

Red blood cells regulate eosinophil chemotaxis by scavenging RANTES secreted from endothelial cells

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Summary

Background Eosinophils play a critical role in the pathogenesis of allergic diseases. CC chemokines, such as regulated on activation, normal, T cell expressed, and secreted (RANTES), are key regulators of eosinophil locomotion. Although eosinophils migrate from the bloodstream into tissues, mechanisms that generate a chemogradient across the endothelium remain to be fully elucidated.

Objective We first examined the polar secretion of RANTES by endothelial cells. We also studied the functional scavenging effect of red blood cells (RBCs) on RANTES secreted into the intravascular side.

Methods and results Endothelial cells were cultured in a transwell chamber with a membrane pore size of 0.45, 3.0, and 8.0 μm and stimulated with TNF- α , IL-1 β , or IFN- γ from the apical or basolateral side for 16 h. The measurement of RANTES in the supernatant was performed by ELISA. We did not see any difference in the amount of RANTES secreted from the cytokine-stimulated endothelium between inner (intravascular side) and outer (extravascular side) wells separated by the 8.0- μm membrane, although apical polarization was observed with the 0.45- μm membrane. The addition of RBCs (hemoglobin (Hb): 0.5–15 g/dL) to the apical supernatant of TNF- α -stimulated endothelial cells reduced the RANTES level in a concentration-dependent manner. The treatment of supernatant on the intravascular side with RBCs significantly enhanced the migration of eosinophils.

Conclusion RBCs possess a scavenging effect on intravascular RANTES, and thereby regulate transendothelial migration of eosinophils. Our findings suggest a new role of RBCs in allergic inflammation.

Keywords endothelial cells, polarity, RANTES, RBC, scavenger

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Introduction

Eosinophils, as well as T-helper type 2 (Th2) cells and mast cells, are key players in the mechanism of allergic diseases including asthma [1]. Chemotaxis of eosinophils is one of the important events in the pathogenesis of allergic inflammation. RANTES (regulated on activation, normal T cell expressed and secreted) plays an important role in various immune and allergic disorders [2, 3] since it is a potent chemoattractant for inflammatory cells such as eosinophils [4], memory T cells, and monocytes [2], and is also involved in the activation of eosinophils [5–7]. The levels of monocyte chemoattractant protein 1 (MCP-1), RANTES, macrophage inflammatory protein 1 α (MIP-1 α), and eotaxin are significantly higher in asthma patients than in control subjects [8, 9]. Although the main source of chemokines in bronchoalveolar lavage fluid (BALF) is bronchial epithelial cells, an important issue is

whether the direction of chemokine release is into the apical or basal side. In this regard, Taguchi et al. [10] have reported that RANTES is preferentially secreted through the apical cell surface of epithelium accompanied with intercellular adhesion molecule-1 (ICAM-1) expression along the apical and basolateral sides. Kato et al. [11] have also demonstrated that eosinophil transmigration was promoted by oversecretion of chemokines to the apical side in a three-dimensional model. Thus, these findings indicated that inflammatory cells may be driven to the mucosal surface by the apical-to-basal chemogradient, which is reasonable for the process of asthma.

Endothelial cells, a main source of RANTES [12], possess a number of specific functions that are important for the regulation of allergic response including eosinophil adhesion and transmigration [13]. Although transendothelial migration of eosinophils is regulated by similar molecular mechanisms to transepithelial movement, the most critical difference between endothelial and epithelial migration is the direction of cell movement. In other words, the former direction is apical-to-basal, whereas the latter is basal-to-apical. If the concept of transepithelial migration suggested by Taguchi

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et al. [10] is applied, endothelial cells should preferentially secrete RANTES through the basolateral cell surface, thereby establishing a chemical gradient for eosinophil chemotaxis. The authors did not, however, observe the polar secretion of RANTES by endothelial cells treated with TNF- α plus IFN- γ . Unless endothelial cells themselves polarize it, abundant RANTES secreted into the bloodstream needs to be scavenged or inactivated in order to allow eosinophils to migrate into the tissues. One possible candidate is red blood cells (RBCs), whose Duffy antigen/receptor for chemokines (DARCs) serves as a promiscuous chemokine receptor for both CXC and CC chemokines. The physiological relevance of RBCs in eosinophil trafficking, however, remains unclear.

