7, D8	MSK2	RSK β , RPS6KA4	S360
D15, D16	HSP27	HSPB1, SRP27	S78/S82
D17, D18	p70 S6 kinase	S6K1, p70 α , RPS6KB1	T421/S424
E3, E4	Rabbit IgG	Control (-)	-
E5, E6	Mouse IgG ₁	Control (-)	-
E7, E8	Mouse IgG _{2A}	Control (-)	-
E9, E10	Mouse IgG _{2B}	Control (-)	-
E11, E12	Goat IgG	Control (-)	-
E13, E14	PBS	Control (-)	-
F1, F2	Positive control	Control (+)	-

regulation of surface ICAM-1 expression by a novel Th2 cytokine IL-25 could lead to subsequent adhesion of eosinophils onto fibronectin [30], thereby shedding light on the role of enhanced level of surface ICAM-1 expression on the cells.

Unlike ICAM-1, ICAM-3 is highly expressed on resting eosinophil surface. However, the significance of this high expression level remains unclear. A previous study has shown that the expression level of ICAM-3 on eosinophils was significantly reduced after exposure to the combination of granulocyte-macrophage colony-

stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- α [31]. Moreover, ligation of ICAM-3 on eosinophils could suppress GM-CSF synthesis, suggesting that ICAM-3 might possess anti-inflammatory function [31]. From our present results, eosinophils exposed to leptin appeared to switch to a “suppressed ICAM-3 and enhanced ICAM-1” status, favoring the proinflammatory responses induced by up-regulation of ICAM-1.

In addition to ICAM-3, we found that L-selectin expression was also significantly suppressed by leptin (Fig. 1). This finding concurs with several other studies

