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The role of the ribosomal protein S19 C-terminus in altering the chemotaxis of leucocytes by causing functional differences in the C5a receptor response

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Ribosomal protein S19 (RP S19) oligomers have been discovered as the first chemoattractant of migrating monocytes/macrophages to apoptotic cells via the C5a receptor (C5aR). In contrast to C5a, a fusion of the C-terminus (I_{134}–H_{145}) of RP S19 to C5a, the C5a/RP S19 chimera, substitutes for the RP S19 oligomers and is able to replicate C5aR antagonist-induced and agonist-induced dual effects on neutrophil and monocyte chemotactic responses, respectively. We recently discovered a gain of binding affinity when the I_{134}–H_{145} inhibited the activation of neutrophil C5aR-mediated chemotactic pathways. However, the opposing ligand-dependent chemotactic mechanisms are not fully understood. In this study, a loss of this additional binding affinity appeared to cause the monocyte C5aR to activate an alternative signalling pathway. The p38 mitogen activated-protein kinase (MAPK) pathway was linked to cell migration rather than a classical extracellular-regulated kinase 1/2 pathway commonly used by C5a. C5aR internalization was not involved in the alternative chemotactic pathway. We propose a model of activation involving a C5aR co-molecule that interferes with the C5aR–Gi protein interaction upon binding to the I_{134}–H_{145} in neutrophils; however, a free I_{134}–H_{145} from the C5aR co-molecule can guide the alternative activation of the chemotactic p38MAPK pathway in monocytes/macrophages.

Keywords: C5a receptor/chemotaxis/intracellular signal pathway/macrophages/ribosomal protein S19.

Abbreviations: C5aR, C5a receptor; C5L2, C5a receptor-like 2; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PLA_2, phospholipase A_2; PMA, phorbol-12 myristate 13-acetate; RP S19, ribosomal protein S19; TRPM, melastatin-type transient receptor potential Ca^{2+} channel.

G protein-coupled receptors (GPCRs) are ubiquitous mediators of the signalling of hormones and neurotransmitters and of sensing. The old dogma is that a one ligand–one receptor interaction constitutes the functional unit of GPCR-mediated signalling (*1*). However, accumulating evidence is challenging this concept. Formyl-peptide receptor (FPR) binds not only f-Met-peptides but also annexin 1 (*2*). The known endogenous ligands interacted with C5a receptor (C5aR) and C5aR-like 2 (C5L2) are the complement C5a anaphylatoxin and a cross-linked oligomer formed by transglutaminases ribosomal protein S19 (RP S19) oligomers (*3*, *4*). Information about this ligand-receptor interaction is shown in Fig. 1. C5a behaves as an agonist to C5aR in a variety of leucocytes (*5*). Notably, RP S19 oligomers were discovered to be the first chemoattractant for migrating monocytes/macrophages to apoptotic cells via the C5aR (*6*). RP S19 oligomers exhibit dual effects on the C5aR of phagocytic leucocytes; the oligomers cause an agonist-induced effect on the monocyte C5aR but an antagonist-induced effect on the neutrophil C5aR (*7*). Furthermore, RP S19 oligomers exhibit both of these effects on the C5aR of a long-term human mast cell line, HMC-1, depending on the ligand concentration. RP S19 oligomers have an antagonist-induced effect at low concentrations but an agonist-induced effect at high concentrations (*8*). In contrast to C5aR, C5L2 is thought to not couple with the G protein. We found that C5L2 on HMC-1 cells works as a decoy receptor, as previously shown in monocytes/macrophages (*9*).

