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ORIGINAL ARTICLE

Activation of Eosinophils by Lipopolysaccharide-Induced Monocyte-Derived Cytokines

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ABSTRACT

Background: Interactions between eosinophils and monocytes after lipopolysaccharide inhalation are yet to be investigated. The mechanism of eosinophil activation induced by lipopolysaccharide in the presence of monocytes was investigated.

Methods: Expression of ICAM-1 and Mac-1 on eosinophils was evaluated after lipopolysaccharide stimulation in the presence of monocytes or monocyte culture supernatants. Cytokines in the supernatant of lipopolysaccharide-stimulated monocytes were measured using a cytokine array.

Results: Expression of ICAM-1 and Mac-1 on eosinophils was up-regulated after lipopolysaccharide stimulation in the presence of monocytes or monocyte culture supernatant. Lipopolysaccharide induced secretion of ENA-78, GMCSF, GRO, IL-1beta, IL-6, IL-10, MCP-1, TNF-alpha and MIP-3 alpha from monocytes. The up-regulation of ICAM-1, but not Mac-1, on eosinophils was attenuated by anti-TNF-alpha neutralizing antibody.

Conclusions: Monocyte-derived TNF-alpha plays an important role in the up-regulation of ICAM-I on eosinophils induced by lipopolysaccharides.

KEY WORDS

allergy, chronic lung disease, eosinophils, lipopolysaccharide (LPS), monocytes

INTRODUCTION

Lipopolysaccharides (LPS) inhalation exacerbates allergic inflammation and is responsible for airway remodeling.[1] Eosinophils and monocytes are included in the major inflammatory cells that accumulate in the inflammatory focus after LPS inhalation. It is suggested that CD14 and toll-like receptor 4 (TLR4) expressed on monocytes play important roles in the LPS-induced physiopathological response in the airway; however, interactions between eosinophils and monocytes after LPS inhalation are yet to be investigated. The mechanism of eosinophil activation induced by LPS in the presence of monocytes was investigated in vitro using intercellular adhesion molecule-I (ICAM-1: CD54) and Mac-1 (CD11b/CD18) expressed on eosinophils as activation markers.

METHODS

CELL PURIFICATION

Eosinophils

Peripheral blood was obtained from subjects with mild eosinophilia. All of these subjects had never been diagnosed with allergic diseases nor helminthic infections and were taking no medications at the blood sampling. Eosinophils were isolated from heparinized venous blood using two different purification methods. The first method was a modified CD16 negative selection method, as previously described.[2] In brief, cells obtained from the buffy coat were incubated with anti-CD3, anti-CD14, anti-CD16 and anti-CD19 monoclonal antibodies (mouse IgG; Nichirei, Tokyo, Japan), and subsequently reacted with anti-mouse IgG magnetic beads (Dynal, Oslo, Norway). CD3-, CD14-, CD16- and CD19-negative eosinophils were obtained using a magnetic cell-sorting system

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(MACS; Miletenyl Biotec, Bergisch Gladbach, Germany). In the second method, eosinophils were isolated by sedimentation with 6% dextran, followed by centrifugation on 1.088 Percoll (Pfizer, NY, USA) density gradients. The cells were additionally purified by negative selection using only anti-CD16 immunomagnetic beads and MACS. Eosinophils were isolated with a high purity (>97%), viability (>99%) and yield in both methods.

Human Eosinophilic Cell Line

EoL-1 established from the peripheral blood of a patient with eosinophilic leukemia by Saito H *et al.*[3] was used as a human eosinophilic cell line.

Monocytes

Circulating peripheral blood mononuclear cells (PBMC) were isolated by means of Ficol-Hypaque separation. For depletion of T cells, NK cells, B cells, dendritic cells and basophils from PBMC, these cells were indirectly magnetically labeled using a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies and magnetic beads coupled to an anti-hapten monoclonal antibody. The magnetically labeled cells were depleted by MACS system. The purity of the monocyte fractions was more than 98% as analyzed by morphology using Diff-Quick stain (Baxter Scientific, Florida, USA).