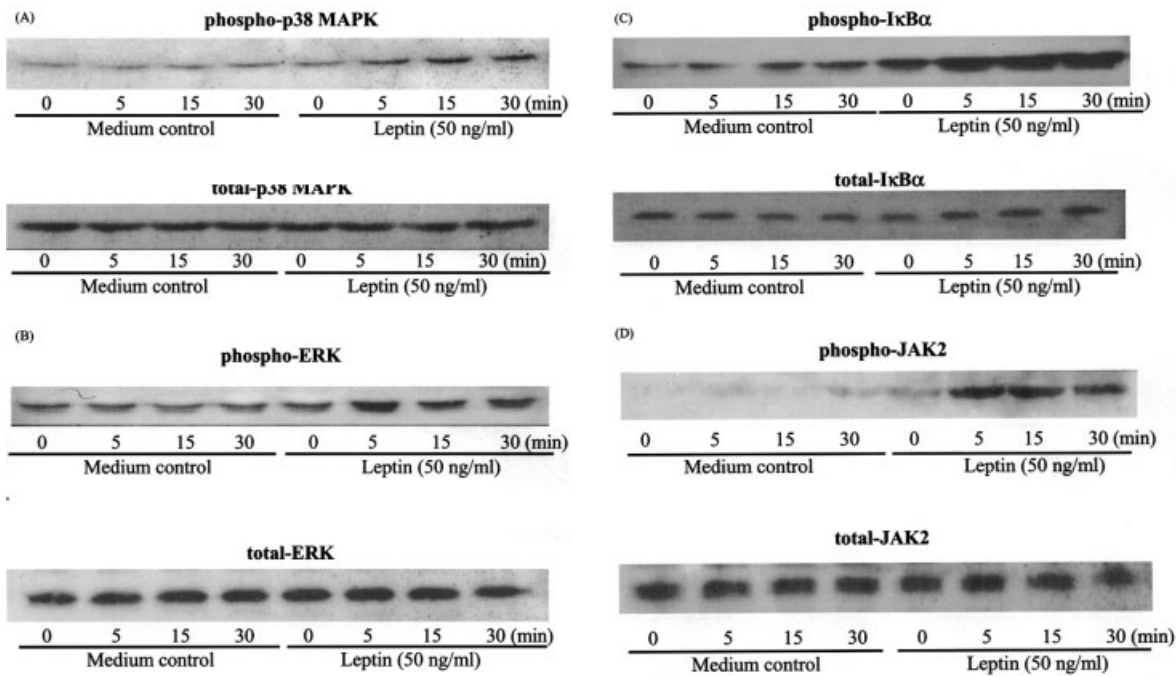


Figure 6. Activation of ERK, p38 MAPK, JAK2 and NF- κ B activities in eosinophils by leptin. Eosinophils (1×10^7 /well) were cultured with or without leptin (50 ng/mL) for different indicated incubation times. Total cellular proteins were extracted from eosinophils for the measurement of (A) phosphorylated and total p38 MAPK, (B) phosphorylated and total ERK, (C) phosphorylated and total I κ B α , and (D) phosphorylated and total JAK2 by Western blot analysis. Experiments were performed in three independent replicates with essentially identical results, and representative results are shown.

showing that activated eosinophils could down-regulate L-selectin [32–34]. Unlike most of the other adhesion molecules, the function of the selectin family is uniquely restricted to the interaction of leukocyte with the

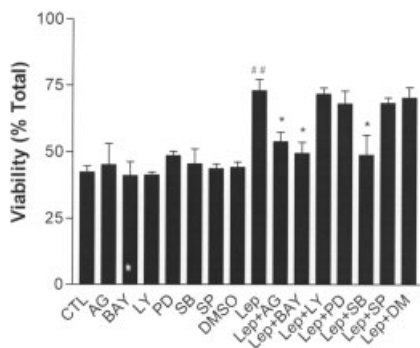


Figure 7. Effects of AG490, BAY11–7082, LY294002, PD98059, SB203580 and SP600125 on viability of leptin-treated eosinophils. Eosinophils (5×10^5 /well) were pretreated with AG490 (10 μ M), BAY11–7082 (1 μ M), PD98059 (10 μ M), LY294002 (5 μ M), SB203580 (7.5 μ M) or SP600125 (3 μ M) for 1 h followed by incubation with or without leptin (50 ng/mL) for 24 h in a 24-well plate. Apoptosis of eosinophils was assessed by the TACSTM Annexin V-FITC assay using flow cytometry. Results were expressed as arithmetic mean plus SD from triplicate experiments. ^{##} $p < 0.01$ when compared with medium control; ^{*} $p < 0.05$ when compared with the leptin control. AG: AG490; BAY: BAY11–7082; PD: PD98059; LY: LY294002; SB: SB203580; SP: SP600125; DM: DMSO.

vascular endothelium. L-selectin mediates the initial attachment of eosinophils onto endothelial cells before their firm adhesion and diapedesis at sites of inflammation [35]. As eosinophils pass through the diverse beds of vascular endothelium, a differential utilization of various adhesion molecules is observed [24]. Based on our observation, we postulate that leptin may play a role in eosinophil flattening and transmigration by lowering the expression of L-selectin. However, further studies with appropriate functional assays for L-selectin are needed to support this hypothesis. Because of the up-regulation of the surface expression of ICAM-1 and CD18, down-regulation of ICAM-3 and L-selectin, and the subsequent dose-dependent induction of chemokinesis and RANTES-mediated chemotaxis of leptin-activated eosinophils, we suggest that leptin may induce eosinophilia and accumulation at inflammatory sites such as lung and airway by enhancing the adhesion of eosinophils onto bronchial epithelial cells and the subsequent transmigration into the inflammatory sites.