The aim of our current research was to further study the opposing effects of RP S19 oligomers in leucocyte chemotaxis. A key moiety in RP S19 oligomers controlling the opposing ligand-dependent functions is the C-terminal region (I_{134}–H_{145}) of RP S19 (*10*). When the sequence of I_{134}–H_{145} was fused to the C-terminus of C5a, the C5a/RP S19 chimera behaved similarly to the RP S19 oligomers (*11*, *12*). We hypothesized that the combinatorial use of C5a and C5a/RP S19 would reveal the mechanism underlying the opposite effects of RP S19 oligomers. The conflicting responses of HMC-1 cells are due to the expression of the neutrophil type C5aR and the monocyte type C5aR on the cell surface, assuming that there are different types of C5aR activation. We initially examined this hypothesis by investigating potential splice variations of the C5aR mRNA. The C5aR gene structure indicates that there is no splice variation in the C5aR gene message (*13*).

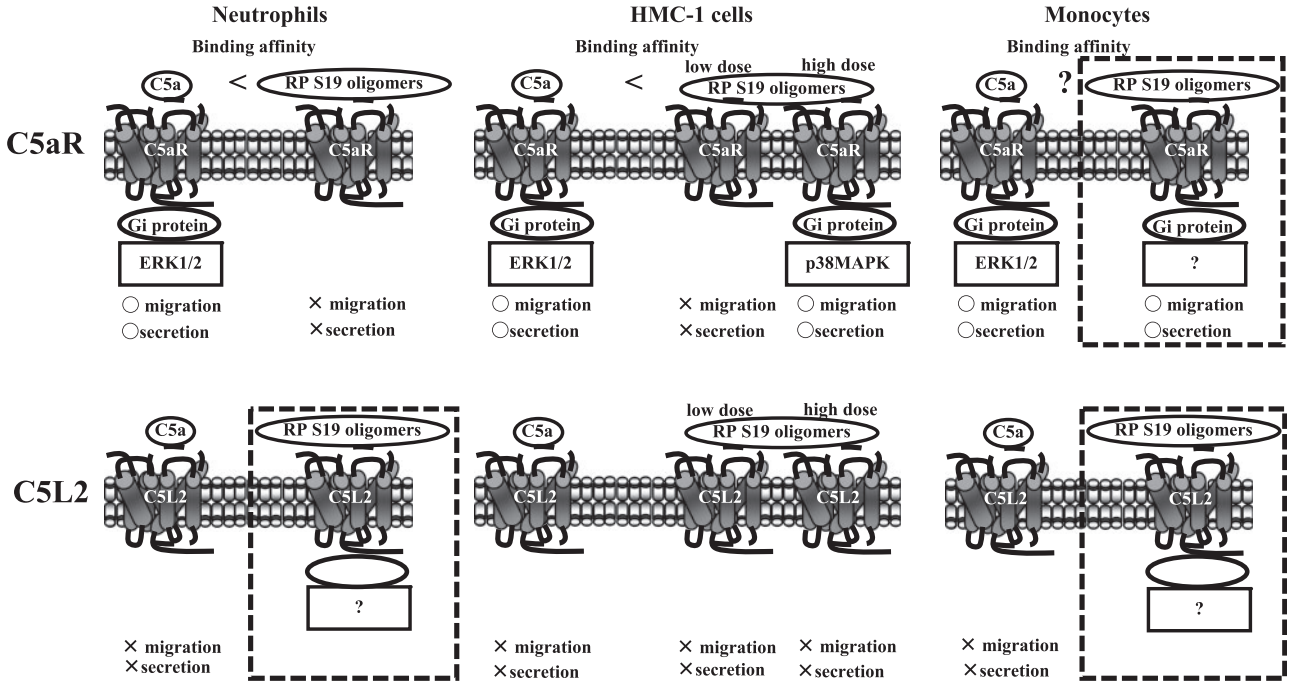

Fig. 1 Schematic illustrations of the ligand-dependent C5aR-mediated and C5L2-mediated responses among leucocytes. C5a causes chemotactic migration and secretion via the C5aR-mediated classical ERK1/2 pathway. RP S19 oligomers do not induce chemotaxis or secretion in neutrophils or at low concentrations in HMC-1 cells. At high concentrations, RP S19 oligomers cause chemotaxis and secretion via the alternative p38MAPK pathway in HMC-1 cells. Currently, a C5L2-mediated leucocyte response is not known. Squares with a dotted line denote unresolved data points when the current study was started.