In the present study, we investigated the polarized secretion of RANTES by endothelial cells stimulated with TNF- α , IL-1 β , IFN- γ , or TNF- α plus IFN- γ . The polar secretion of cytokine-induced RANTES by endothelial cells was not observed. Moreover, we revealed that RBCs scavenged RANTES secreted through the intravascular side, resulting in the initiation of transendothelial eosinophil migration as a result of the subsequent chemogradient generated across the endothelium.

Materials and methods

Endothelial cell cultures

The endothelial cell line from human umbilical vein endothelial cells (HUVECs) transformed with SV-40 [14] was supplied by Drs Ikuro Maruyama and Yasuko Soejima (Department of Laboratory Medicine, Kagoshima University School of Medicine, Kagoshima, Japan). The cells were cultured in 70 cm² Collagen I Cellware flasks (Becton Dickinson Labware, Bedford, MA, USA) in MCDB107 (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 10% fetal bovine serum (FBS) (Filtron Pty Ltd, Brooklyn, Australia), penicillin (100 IU/mL), streptomycin (100 μ g/mL), 10 μ g/mL transferrin, 5 μ g/mL insulin, and 150 μ g/mL endothelial cell growth supplement (ECGS) (Becton Dickinson Labware) at 37 °C in a humidified 5% CO₂ atmosphere. The culture medium was replaced every other day. After the cells reached confluence, they were harvested with 0.05% trypsin and 0.02% EDTA dissolved in phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and seeded on the collagen type I (Becton Dickinson Labware)-coated membrane (0.45, 3.0, 8.0 μ m pore size, 10.5 mm diameter) of 12-well Cell Culture Inserts (Becton Dickinson Labware). Five hundred microlitres of cell suspension (5×10^3 cells/mL) was placed in the inner well, with the outer well containing 1.5 mL of culture medium alone. After the cells had almost grown to reach confluence, the culture medium was replaced with medium depleted of all supplements followed by additional culture for 24 h before each experiment.

Eosinophil preparation

Peripheral venous blood was obtained from healthy volunteers after obtaining oral informed consent. Eosinophils were isolated by sedimentation with 6% dextran followed by

centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients as modified from the method of Chihara et al. [15] and Hansel et al. [16]. The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a magnetic-activating cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). The eosinophils (>99% purity) were then suspended in Hank's balanced salt solution (HBSS) with 1% fetal calf serum (FCS).

Measurement of RANTES

In order to study the polar secretion of RANTES, the endothelial cells were stimulated with cytokines including TNF- α , IL-1 β , or IFN- γ (Genzyme TECHNE, Minneapolis, MN, USA) from the apical or basolateral side for 16 h. After the supernatants of the inner and outer well were obtained, the concentration of RANTES in each well was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

Scavenging effect of red blood cells on RANTES secretion

Irradiated human RBC concentrates in mannitol-adenine-phosphate were obtained from the Japan Red Cross Society (Tokyo, Japan). The RBC suspension was washed more than twice in PBS before each experiment. The supernatants stimulated with TNF- α for 16 h in the inner well were mixed with medium or a serial concentration of RBCs (hemoglobin (Hb) concentration: 0.5–15 g/dL) for 10 min. In some experiments, the endothelial cells were stimulated with TNF- α for 16 h in the presence of RBC suspension. Then, each supernatant was collected by centrifugation for the measurement of RANTES.

Chemotaxis assay

Chemotaxis assay of eosinophils was conducted in duplicate using Boyden chambers (Neuro Probe, Bethesda, MD, USA). Purified eosinophils (2×10^6 cells/mL) were suspended in the supernatant of the inner well that had been incubated with or without RBCs and placed on the upper Boyden chamber. The supernatant from the outer well was applied to the lower chamber, which was separated from the upper one by a 5 μ m pore size polycarbonate, polyvinylpyrrolidone-free membrane (Nucleopore, Pleasanton, CA, USA). The loaded chambers were incubated at 37 °C in humidified air with 5% CO₂ for 1 h. Then, the membrane was removed followed by fixation and staining for 3 min in May-Grünwald solution. The cells that had migrated and adhered to the lower surface of the membrane were counted in 10 fields by light microscopy.