Chemokines and cytokines have been shown to play important pathogenetic roles in allergic inflammation [36, 37]. In the present study, we firstly used a cytokine protein array to screen for the induction of 79 different cytokines and chemokines from eosinophils by leptin. The results of the array analysis in conjunction with those of ELISA (Fig. 3) confirmed that leptin could selectively induce the significant *in vitro* release from

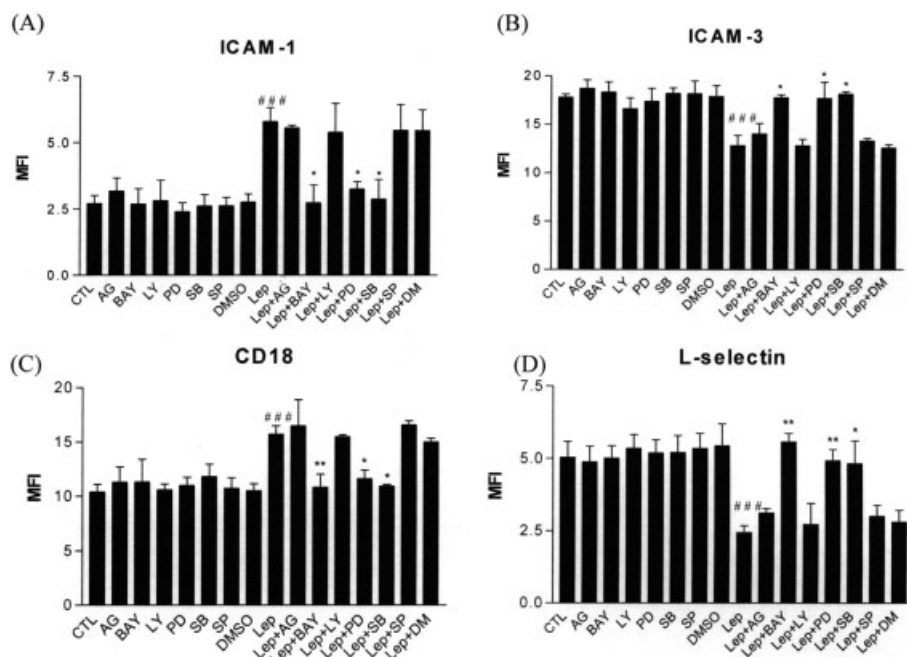


Figure 8. Effects of AG490, BAY11-7082, LY294002, PD98059, SB203580 and SP600125 on leptin-induced cell surface expression of (A) ICAM-1, (B) ICAM-3, (C) CD18, and (D) L-selectin on eosinophils. Eosinophils (5×10^5 /well) were pretreated with AG490 (10 μ M), BAY11-7082 (1 μ M), PD98059 (10 μ M), LY294002 (5 μ M), SB203580 (7.5 μ M) or SP600125 (3 μ M) for 1 h followed by incubation with or without leptin (50 ng/mL) for further 16 h. Surface expression of the adhesion molecules of 10 000 cells was assessed by flow cytometry as MFI. Results are expressed as the arithmetic mean plus SD from five independent experiments. DMSO (0.1%) was used as the DMSO control. ### $p < 0.001$ when compared with medium control; * $p < 0.05$, ** $p < 0.01$ when compared with the leptin control.

eosinophils of inflammatory cytokine IL-1 β and IL-6 for macrophages and lymphocytes, CXC chemokine IL-8 and GRO- α for neutrophils, and CC chemokine MCP-1 for monocytes, macrophages, eosinophils and Th2 cells. Moreover, both the protein synthesis inhibitor cyclohex-

imide and transcriptional inhibitor actinomycin D significantly suppressed the release of chemokines/cytokines upon leptin stimulation from eosinophils at 24 h (data not shown). The above results therefore demonstrated that leptin actually induced newly synthesized IL-1 β , IL-6 and chemokines rather than the release of preformed chemokines and cytokines in eosinophils.

A previous study has shown that chemokine IL-8 could be up-regulated in body fluids or tissues 4–12 h after allergen challenge, and IL-6 was consistently increased after 18–24 h [38]. MCP-1 can trigger chemotaxis of eosinophils via the CCR2 receptor [39]. MCP-1 has also been shown to provoke aggregation and release of histamines and prostaglandin D2 from mast cells for IgE-mediated hypersensitivity [40], and its production is increased in allergic asthma [41]. Our present study of the induction of Th2 and macrophage chemokine MCP-1 and Th2 cytokine IL-6 from eosinophils suggested that leptin may be involved in mediating Th2 immune responses. The induction of IL-1 β can activate macrophages, and IL-8 and GRO- α can mediate the infiltration of neutrophils and basophils into the inflammatory sites in the bronchial airways, thereby amplifying inflammatory responses in allergic diseases such as allergic asthma and allergic dermatitis.