We next examined the possible involvement of C5L2. This possibility was also rejected, because anti-C5L2 antibodies did not change the responses in HMC-1 cells (*8*). For these reasons, we have not fully investigated the differences between the neutrophil C5aR and the monocyte C5aR.

In our previous study of HMC-1 cells, C5a/RP S19 exhibited the agonist-induced effects through an alternative intracellular signalling transduction pathway that caused cellular chemotaxis and histamine release. The p38 mitogen-activated protein kinase (p38MAPK) and melastatin-type transient receptor potential Ca^{2+} channel (TRPM) were involved in the C5a/RP S19-guided responses, whereas the extracellular signal-regulated kinase 1/2 (ERK1/2), the inositol 1,4,5-trisphosphate receptor-dependent Ca^{2+} channel and the Ca^{2+} release-activated Ca^{2+} channel were involved in the C5a-guided responses (*8*). We are also currently comparing the ligand-dependent activation of the classical pathway and the alternative pathway in monocytes/macrophages and neutrophils, respectively. We report here that the monocyte C5aR selectively activates the classical pathway with the binding of C5a and the alternative pathway with the binding of C5a/RP S19. The C5a/RP S19-stimulated alternative pathway activation is somehow selectively blocked through the neutrophil C5aR.

Experimental procedures

Cells

HMC-1 cells and the leukaemia-derived cell lines, HL-60 and THP-1, were obtained from the Mayo Clinic, USA (Dr J. H. Butterfield) and the RIKEN BioResource Center, Japan, respectively. HL-60 cells and THP-1 cells were differentiated into macrophage-like cells in the presence of 1.6 nM phorbol-12 myristate 13-acetate (PMA) (Sigma Chemical. St Louis, MI, USA) for 5 days (*14*). We called these cells the HL-60 and THP-1 macrophages. Mononuclear cells and neutrophils were isolated from heparinized human venous blood using Ficoll-Paque™ PLUS (Amersham Biosciences KK, Tokyo, Japan) (*15*, *16*). The mononuclear cell fraction contained ~20% monocytes and the neutrophil fraction contained nearly all neutrophils. The mononuclear cells were further enriched using MACS column purification with anti-CD14 antibody beads (Miltenyi Biotec K.K., Tokyo, Japan). Satisfactory purities of monocytes (>95%) or neutrophils (>98%) were confirmed by fluorescence-activated cell sorting (FACS) with either anti-CD14 antibody or anti-CD16b antibody.

Binding assay with radio-labelled C5aR ligand

Cells (1×10^6 cells/ml) in the Ca^{2+} buffer (HBSS containing 20 mM HEPES and 0.5% BSA, pH 7.4) were pretreated with 2 μg/ml control rabbit IgG or anti-C5aR rabbit IgG; control goat IgG or anti-C5L2 goat IgG; and control PBS, C5aR antagonistic peptides (10^{-7} M PMX-53 or W-54011) (Merck KGaA, Darmstadt, FRG) (*17*, *18*) or a C3aR antagonistic/agonistic peptide (2×10^{-7} M SB290157) (*19*) for 30 min on ice. The cells were then mixed with the ^{125}I-C5aR ligand at a putative final concentration of 10^{-8} M (*8*). After incubation on ice for 60 min, the radioactivity of cellular bound ^{125}I-C5aR ligand was measured for 2 min in a gamma counter (PerkinElmer, Yokohama, Japan).