CELL CULTURE AND FLOW CYTOMETRIC ANALYSIS

Eosinophils (2×10^6/ml) and/or monocytes (1×10^6/ml) were cultured in RPMI containing 10% fetal calf serum with or without LPS (10 ng/ml) for 18 hours. In some experiments, eosinophils (4×10^6/ml × 500 µl) and monocytes (2×10^6/ml × 500 µl) were cultured in a well separated by Millicell HA (Millipore, Billerica, MA, USA), a membrane with pores with a size of 0.45 µm allowing the medium to be mixed and isolating eosinophils in the upper chamber from monocytes in the lower chamber. In other experiments, eosinophils were cultured in 1 ml of RPMI containing 10% fetal calf serum and 100 µl culture supernatant of monocytes treated with LPS for 18 hours. Eosinophils were analyzed for their expression of ICAM-1, Mac-1, TLR4, and CD14 using a flow cytometer (FACScan, Becton Dickinson, Cockeysville, MD, USA). Monoclonal antibodies against ICAM-1 (84H10, Beckman Coulter, MN, USA), Mac-1 (Bear-1 Nichirei, Tokyo, Japan), TLR4 (HTA125, Becton Dickinson), CD14 (CLB-Mon/1, Nichirei), mouse IgG1 (x0931, DAKO, Denmark) and mouse IgG2a (x0943, DAKO) were used as primary antibodies. Phycoerythrine-conjugated donkey antimouse IgG (715-116-151, Jackson Immuno Research Laboratories, Pennsylvania, USA) was used as a secondary antibody. Dexamethasone (DX) was purchased from Wako Pure Chemical Industries (Japan). Expression of ICAM-I and Mac-1 was expressed in delta-mean fluorescence intensity (delta-MFI), the difference in MFI between the sample and the control.

MEASUREMENT OF CYTOKINE LEVELS IN CELL CULTURE SUPERNATANT

The cell culture supernatants were analyzed for cytokine levels by an enzyme-linked immunosorbent assay (ELISA, R & D Systems). And a human cytokine antibody array (Ray Bioteck, Norcross, GA, USA), which had epithelial neutrophil-activating protein-78, Granulocyte-macrophage colony stimulating factor (GMCSF), growth-related oncogene (GRO), GRO-alpha, I-309, interleukin (IL)-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40p70, IL-13, IL-15, interferon (IFN)-gamma, monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, MCP-4, macrophage-colony stimulating factor (MCSF), macrophage-derived chemokine (MDC), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1 beta, MIP-1 delta, MIP-3 alpha, regulated upon activation, T-cell expressed, and presumably secreted (RANTES), stem cell factor (SCF), stromal cell-derived factor (SDF)-1, thymus and activation-regulated chemokine (TARC), tumor necrosis factor (TNF)-α, TNF-β, epidermal growth factor (EGF), insulin-like growth factor (IGF)-4, angiotensin, oncostatin, thrombopoietin, vascular endothelial growth factor (VFGF), platelet-derived growth factor (PDGF)-BB, leptin, brain-derived neurotrophic factor (BDNF), B-lymphocyte chemoattractant (BLC), Ck beta 8-1, eotaxin, eotaxin-2, eotaxin-3, fibroblast growth factor (FGF)-4, FGF-7, FGF-9, Fms-like tyrosine kinase (Flt)-3 ligand, fractalkine, granulocyte chemotactic protein (GCP)-2, glial-derived neurotrophic factor (GDNF), hematopoietic growth factor (HGF), insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, IGFBP-3, IGFBP-4, IL-16, IP-10, leukemia inhibitory factor (LIF), homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), mesoderm-inducing factor (MIF), neutrophil activating peptides (NAP)-2, neurotrophin (NT)-3, NT-4, osteoprotegerin, pulmonary and activation-regulated chemokine (PARC), placenta growth factor (PlGF), transforming growth factor (TGF)-beta 1, TGF-beta 2, TGF-beta 3, tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 on the map was utilized to know which of these cytokines show apparent increase in the supernatant of monocytes after LPS stimulation. The maps obtained from the monocyte culture supernatants with or without LPS stimulation were compared by three laboratory technicians without any knowledge of the mapping. The spots judged as newly emerged or remarkably enhanced with LPS stimulation were identified, and as a consequence, the cytokines located on the map were judged as "apparently increased".