Statistical analysis

Data are presented as means $\pm$ SEMs. One-way ANOVA with repeated measures was used for the comparison of more than two variables. When the initial *P*-value was less than 0.05, the Bonferroni/Dunn post hoc test was used to determine the significance among groups. Significance was established at the *P* < 0.05 level.

Results

Direction of RANTES secretion by endothelial cells

To investigate whether endothelial cells polarize RANTES secretion, we measured the RANTES concentration in the inner (intravascular side) and outer (extravascular side) wells on the 0.45 µm pore size membrane of the transwell chamber. Endothelial cells were stimulated with medium, TNF-α (10 ng/mL), IL-1β (10 ng/mL), IFN-γ (100 U/mL), or TNF-α (10 ng/mL) plus IFN-γ (100 U/mL) from the apical or basolateral side for 16 h followed by collection of the supernatant. The level of RANTES was measured by ELISA. RANTES released in the intravascular side was significantly increased in the presence of TNF-α, IL-1β, IFN-γ, or TNF-α plus IFN-γ in the apical and basolateral sides compared with that in medium alone (Fig. 1). Moreover, the level and the amount of RANTES secreted from the cytokine-stimulated endothelium were significantly higher in the intravascular side than extravascular side. These data indicate that endothelial cells cultured on the 0.45 µm pore size membrane of the transwell chamber preferentially secreted RANTES to the intravascular side.

To assess whether polar secretion of RANTES by endothelium depends on pore size since permeability is increased by enlarged pore size, we next performed similar experiments using the 3.0 and 8.0 µm pore size membranes of the transwell chamber. No differences were observed in the amount of cytokine-induced RANTES between the intravascular and extravascular sides in the 8.0 mm pore size membrane of the transwell chamber (Fig. 2). Additionally, to rule out the possibility that RANTES leaked from the apical to the basal side, we applied albumin (100 mg/dL) to the apical side of an endothelial culture and measured the concentration in the supernatant after 16 h. No albumin was detected in the outer well separated by the 0.45, 3.0, and 8.0 µm pore size membranes (data not shown). Taken together, the polarity of RANTES secretion by endothelial cells appears to be pore size-dependent.

Scavenging effect of red blood cells on the secreted RANTES

To study whether abundant RANTES in the intravascular side was scavenged by RBCs, the supernatants in the inner well were incubated with RBC suspension (Hb: 0.5–15 g/dL) for 10 min after stimulation of endothelial cells with TNF-α (10 ng/mL) for 16 h. As shown in Fig. 3, RBCs significantly scavenged the TNF-α-induced RANTES secretion at least less than Hb 2.5 g/dL in a concentration-dependent manner. Moreover, to assess whether RBCs directly inhibit RANTES production by endothelial cells, RBCs were incubated together with endothelial cells in the presence of TNF-α for 16 h and the RANTES level was measured in the collected supernatants. No difference was observed in the RANTES levels in the supernatant compared with the case that RBCs were added to the endothelial culture supernatant (Fig. 4), indicating that RBCs may scavenge RANTES simply by surface binding.