Calcium imaging

Cells (2×10^6 cells/ml) loaded with 1 μM Fura 2-AM (Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37°C were mixed with the C5aR ligand in the Ca^{2+} buffer or the Ca^{2+} free buffer (2 mM EGTA) after a baseline recording for 5 min. The recordings were made with an F-2500 calcium imaging system with FL Solutions (Hitachi, Tokyo, Japan). The maximal potency of each cell-type was measured and calculated relative to the response ratio using the following formula: the maximal potency of each cell-type induced by C5a/RP S19/a maximal potency of each cell-type induced by C5a × 100%.

Western blotting analysis

Proteins were separated by gel-electrophoresis on a 12% polyacrylamide vertical slab gel (*8*) and then transferred to a membrane (Millipore, Billerica, MA, USA), using a Semi Dry Electroblotter (Sartorious) for 90 min with an electric current of 15 V (*8*). Primary antibodies from rabbit IgGs (Cell Signaling Technology®, Boston, USA) were used at a concentration of 100 ng/ml for 1 h at 22°C, and HRP-conjugated anti-rabbit IgG goat IgG (Santa Cruz, CA, USA) secondary antibody was added at 20 ng/ml for 30 min. Reaction products were visualized with a chemiluminescence reaction using a ECL Plus Western Blotting Detection System™ (Amersham Biosciences KK, Tokyo, Japan).

Chemotaxis assay

Cells (2×10^6 cells/ml) pretreated with either an inhibitor of p38MAPK (10 μM SB203580) or phospholipase A_2 (PLA_2) and PLA_2-dependent TRP [10 μM N-(*p*-amylcinnamoyl) anthranilic acid (ACA)] (Sigma Chemical, St. Louis, MI, USA) (*8*) in the Ca^{2+} buffer or with the Ca^{2+}-free buffer at 37°C for 60 min were used for the multi-well chamber (Neuro Probe, Bethesda, MD, USA) assay using a Nucleopore filter (Pleasant, CA, USA) (*8*). After incubation for 90 min, the total number of cells that migrated beyond the lower surface of the membrane was counted in five microscopic high-power fields.

Statistical analysis

Statistical significance was calculated by the non-parametric or parametric tests offered in two-way analysis of variance window ($^*P < 0.05$ and $^{**}P < 0.01$).

Results and discussion

C5a/RP S19 binds to the C5aR on macrophage-like cells

We have demonstrated that C5a/RP S19 attracts monocytes/macrophages but not neutrophils. In the current study, we examined whether the HL-60 macrophages exhibit a chemotactic response to C5a/RP S19. To determine selective interaction with C5a/RP S19, the HL-60 macrophages were pre-treated with either anti-C5aR rabbit IgG, anti-C5L2 goat IgG, non-specific control IgGs, the C5aR antagonistic peptides (PMX-53 and W-54011), the C3aR antagonistic/partial agonistic peptide (SP-290157) or the control vehicle buffer (PBS) and subjected to the chemotaxis chamber assay. As shown in Fig. 2A, C5a/RP S19 attracted the HL-60 macrophages in a C5aR-dependent but C5L2- and C3aR-independent manner. Moreover, the inhibition of the C5aR with the antibodies or the antagonists completely suppressed both the C5a- and C5a/RP S19-dependent migration of the HL-60 macrophages (Fig. 2B). These data indicate that C5a/RP S19 attracts the HL-60 macrophages through the C5aR. The same results were obtained with the THP-1 macrophages in the chemotaxis chamber assay (data not shown).

We confirmed the above results by measuring the binding affinity of a radio-labelled ligand to the C5aR on the HL-60 macrophages. As shown in Fig. 2C, our results were consistent with the chemotaxis assay; however, we did see attenuated binding in the presence of anti-C5L2 goat IgG. Although the ligand binding was partially inhibited by the anti-C5L2 IgG, the chemotactic response of the cells was not affected by this blocking antibody. These results indicate that the HL-60 macrophages express the C5L2 as a decoy receptor, as previously shown in monocytes (*20*).