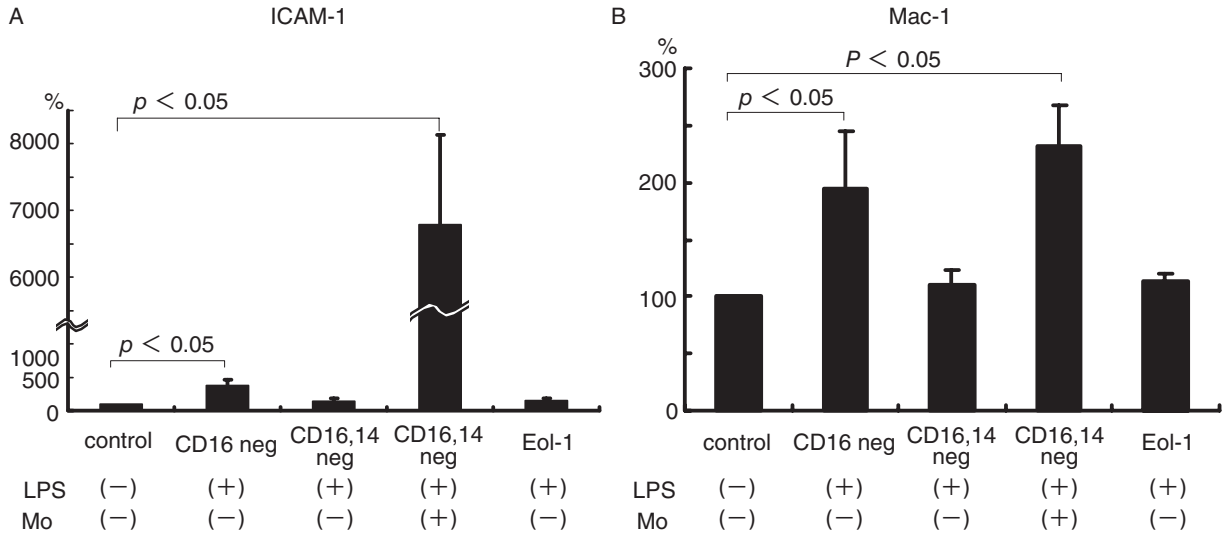


Fig. 1 Expression of ICAM-1 and Mac-1 on eosinophils after LPS stimulation. Eosinophils cultured with monocytes (CD16,14 neg, Mo (+)) showed a marked increase in ICAM-1 (6790.3 ± 1347.3%). Eol-1 and eosinophils purified using anti-CD16 and anti-CD14 magnetic beads (CD16,14 neg) showed no significant increase (149.9 ± 28.7% and 142.6 ± 42.0%, respectively). Eosinophils purified by only CD16-negative selection (CD16 neg) also showed a significant increase in ICAM-1 after LPS stimulation (380.2 ± 80.4%) (**A**) ($n = 7$).

Expression of Mac-1 increased in eosinophils purified by only CD16-negative selection (CD16 neg; 195.3 ± 51.2%) and eosinophils cultured with monocytes (CD16,14neg, Mo (+), 232.3 ± 35.6%). Eol-1 and eosinophils purified using anti-CD16 and anti-CD14 magnetic beads (CD16,14 neg) showed no significant increase in Mac-1 (112.9 ± 7.3% and 110.6 ± 12.8%, respectively) (**B**) ($n = 7$).

STATISTICAL ANALYSIS

All measured values were presented as the means ± SEs. When comparing three or more groups of data, the Scheffe F-test was used as a post hoc test, and significance was set at a value of less than 0.05 after analysis of variance.

ETHICAL ASPECT

The ethical committee of the Akita University School of Medicine approved the methods and design of this study.