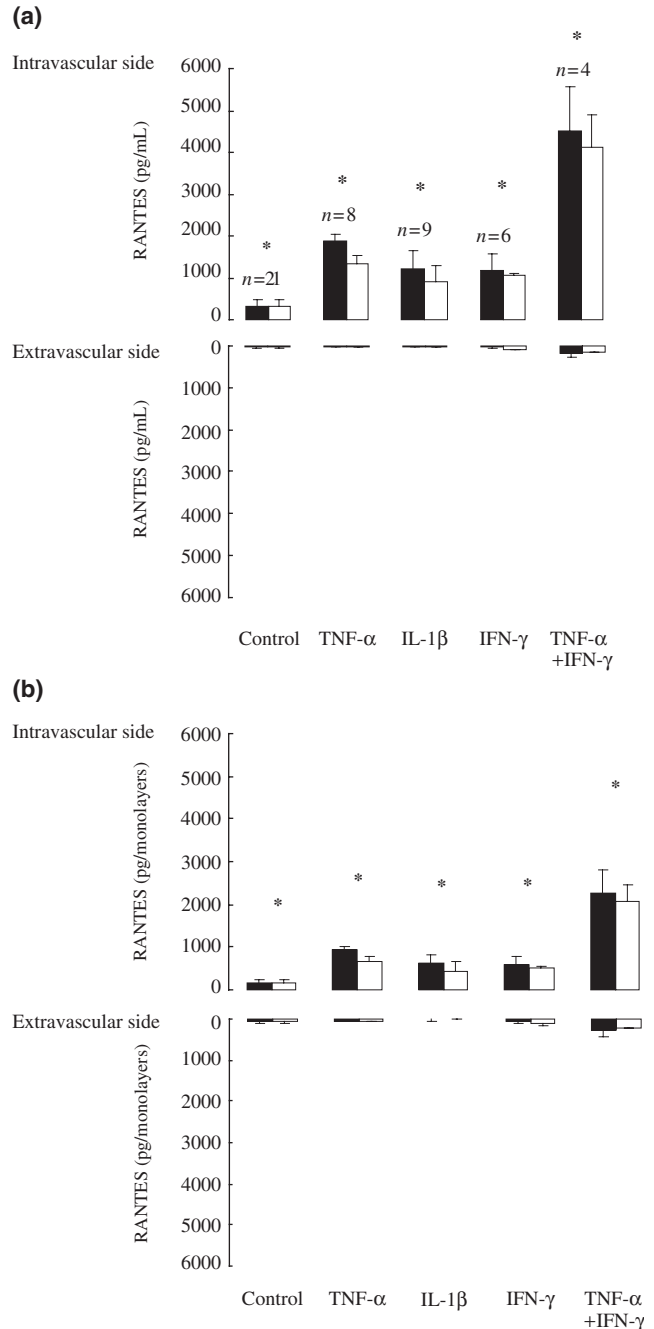


Fig. 1. Apical and basolateral secretion of RANTES by endothelial cells on the 0.45 µm pore size membrane of the transwell filter. The endothelial cells were stimulated from the apical side (filled bars) or basolateral side (open bars; $n = 3$) with TNF-α (10 ng/mL), IL-1β (10 ng/mL), IFN-γ (100 U/mL), or TNF-α plus IFN-γ for 16 h. The measurement of RANTES in the intravascular and extravascular sides was performed by ELISA. Each value is presented as the concentration (a) and the amount (b) of RANTES in each side. Data are expressed as the mean ± SEM. * $P < 0.05$ vs. extravascular side (ANOVA).

Functional effect of red blood cells on eosinophil chemotaxis

Finally, the functional involvement of RBCs in eosinophil migration was assessed. The migration of eosinophils from the upper to lower chamber was studied according to the procedure described in Material and methods. The pretreatment