C5aR-mediated chemotactic signalling pathways in monocytes/macrophages and neutrophils

In the previous study with C5a and C5a/RP S19 in HMC-1 cells, we observed ligand-guided selections of intracellular signal pathways through the C5aR (*5*). We examined the ligand-guided selections between the classical pathway and the alternative pathway upon the interaction of the monocyte C5aR or the neutrophil C5aR with C5a and C5a/RP S19, respectively. For this experiment, monocytes, HL-60 macrophages and neutrophils were used to determine the phosphorylation of various protein kinases, which served as an indicator of the activation of a signal pathway, as assayed by western blotting. The phosphorylation of Akt and ERK1/2 is a marker of the classical pathway and the phosphorylation of

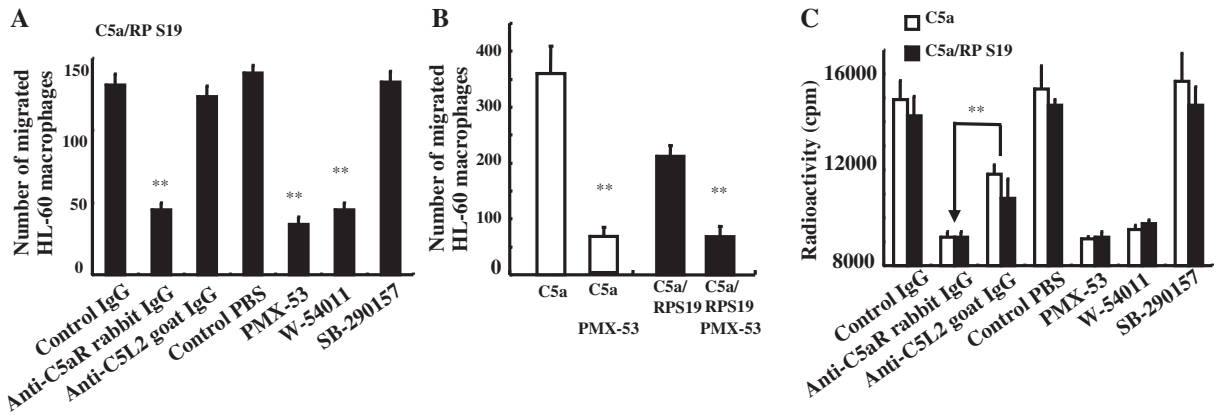

Fig. 2 C5a/RP S19-dependent C5aR-mediated responses in the HL-60 macrophages. (A) The HL-60 macrophages pre-treated with various inhibitors were attracted by C5a/RP S19 (the closed columns) in a 48-well chemotaxis chamber. Data are expressed as the mean ± SD ($n = 4$). $P < 0.05$ were considered statistically significant ($^{**}P < 0.01$). (B) The HL-60 macrophages pre-treated with or without the C5aR antagonistic peptide (PMX-53) were attracted by C5a (white columns) and C5a/RP S19 (black columns), respectively ($n = 4$). (C) The HL-60 macrophages pre-treated with various anti-receptor antibodies or with various receptor antagonistic peptides were mixed with the ^{125}I-labelled C5a (the open columns) or C5a/RP S19 (the closed columns), respectively ($n = 4$).

p38MAPK and c-Jun N-terminal kinase (JNK) is a marker of the alternative signalling pathway. As shown in Fig. 3, the classical pathway was selectively activated upon the C5a-C5aR interaction in monocytes and neutrophils. In the HL-60 cells, small amounts of p38MAPK and JNK phosphorylation were also detected. In contrast, the C5a/RP S19-C5aR interaction showed selective activation of the alternative pathway in monocytes and no activation of either pathway in neutrophils.