RESULTS

EXPRESSION OF ICAM-1 AND MAC-1 ON EOSINOPHILS AFTER LPS STIMULATION

Eosinophils cultured with monocytes showed a significant increase in ICAM-1 and Mac-1 expression. Interestingly, eosinophils purified by only CD16-negative selection also showed a significant increase in these adhesion molecules after LPS stimulation in the absence of monocytes (Fig. 1); however, these eosinophils expressed neither CD14 nor TRL4 before and after the stimulation (Fig. 2). To exclude the possibility of the direct effect of LPS on the eosinophils, eosinophils purified by CD3-, CD14-, CD16- and CD19-negative selections and eosinophilic cell line EoL-1 were stimulated with LPS in the absence of monocytes. The eosinophils purified by CD3-, CD14-, CD16- and CD19-negative selections and Eol-1 showed no increase of ICAM-1 and Mac-1 expression (Fig. 1), which suggested that a very low percentage of monocytes contaminating eosinophils purified by only CD16-negative selection caused eosinophil activation following LPS stimulation.

INFLUENCE OF DIRECT EOSINOPHIL-MONOCYTE CONTACT ON UP-REGULATION OF ICAM-1 AND MAC-1 ON EOSINOPHILS AFTER LPS TREATMENT

Eosinophils mixed with monocytes and those separated from monocytes by means of Millicell HA both showed up-regulations of ICAM-1 and Mac-1 after LPS treatment (Fig. 3). The up-regulatory effect of the monocytes on the ICAM-1 expression of eosinophils was augmented by the direct contact of monocytes and eosinophils. Furthermore, eosinophils cultured in the culture supernatant of monocytes treated with LPS showed up-regulations of ICAM-1 and Mac-1 (Fig. 4). These results indicated that the direct contact of monocytes was not essential for eosinophil activation, and the culture supernatant of monocytes stimulated with LPS contained an eosinophil-activating substance(s).

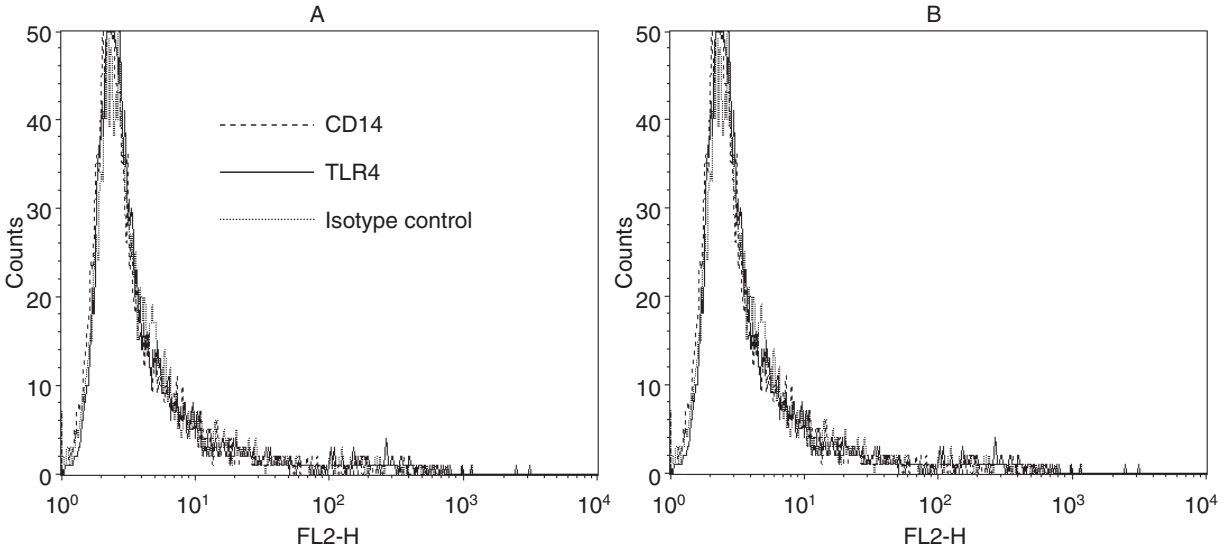


Fig. 2 Expression of CD14 and TLR4 on eosinophils. Eosinophils expressed neither CD14 nor TLR4 before (**A**) and after (**B**) LPS stimulation.