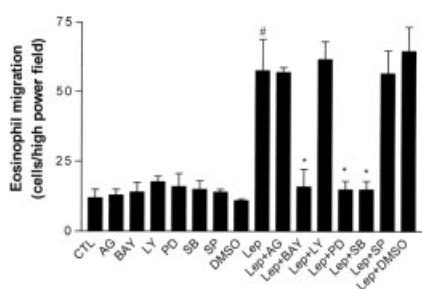


Figure 9. Effects of AG490, BAY11-7082, LY294002, PD98059, SB203580 and SP600125 on the migration of eosinophils induced by leptin. Eosinophils (5×10^4 cells) were pretreated with AG490 (10 μ M), BAY11-7082 (1 μ M), PD98059 (10 μ M), LY294002 (5 μ M), SB203580 (7.5 μ M) or SP600125 (3 μ M) for 1 h followed by incubation with or without leptin (50 ng/mL) for further 16 h. Eosinophils were then incubated in a 48-well microchamber in the upper chambers, while the RPMI medium was placed in the lower chambers. Migration was carried out for 6 h. Results are expressed as cell number/high power field with arithmetic mean plus SD of three independent experiments. # $p < 0.05$ when compared with medium control; * $p < 0.05$ when compared with the leptin control.

It has been shown that eosinophils may up-regulate their chemokine receptors upon cytokine activation and use them during the progression of allergic inflammation for eosinophilic migration and activation [42]. Our results indicated that cell expression of CCR3 on eosinophils was not affected by leptin (data not shown). Since eosinophils express CCR2 for MCP-1, the modulation of other chemokine receptors on eosinophils by leptin also require further investigation.

Leptin has been reported to regulate many signaling pathways including JAK/STAT, src-like homology 2 domain containing protein tyrosine phosphatase, MAPK and PI3K, Akt and protein kinase C (PKC), often in a tissue-specific manner [7]. Our former study has shown that p38 MAPK and NF- κ B play crucial roles in the TNF- α - and IL-25-mediated release of cytokines, chemokines and expression of adhesion molecules from eosinophils [30, 43, 44]. The present intracellular mechanistic study indicates that leptin can activate ERK1, p38 δ , JAK2 and NF- κ B activities (Fig. 4 and 5). We used inhibitors PD98059, SB203580, BAY11–7082 to

elucidate the intracellular signaling mechanisms regulating the induction of adhesion molecules and cytokines/chemokines. Following previous publications [30, 43], we used the optimal concentration of PD98059 (10 μ M), SB203580 (7.5 μ M) and BAY11–7082 (1 μ M), with the highest inhibitory effect without any cell toxicity. The inhibition experiments demonstrated that the leptin-induced up-regulation of ICAM-1 and CD18, down-regulation of ICAM-3 and L-selectin, and chemokinesis were regulated by the activation of intracellular ERK1, p38 δ and NF- κ B (Fig. 6 and 7). The results are in concordance with previous studies that p38 MAPK and NF- κ B are involved in the regulation of adhesion molecules on eosinophils, while ERK and p38 MAPK are involved in the regulation of chemotaxis [28, 45]. Regarding the cytokine release, the induction of IL-1 β and IL-6 were mediated by both p38 δ and NF- κ B, while IL-8, GRO- α and MCP-1 was mediated by the ERK1, p38 δ and NF- κ B activities. It is because p38 MAPK and NF- κ B are commonly signal transduction molecules, by which the expression of cytokines can be regulated in