We confirmed the involvement of the positive feed back loop between p38MAPK and TRPM in the alternative pathway in the C5a/RP S19-induced chemotaxis of monocytes/macrophages. In this experiment, the HL-60 macrophages and monocytes were pretreated with either the p38MAPK inhibitor (SB203580), the TRPM inhibitors (ACA or 2.5 mM $MgCl_2$) or an extracellular Ca^{2+}chelator (2 mM EGTA) before the chemotaxis assay with C5a and C5a/RP S19. As shown in Fig. 4A, the C5a/RP S19-induced chemotactic response was completely suppressed by all of the inhibitors. In contrast, the C5a-induced chemotaxis was only partly inhibited by EGTA. Both extracellular and intracellular Ca^{2+} is required in the classical pathway, whereas only extracellular Ca^{2+} is involved in the alternative pathway.

We further confirmed the absence of the C5aR internalization in the alternative pathway in the HL-60 macrophages, THP-1 macrophages and monocytes. Because the alternative pathway lacks certain molecules required for C5aR internalization, such as C5aR kinases, C5aR should remain on the cell surface even after the binding of C5a/RP S19, as previously observed in HMC-1 cells. We examined the expression of the C5aR using FACS analysis. The results are shown in Fig. 4B. In all of the monocyte/macrophage types, the cell surface expression of C5aR was decreased upon the C5a-C5aR interaction but was not decreased with the C5a/RP S19-C5aR interaction. Furthermore, the C5a-induced C5aR internalization was competitively inhibited by a simultaneous addition of C5a/RP S19.

The activation of the alternative pathway seems to be determined by the C-terminal moiety (I_{134}–H_{145}) of RP S19 oligomers and C5a/RP S19. C5aR is a GPCR that catalyses guanine nucleotide exchange from GDP to GTP on the Gα protein. We believe that the guidance by the I_{134}–H_{145} would occur by the choice of Gα protein as the substrate of catalytic C5aR. As previously demonstrated, the chemoattraction of HMC-1 cells by C5a/RP S19 and C5a is blocked with pertussis toxin. We examined the chemoattraction in the HL-60 macrophages and monocytes and obtained the same results as the HMC-1 cells (data not shown). These findings indicate that the Gα proteins chosen by the C5a-C5aR and the C5a/RP S19-C5aR interaction must be family members of Gαi/o/t proteins. A complete study of these Gα protein interactions with the ligand-activated C5aR does not exist for any type of leucocytes. Therefore, it is difficult to further discuss the mechanism of ligand-guided selections of signal pathways via C5aR from the perspective of Gα protein selection.

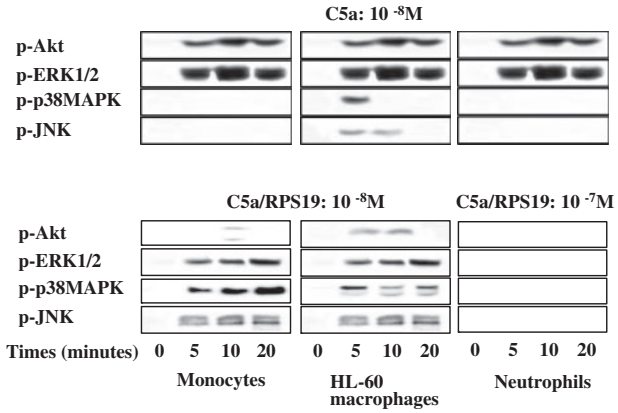

Fig. 3 Ligand-dependent selection of C5aR-mediated downstream signals in monocytes/macrophages. Akt, ERK1/2, p38MAPK and JNK phosphorylation in leucocytes was assayed by western blotting at several time points after stimulation with C5a and C5a/RP S19, respectively ($n = 4$).