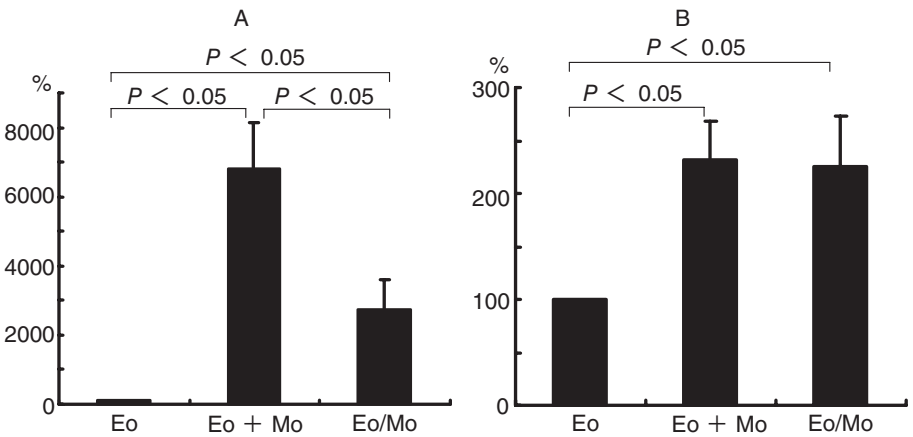


Fig. 3 Influence of direct eosinophil-monocyte contact on up-regulation of ICAM-1 and Mac-1 on eosinophils after LPS treatment. Eosinophils mixed with monocytes (Eo + Mo) and those separated from monocytes by means of Millicell HA (Eo/Mo) showed up-regulation of ICAM-1 (**A**; 6790.3 ± 1347.3 and 2723.0 ± 876.6%, respectively) and Mac-1 (**B**; 232.3 ± 35.6 and 225.8 ± 46.7%, respectively) ($n = 3$).

ANALYSIS OF CYTOKINES IN CULTURE SUPERNATANT OF MONOCYTES STIMULATED WITH LPS

The culture supernatant of monocytes stimulated with LPS was analyzed by a human cytokine antibody array. Among the 79 cytokines and chemokines, ENA-78, GMCSF, GRO, IL-1beta, IL-6, IL-10, MCP-1, TNF-alpha and MIP-3 alpha showed an apparent increase after LPS stimulation (Fig. 5).

EFFECT OF ANTI-TNF-α NEUTRALIZING ANTIBODY ON ICAM-1 AND MAC-1 UP-REGULATION IN EOSINOPHILS

Eosinophils express no, or if any, negligible amount of ligands against ENA-78 (CXCR1), GRO (CXCR2) and MCP-1 (CCR2),[4] and IL-1beta, GMCSF and IL-6 are reported to have little effect on ICAM-1 and Mac-1 expression on eosinophils.[5,6] As a possible contributing factor, TNF-alpha, which may cause up-regulation of these adhesion molecules on human eosinophil,[7] was checked. The effect of anti-TNF-alpha neutralizing antibody on ICAM-1 and Mac-1 up-regulation in eosinophils induced by LPS-stimulated