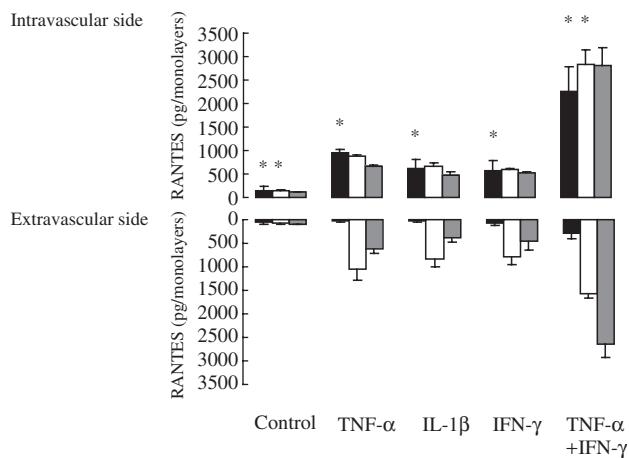


Fig. 2. The amount of apical and basolateral secretion of RANTES by endothelial cells on the 0.45, 3.0, 8.0 μm pore size membrane of the Transwell filter. The endothelial cells on the 0.3 (filled bars), 3.0 (open bars), and 8.0 μm (diagonal stripes bars) pore size membranes of the Transwell filter were stimulated from the apical side with $\text{TNF-}\alpha$ (10 ng/mL), $\text{IL-1}\beta$ (10 ng/mL), $\text{IFN-}\gamma$ (100 U/mL), or $\text{TNF-}\alpha$ plus $\text{IFN-}\gamma$ for 16 h. The measurement of RANTES in the well was performed by ELISA. Data are expressed as the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. extravascular side (ANOVA).

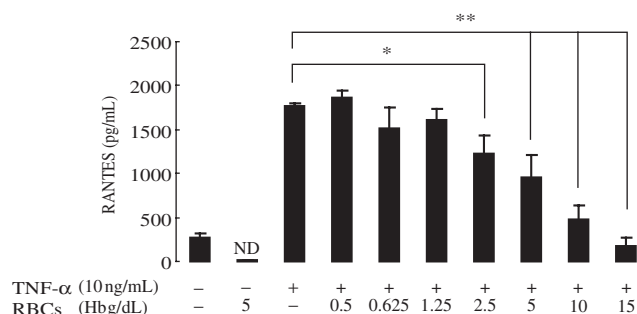


Fig. 3. Scavenging effect of red blood cells (RBCs) on RANTES secreted by endothelial cells. Endothelial cells were stimulated with $\text{TNF-}\alpha$ for 16 h and the supernatant of the intravascular side was collected. After serial concentrations of RBCs were added to the supernatant for 10 min, the RANTES level was measured. Data are expressed as the mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.001$ vs. without RBCs.

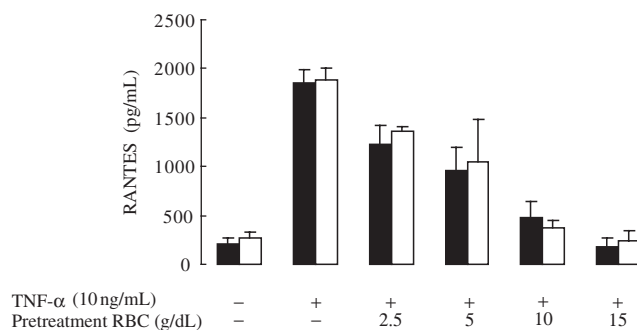


Fig. 4. Mechanism of the scavenging effect of red blood cells (RBCs) on the secretion of RANTES by the endothelium. The endothelial cells were incubated with incremental concentrations of RBCs for 16 h in the presence of $\text{TNF-}\alpha$, and the RANTES level was measured (open bars). In some experiments, endothelial cells were stimulated with $\text{TNF-}\alpha$ for 16 h and the supernatant of the intravascular side was collected. After serial concentrations of RBCs were added to the supernatant for 10 min, the RANTES level was measured (filled bars). Data are expressed as the mean $\pm$ SEM ($n = 3$).