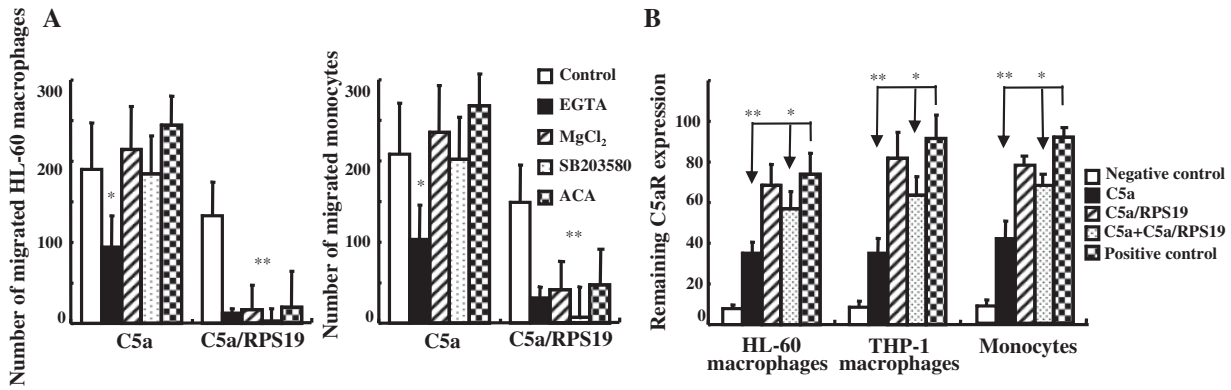

Fig. 4 Involvement of the positive feedback loop in the C5a/RP S19-induced chemotaxis of monocytes/macrophages. (A) Inhibition of chemotactic enhancement by various inhibitors of the positive feedback loop in the intracellular signal pathway. The HL-60 macrophages and monocytes pre-treated with either PBS (open columns), EGTA (closed columns), $MgCl_2$ (hatched columns), SB203580 (dotted columns) or ACA (checked columns) were attracted by C5a and C5a/RP S19, respectively ($n = 4$). (B) The HL-60 macrophages, the THP-1 macrophages and monocytes collected at 20 min after stimulation with C5a (closed columns), C5a/RP S19 (hatched columns) or the mixture of both (small dotted columns) were stained with PE-conjugated anti-C5aR mouse IgG ($n = 4$).

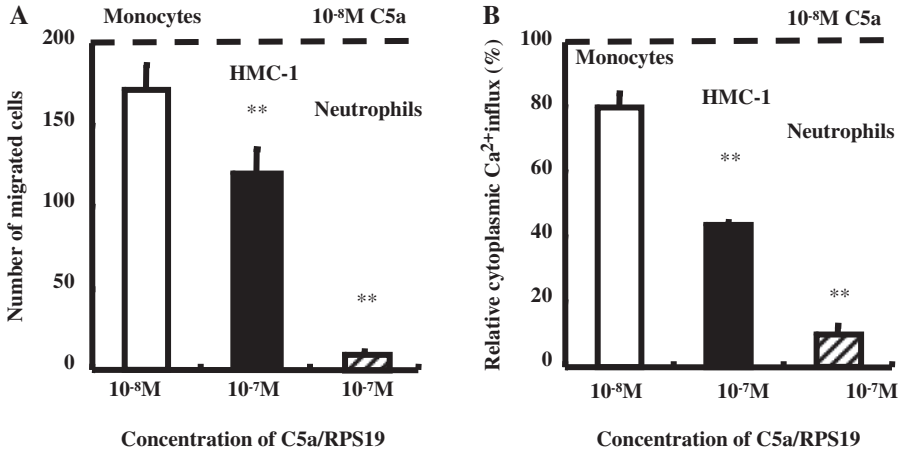

Fig. 5 Differential utilization of Ca^{2+} among leucocytes. (A) Monocytes (white columns), HMC-1 cells (black columns) and neutrophils (hatched columns) were attracted by C5a/RP S19 and C5a, respectively ($n = 4$). (B) Monocytes (white columns), HMC-1 cells (black columns) and neutrophils (hatched columns) pretreated with Fura 2-AM were stimulated by C5a/RP S19 and C5a, respectively ($n = 4$).