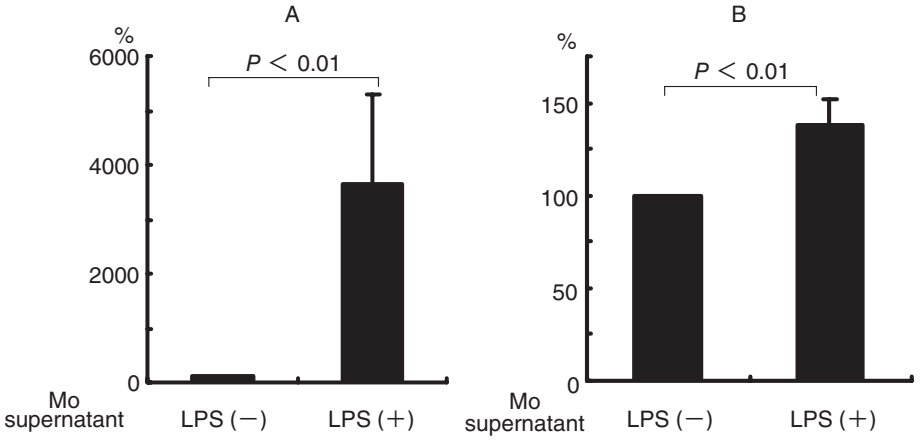
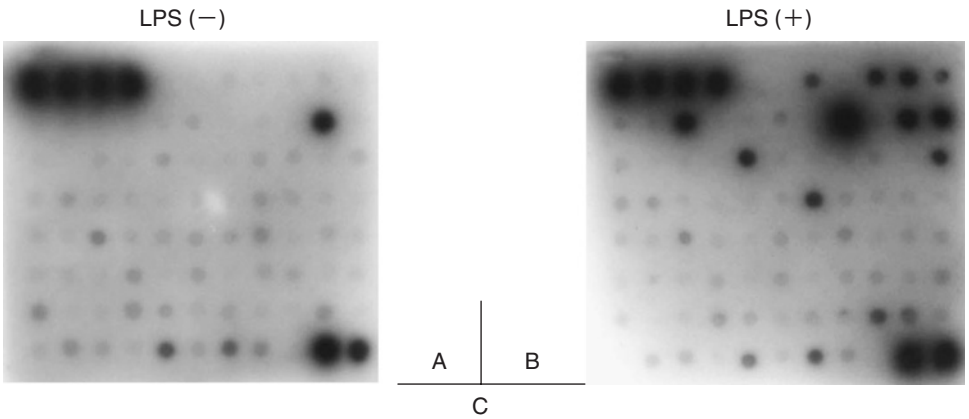


Fig. 4 Up-regulation of ICAM-1 and Mac-1 on eosinophils by monocyte culture supernatant after LPS treatment. The culture supernatant of monocytes treated with LPS induced up-regulation of ICAM-1 (**A**; 3664 ± 1640.7%) and Mac-1 (**B**; 138.2 ± 14.2%) on eosinophils ($n = 8$).



	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	FEG	IGF-1	Angiogenin
5	Oncostatin M	Thrombo-poietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ckβ8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalking	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Octeo-protogerin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Neg	Pos	Pos

Fig. 5 Analysis of cytokines in the culture supernatant of monocytes stimulated with LPS. Cytokines in the supernatant of monocytes cultured with (**B**) or without (**A**) LPS were detected with a human cytokine antibody array. An array of cytokines was shown on the table (**C**).

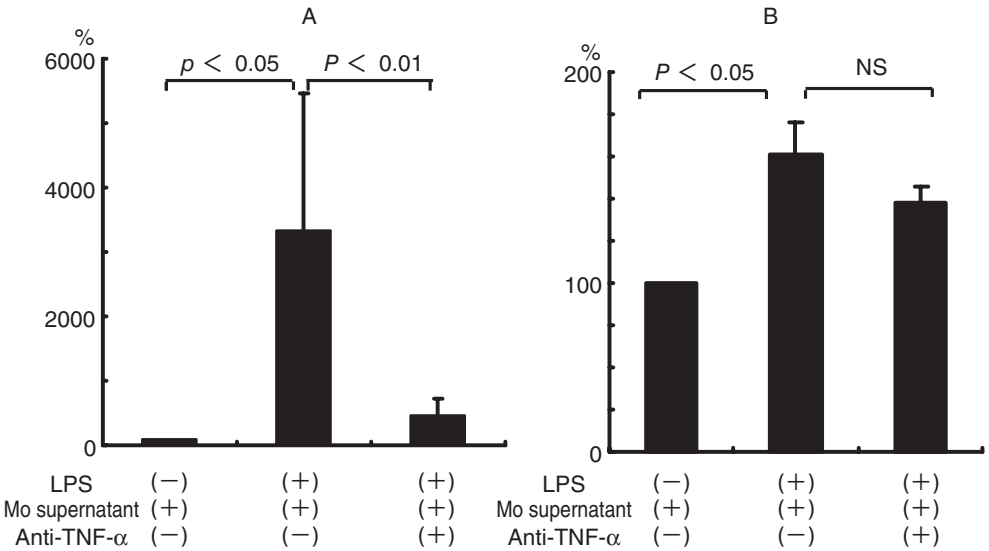


Fig. 6 Effect of anti-TNF-α neutralizing antibody on ICAM-1 and Mac-1 up-regulation in eosinophils. Anti-TNF-alpha neutralizing antibody reduced up-regulation of ICAM-1 (**A**) by 89% and Mac-1 (**B**) by 37.0% ($n = 3$).