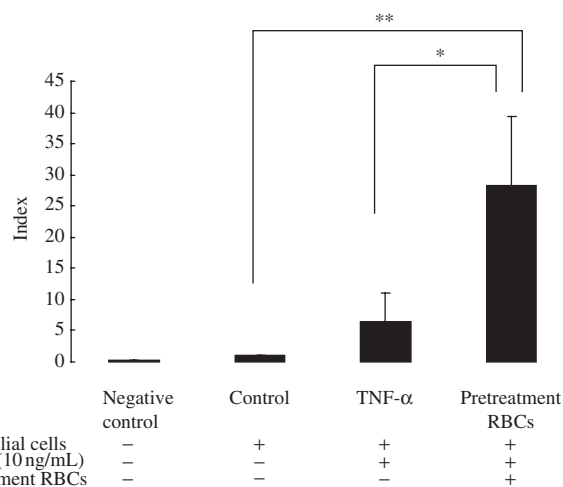


Fig. 5. Functional effect of red blood cells (RBCs) on eosinophil chemotaxis. The supernatant of the intravascular side stimulated with tumor necrosis factor- α for 16 h was mixed with medium or RBC suspension (hemoglobin (Hb): 15 g/dL) for 10 min at 37 $^{\circ}\text{C}$. The supernatant in the intravascular side obtained after centrifugation and that in the outer well were placed in the upper and lower Boyden chamber, respectively. Eosinophils were added to the upper chamber and allowed to migrate for 60 min at 37 $^{\circ}\text{C}$. The membrane was removed followed by fixation, and the migrated eosinophils were counted. The index indicates the ratio of control. Data are expressed as the mean $\pm$ SEM ($n = 3$). * $P < 0.01$, ** $P < 0.001$ vs. without RBCs.

with RBCs significantly augmented eosinophil chemotaxis more than 36-fold compared with control (Fig. 5). These data suggest that RBCs scavenge chemokines secreted into the intravascular side, resulting in a basal-to-apical chemokine gradient that allows eosinophils to migrate.

Discussion

In the present study, we first studied the polar secretion of RANTES by endothelial cells. We did not observe dissociated levels of RANTES between the apical and basal sides after the stimulation with cytokines, which is consistent with results reported previously [10]. In contrast, bronchial epithelial cells appear to preferentially release RANTES into the apical side in the Transwell system regardless of the epithelial culture being carried out in an upright or inverted manner [10]. The physiological difference in protein secretion between the epithelial and endothelial cells (i.e. polarized and non-polarized cells) may be explained by a sorting mechanism [17, 18]. In the models of polarized epithelium such as Madin-Darby canine kidney (MDCK) and CaCo-2 cells, membrane and secretory proteins are delivered to the apical surface via exocytic or endocytic routes. The former represents the apical pathway from the *trans*-Golgi network, and the latter is transcytosis after endosomal recycling from the basolateral membrane. These pathways to the cell surface are likely to require signal-mediated recognition in polarized cells. Although the definitive identification of sorting signals has not been fully clarified, one possibility is that glycosphingolipids and proteins form clusters anchoring to the membrane. This mechanism allows the glycolipid patches to

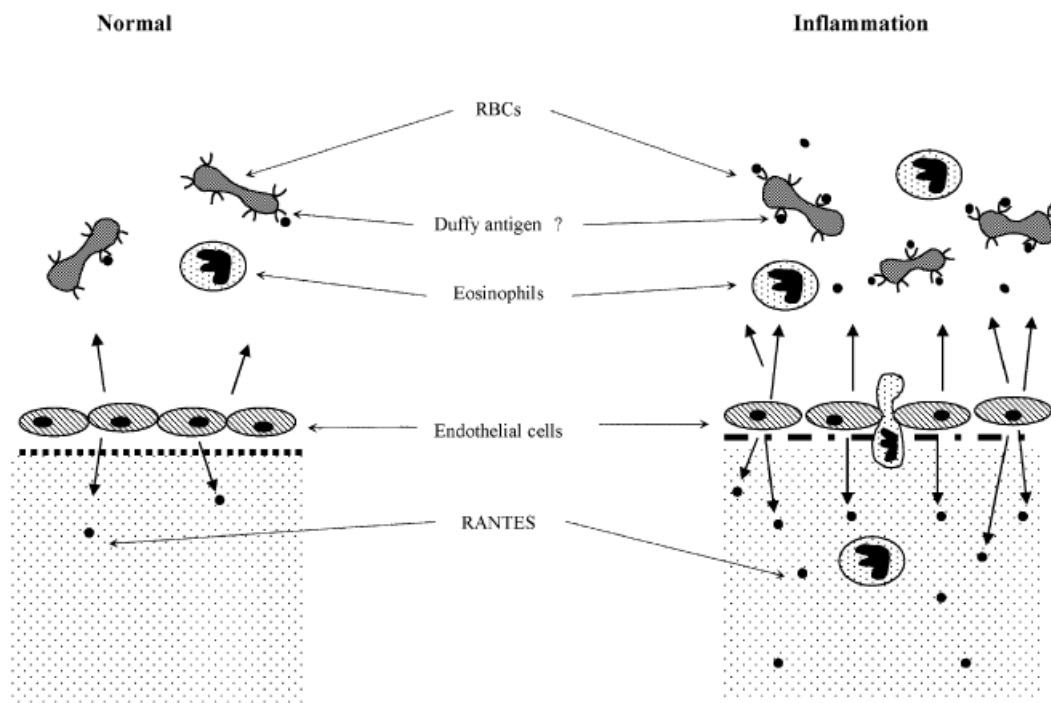


Fig. 6. The speculated contribution of red blood cells (RBCs) in transendothelial migration of eosinophils. Left and right figures indicate normal and inflammatory conditions, respectively.