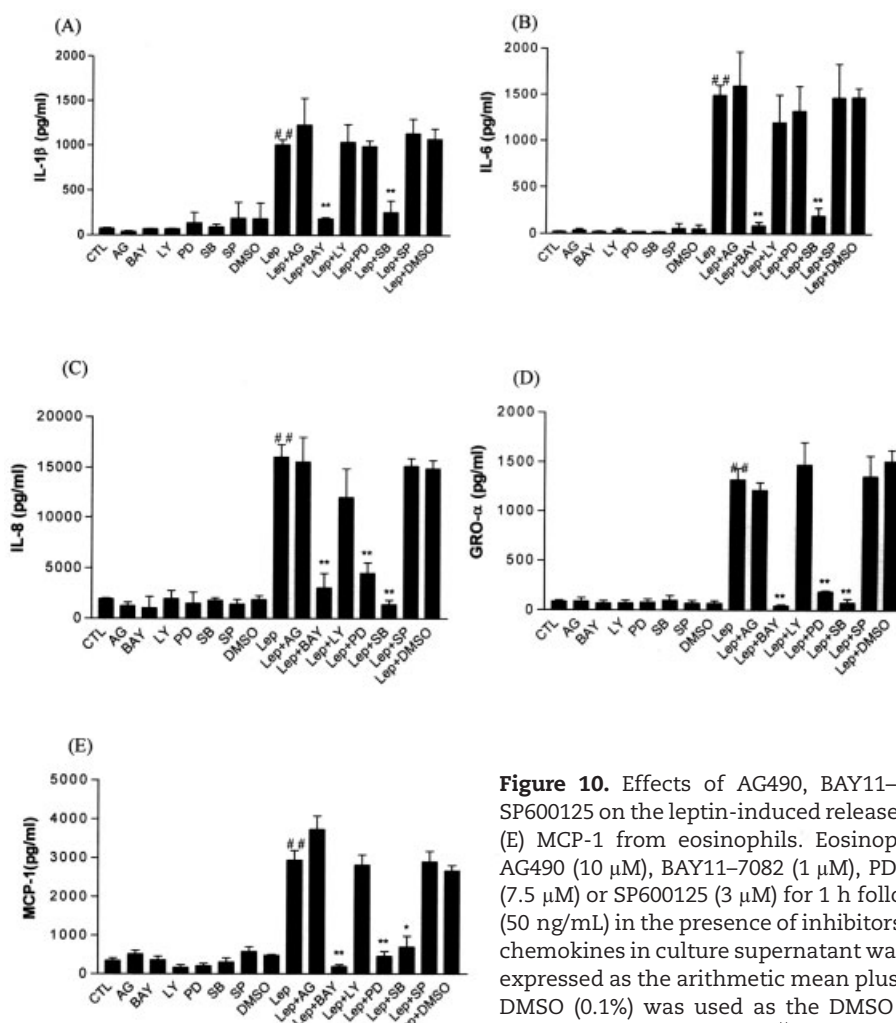


Figure 10. Effects of AG490, BAY11–7082, LY294002, PD98059, SB203580 and SP600125 on the leptin-induced release of (A) IL-1 β , (B) IL-6, (C) IL-8, (D) GRO- α and (E) MCP-1 from eosinophils. Eosinophils (5×10^5 /well) were pretreated with AG490 (10 μ M), BAY11–7082 (1 μ M), PD98059 (10 μ M), LY294002 (5 μ M), SB203580 (7.5 μ M) or SP600125 (3 μ M) for 1 h followed by incubation with or without leptin (50 ng/mL) in the presence of inhibitors for further 36 h. Release of cytokines and chemokines in culture supernatant was determined by human ELISA. Results are expressed as the arithmetic mean plus SD from three independent experiments. DMSO (0.1%) was used as the DMSO control. ## $p < 0.01$ when compared with medium control; * $p < 0.05$, ** $p < 0.01$ when compared with the leptin control.

eosinophils [46], that it is reasonable that the above signaling molecules are commonly involved in leptin-mediated release of IL-8, GRO- α , MCP-1, IL-1 β and IL-6 from eosinophils. Our results also indicated that leptin-mediated intracellular signal transduction could be different between eosinophils and other cell types such as T cells, macrophages and epithelial cells because JAK-STAT and PI3K pathways were not involved in the regulatory mechanisms for the above-studied biological functions of eosinophils. However, we found that activation of JAK2 was involved in the leptin-enhanced survival of eosinophils (Fig. 7).