monocyte culture supernatant was studied. The culture supernatants of monocytes (1×10^6 cells/ml) stimulated with LPS contained 13,800 pg/ml of TNF-alpha at the highest concentration. In consequence with the highest concentration of TNF-alpha, the final concentration of anti-TNF-alpha neutralizing antibody was adjusted at 200 ng/ml according to the manufacturer's instructions. Anti-TNF-alpha neutralizing antibody blocked up-regulation of ICAM-1 expression on eosinophils (Fig. 6). Mac-1 expression was also reduced by anti-TNF-alpha neutralizing antibody; however, there was no statistical significance and the effect was far less marked than that on ICAM-1.

EFFECT OF DEXAMETHASONE ON ICAM-1 AND MAC-1 UP-REGURATION IN EOSINOPHILS

Purified eosinophils contaminated with 3% of monocytes were stimulated with LPS under the presence of DX at concentrations of 0, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} nM for 18 hours to determine the effect of DX on the up-regulation of ICAM-1 and Mac-1 on eosinophils. The up-regulatory effect of LPS on expression of ICAM-1 and Mac-1 on eosinophils in the presence of monocytes was cancelled by DX in a dose-dependent manner (Fig. 7).

DISCUSSION

LPS is a biologically active substance that triggers inflammatory reactions. The chance of inhaling this common substantial bio-hazard is not rare. For example, LPS is contained in cigarette smoke[8] and grain dust[9,10] in the environment. Chronic inhalation of LPS exacerbates allergic inflammation and is responsible for remodeling of the airway.[1]

We measured ICAM-1 and Mac-1 as activation markers of eosinophils after the stimulation of LPS in this study, since these molecules trigger activation of eosinophils such as superoxide production[11] and degranulation.[12,13] Eosinophils mixed with monocyte showed up-regulation of ICAM-1 and Mac-1 after LPS stimulation, and in addition, this up-regulation was also observed without direct contact of eosinophils with monocytes. This means that the direct contact of eosinophils and monocytes is not essential for eosinophil activation, and molecules secreted by monocyte play important roles in the activation of eosinophils after LPS stimulation. LPS augmented the secretion of ENA-78, GMCSF, GRO, IL-1 beta, IL-6, IL-10, MCP-1, TNF-alpha and MIP-3 alpha from monocyte. Of these cytokines, we focused TNF-alpha which had been proven to enhance ICAM-1 expression on human eosinophilic leukemia Eol-1 cells.[7] As expected, anti-TNF-alpha neutralizing antibody blocked up-regulation of ICAM-1 expression on eosinophils. These results suggest that TNF-alpha secreted by monocytes plays a principal role in the up-regulation of ICAM-1 on eosinophils after LPS stimulation. Supporting our results, recently anti-TNF-alpha therapy has been reported to be a novel therapy for asthma.[14] On the other hand, Mac-1 expression was not significantly reduced by anti-TNF-alpha neutralizing antibody. It is possible that the other factors such as GMCSF[5] play roles in up-regulation of Mac-1 on eosinophils after LPS stimulation. These results suggest that TNF-alpha is, at least partly, responsible for exacerbation of asthma.

TLR4[1] and CD14[15,16] are known as essential components for LPS to be biologically active; however, it