act as apical targeting signals. Endothelial cells may not have this mechanism, resulting in the non-polarized protein secretion.

Since endothelial cells do not induce polarized RANTES production, other mechanisms must be involved in the transendothelial migration of eosinophils. One possibility is the contribution of RBCs as indicated by our results showing their scavenging effect on RANTES. RBCs express Duffy antigen, which serves as the scavenger for RANTES. It is identical to the erythroid promiscuous chemokine receptor and has a unique capacity to bind to both CXC and CC chemokines. Binding of chemokines to DARC is reversible and does not result in receptor internalization or signal transduction [19, 20]. Darbonne et al. [20] have shown that IL-8 bound to RBCs is incapable of stimulating Ca^{2+} influx in neutrophils, suggesting that RBCs are a sink for chemokines and thereby down-regulate overstimulation of leucocytes. We showed that incubation of the supernatant of TNF- α -stimulated endothelial cells with incremental concentrations of RBCs resulted in a reduced level of RANTES. This result indicates that RBCs simply bind to RANTES as scavengers, consistent with previous findings [19, 20].

Another possibility is the involvement of dipeptidyl-peptidase IV (CD26) in the modulation of secreted RANTES. Chemokines including RANTES share a highly conserved Xaa-Pro motif on the N-terminus [21]. CD26 is capable of cleaving this N-terminal Xaa-Pro dipeptide, resulting in functional alteration of RANTES. CD26-processed RANTES (3–68) has a much lower activity in monocyte migration compared with full-length RANTES (1–68) [22, 23]. In contrast, the full-length and truncated RANTESs appear to exhibit a similar chemotactic response in T cells [23]. An explanation for this finding is that RANTES (3–68)

has impaired binding and signalling capacity through CCR1 and CCR3 but retains activity on CCR5 [24]. In an *in vitro* system, RANTES (3–68) as well as RANTES (1–68) can be detected in the culture medium of dermal fibroblasts stimulated with TNF- α or IL-1 β [25]. That study further showed that the loss of the two N-terminal residues (Ser and Pro) does not affect the eosinophil chemotactic activity towards RANTES. Although eotaxin (3–74) is another substrate for CD26 and is released from TNF- α -stimulated dermal fibroblasts [26], this truncated eotaxin, unlike RANTES (3–68), exhibits impaired eosinophil chemotaxis compared with full-length eotaxins [27]. Since eosinophils do not express CCR5, the differential effect between the truncated RANTES and eotaxin is unclear. In our system, CD26 expression may be up-regulated on the TNF- α -stimulated endothelial cells and cleave the N-terminus of CC chemokines, resulting in the functional alteration of chemokine balance between the intra- and extra vascular sides.

Our results are unique findings that show the involvement of RBCs in modulating allergic condition, leading us to speculate that the schema shown in Fig. 6 is a possible mechanism. When inflammation such as asthma occurs, the extravascular concentration of RANTES is consequently increased because of enlarged pore size (Fig. 2), a condition like hyperpermeability. In addition, redundant intravascular RANTES seems to be not only washed out simply by blood flow but also sunk by RBCs. As a consequence of these mechanisms, the inflammatory site forms a RANTES gradient that facilitates the endothelial transmigration of eosinophils. Recruitment of eosinophils from the bloodstream into the airway is an important characteristic feature of allergic diseases including asthma. The delineation of the

secretory mechanism of chemokines by endothelial cells will help elucidate the first step in eosinophil accumulation, and it may shed light on how to develop a new strategy to treat allergic disorders.

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