It has been reported that serum leptin concentration can be up to 400 ng/mL and 700 ng/mL in children with chronic renal failure and obese subjects treated with leptin, respectively [47, 48]. Our recombinant leptin concentrations used in the present study were only 0–100 ng/mL, which are similar to previous concentration used in *in vitro* study [11, 49]. In addition, recombinant leptin should have a lower activity than native leptin because of differences in glycosylation [50]. Moreover, leptin concentrations should be significantly higher in local inflammatory sites than that in circulation. Therefore, leptin might activate eosinophils at sites of inflammation but probably not in the circulation.

In conclusion, this is the first report on the activation of eosinophils for the expression profile of chemokines, cytokines and adhesion molecules, and chemokinesis by leptin. Our result suggest that leptin-regulated apoptosis, expression of adhesion molecules, chemokinesis and the release of IL-1 β , IL-6, IL-8, GRO- α and MCP-1 from eosinophils is mediated by the combined activation of MAPK, JAK and NF- κ B pathways. In conjunction with the anti-apoptotic effect of leptin on eosinophils [11], results therefore together provide new clues for the elucidation of immunopathological mechanisms of obesity-related activation and accumulation of eosinophils in allergic inflammation.

Materials and methods

Materials

Recombinant human leptin was purchased from PeproTec Inc (Rocky Hill, NJ, USA). Anti-human leptin neutralization mAb was from R&D Systems (MN, USA) and mouse IgG1 antibody was from BD PharMingen (CA, USA). Cycloheximide and actinomycin D were purchased from Sigma-Aldrich Co. (MO, USA). I κ B- α phosphorylation inhibitor BAY11–7082, ERK inhibitor PD98059, JNK inhibitor SP600125, and p38 MAPK inhibitor SB203580, PI3-kinase inhibitor LY294002 and JAK inhibitor AG490 were purchased from Calbiochem Corp. (CA, USA). SB203580 and LY294002 were dissolved in water, while PD98059, SP600125, AG490 and BAY11–7082 were dissolved

in dimethyl sulfoxide (DMSO). In all studies, the concentration of DMSO was 0.1%.

Isolation of human blood eosinophils from buffy coat and eosinophil culture

Fresh human buffy coat obtained from the healthy volunteers of Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with phosphate-buffered saline (PBS) at 4°C and centrifuged using an isotonic Percoll solution (density 1.082 g/mL; Amersham and Pharmacia Biotech, Uppsala, Sweden) for 30 min at 1000 $\times$ g. The eosinophil-rich granulocyte fraction was collected and washed twice with cold PBS containing 2% fetal bovine serum (FBS). The cells were then incubated with anti-CD16 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 45 min and CD16-positive neutrophils were depleted by passing through a LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through fraction contained eosinophils with a purity of at least 98% as assessed by Hemacolor rapid blood smear stain (E Merck Diagnostica, Darmstadt, Germany). The isolated eosinophils were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 20 mM HEPES (Gibco).

Endotoxin-free solutions

Cell culture medium was purchased from Gibco, free of detectable lipopolysaccharide (LPS) (<0.1 EU/mL). All other solutions were prepared using pyrogen-free water and sterile polypropylene plastic ware. No solution contained detectable LPS, as determined by the Limulus amoebocyte lyase assay (sensitivity limit 12 pg/mL; BioWhittaker Inc, MD, USA).

Apoptosis assay

Apoptosis of eosinophils was assessed by the TACSTM Annexin V-FITC assay (Trevigen Inc, Gaithersburg, MD, USA) using flow cytometry (FACSCalibur, BD Biosciences Corp, CA, USA) on eosinophils gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. The population of viable cells was characterized by low mean fluorescence intensity (MFI) of both Annexin V-FITC and propidium iodide (PI)-PE.