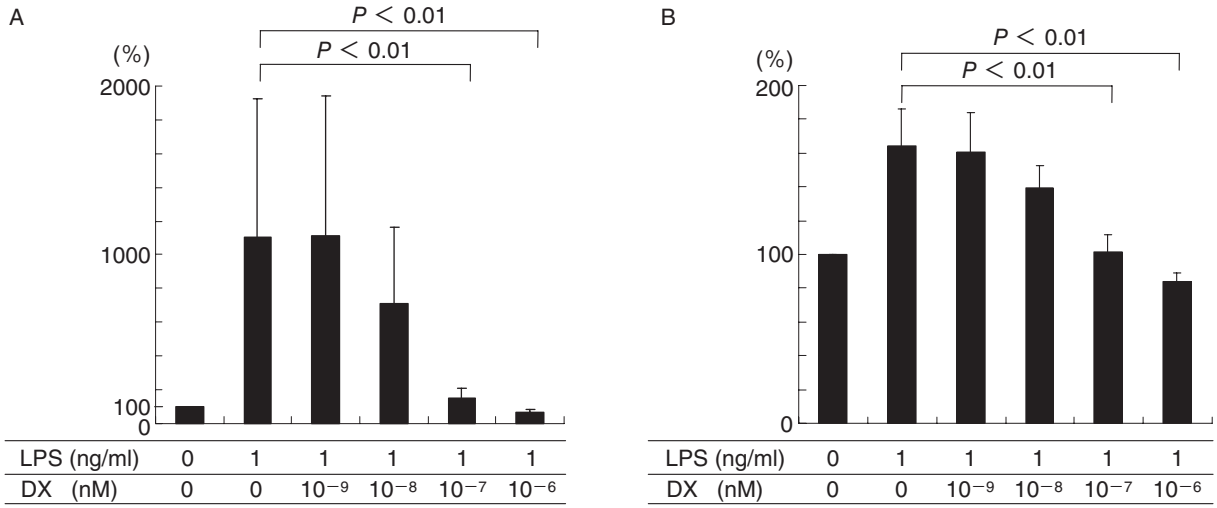


Fig. 7 Effect of dexamethasone on ICAM-1 and Mac-1 up-regulation in eosinophils. The up-regulatory effect of LPS on expression of ICAM-1 (**A**) and Mac-1 (**B**) on eosinophils in the presence of monocytes was cancelled by dexamethasone (DX) in a dose-dependent manner ($n = 5$).

is still controversial whether LPS acts directly[16,17] on eosinophils or indirectly via TLR4- and CD14-expressed cells, such as monocytes and macrophages. Previous studies demonstrated TLR4 and CD14 mRNA in eosinophils.[18,19] But, TLR4 and CD14 are yet to be proven as functioning proteins expressed on eosinophils. We failed to show TLR4 and CD14 expression on eosinophils, which was compatible with the report by Sabroe *et al.*[20] The possibility that the eosinophil purification procedures using antibody cocktails can down-regulate the expression of CD14 or TLR4 on eosinophils was ruled out, because we also failed to show the expression of CD14 and TLR4 on the eosinophils analyzed by whole blood staining method which does not require eosinophil purification procedures (data not shown). These results indicate that eosinophils and Eol-1 express no or, if any, undetectable levels of CD14 and TLR4, and these cells are not activated directly by LPS. Papi A *et al.* reported an increase in eosinophils, neutrophils, RANTES, TNF-alpha, ENA-78 and ICAM-1 in the bronchoalveolar lavage fluid of patients with exacerbation of chronic obstructive pulmonary disease.[21] Eosinophils and monocytes, as well as neutrophils, are included in the major inflammatory cells that accumulate in the airway after LPS inhalation. ENA-78 and GRO induce neutrophil chemotaxis. IL-1 beta augments IL-8 production by accumulating neutrophils as well as resident macrophages.[22] GMCSF activates neutrophils and induces release of matrix metalloprotease (MMP)-9.[23] Thus, monocytes, eosinophils and neutrophils may participate in the pathophysiology of exacerbation of chronic lung disease including bronchial asthma caused by LPS inhalation.

Monocytes, cultured at a concentration of 1×10^6 cells/ml, secreted TNF-alpha up to 13,800 pg/ml after LPS stimulation. This result indicates that 3% of contaminated monocytes can produce enough amounts of TNF-alpha to act on eosinophils in vitro, which is compatible to the result that eosinophils purified by anti-CD16 immunomagnetic beads showed up-regulation of ICAM-1 after LPS stimulation in spite of their high purity.

In conclusion, activation of eosinophils by lipopolysaccharide-induced monocyte-derived cytokines, especially TNF-alpha, plays an important role in the LPS-induced physiopathological response in the airway.

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