Protein array analysis of chemokines and cytokines in culture supernatant of eosinophils

The expression profile of 79 different cytokines in culture supernatant of eosinophils was assessed semi-quantitatively using antibody based RayBioTM human cytokine array V (RayBiotech Inc, GA, USA) [30].

Quantitative analysis of IL-1 β , IL-6, IL-8, GRO- α and MCP-1

Concentrations of pro-inflammatory cytokine IL-1 β and IL-6, and chemokine IL-8 and MCP-1 in culture supernatant were quantitated by ELISA kit from BD Pharmingen. Concentration of GRO- α was measured by ELISA kit from R&D Systems.

Flow cytometry of cell surface expression of adhesion molecules

Eosinophils (5×10^5 cells/0.5 mL), after preceding treatments, were washed and resuspended in cold PBS supplemented with 0.5% BSA. After blocking with 2% human pooled serum for 20 min at 4°C and washing with PBS supplemented with 0.5% BSA, cells were incubated either with FITC-conjugated mouse anti-human adhesion molecule mAb or fluorescein-conjugated mouse IgG₁ and IgG_{2b} isotype for 30 min at 4°C in the dark. After washing, the cells were resuspended in 1% paraformaldehyde in 1× PBS as fixative. Expression of surface adhesion molecule on 10 000 viable cells was then analyzed by flow cytometry (FACSCalibur) as MFI, which includes both the changes of adhesion molecule expression on individual cell and the percentage of cells expressing the adhesion molecules.

Assay of cell migration

The chemotaxis and chemokinetic assay was performed in a 48-well microchamber (Neuro Probe, Bethesda, MD, USA) using a checkerboard analysis as previously described [51]. Briefly, the lower wells of the Boyden chamber were loaded with 25 µL diluted RANTES or leptin (0–100 ng/mL) in RPMI medium. Cell suspension (5×10^4 cells) of eosinophils treated with leptin (0–100 ng/mL) was added to the upper wells of the chamber, which was separated from the lower wells by a 5-µm pore size nitrocellulose membrane (Neuro Probe). The chamber was incubated for 6 h at 37°C in an atmosphere containing 5% CO₂. The cells that had migrated to the lower wells were counted microscopically under 400× magnification. For each of the duplicate wells, the numbers of cells in four random fields were determined, and the arithmetic mean and SD were calculated.

Membrane array for the detection of phosphorylation of intracellular kinases

The expression profile of intracellular phosphorylation of 21 different MAPK and other serine/threonine kinases of eosinophils was assessed semi-quantitatively using an antibody based Human Phospho-MAPK Array Kit (R&D Systems) (Table 2).

Western blot analysis

Eosinophils were washed with ice-cold PBS, and lysed in 0.2 mL lysis buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-mercaptoethanol, 1× protease inhibitors). Cell debris was removed by centrifugation at $14\,000 \times g$ for 15 min, and the supernatant was boiled in Laemmli sample buffer (Bio-Rad Laboratory, CA, USA) for 5 min. An equal amount of proteins was subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis before blotting onto a PVDF membrane (Amersham and Pharmacia Biotech). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20, pH 7.6 for 1 h at room temperature, and probed with primary rabbit anti-human GAPDH, anti-human phospho-ERK, anti-human phospho-p38 MAPK, anti-human

phospho-JAK2, anti-human phospho-IκB-α (Cell Signaling Technology Inc, MA, USA) at 4°C overnight. After washing, the membrane was incubated with secondary donkey anti-rabbit antibody coupled to horseradish peroxidase (Amersham and Pharmacia Biotech) for 1 h at room temperature. Antibody-antigen complexes were then detected using a ECL chemiluminescent detection system according to the manufacturer's instructions (Amersham and Pharmacia Biotech) [30].

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA analysis. A *p* value of <0.05 was considered significantly different. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 10.1.4 (SPSS Inc., Chicago, IL, USA).

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