Differential utilization of C5aR-mediated alternative pathways among leucocytes

(i) Differential utilization of Ca^{2+} between C5a and C5a/RP S19 in chemotaxis among leucocytes.

The current results observed in monocytes/macrophages and neutrophils and the previous results observed in HMC-1 cells strongly suggest differential expression of C5aR co-molecules, which leads to the activation of the alternative signal pathway among these leucocytes with the greatest activation in monocytes/macrophages, then HMC-1 cells and lastly neutrophils. We tested this hypothesis by comparing the chemotactic response and the cytoplasmic Ca^{2+} influx upon the C5a/RP S19-C5aR interaction in these leucocytes. The difference in the chemotactic response between C5a and C5a/RP S19 was minimal in monocytes, marginal in HMC-1 cells and greatest in neutrophils (Fig. 5A). This hierarchy was maintained when the cytoplasmic Ca^{2+} influx was measured between C5a and C5a/RP S19. These findings also reflected the ordered capacities of the alternative signalling pathway among these leucocytes (Fig. 5B).

(ii) Different binding affinities between C5a and C5a/RP S19 to the C5aR among leucocytes.

There are three possible mechanisms for the C-terminus (I_{134}–H_{145}) of RP S19-dependent antagonistic effect on the neutrophil C5aR: absence of the alternative pathway, active blockage of the C5aR and G protein, and a combination of both scenarios. To substantiate the first possibility, there should be some activation of the classical pathway as a substitute, which is not observed (Fig. 3). To address the potential of active blockage between the C5aR and the G protein, we propose that a receptor co-molecule is in association with the neutrophil C5aR and would interfere with the C5aR-Gα protein interaction upon binding to the I_{134}–H_{145}. In our previous study of HMC-1 cells, we determined a peculiar phenomenon: there was a greater binding affinity of the radio-labelled C5a/RP S19 compared to C5a. To support our assumption of the I_{134}–H_{145}-co-molecule interaction increasing this binding affinity, we speculated that the higher binding affinity of C5a/RP S19 would be apparent in neutrophils, but not in monocytes.

Neutrophils, HMC-1 cells and monocytes were pre-treated with anti-C5L2 goat IgG to exclude the involvement of C5L2 and mixed simultaneously with ^{125}I-labelled C5a or C5a/RP S19 and varying concentrations of a C5aR antagonistic peptide, PMX-53. We then determined the concentration of PMX-53 needed to inhibit the radio-labelled C5a or C5a/RP S19 binding to 50% as IC_{50} [$IC_{50} = 10 \log$ (PMX-53 concentration needed to express greater than 50% inhibition/PMX-53 concentration to needed to express lower than 50% inhibition) $\times$ (50% – the lower than 50% value)/(the greater than 50% value – the lower than 50% inhibition value) + log (PMX-53 concentration needed to express the lower than 50%)]. The results are shown in Fig. 6. The apparent IC_{50} of PMX-53 needed to inhibit the C5a-C5aR interaction (4.5×10^{-9} M) was two times less than the IC_{50} needed to inhibit the C5a/RP S19-C5aR interaction (8.8×10^{-9} M) in neutrophils. Similar results were observed in HMC-1 cells, as previously described (*8*). In contrast to these cases, different binding affinities to C5a and C5a/RP S19 were not obvious for the monocyte C5aR.

These results strongly suggested the presence of a receptor co-molecule that is shutting down the C5aR-mediated signal in neutrophils. In addition to monocytes, we used macrophage-like HL-60 cells and THP-1 cells differentiated *in vitro* as indicator cells for monocyte/macrophage chemotaxis. The cells that can be differentiated *in vitro* will be advantageous in helping us identify a C5aR co-molecule. In future studies, we will be able to transform HL-60 or THP-1 cells with an artificial gene expressing each candidate protein. Using this method, separation and identification studies of the C5aR co-molecule are currently under way in our laboratory. Under these assumptions, we

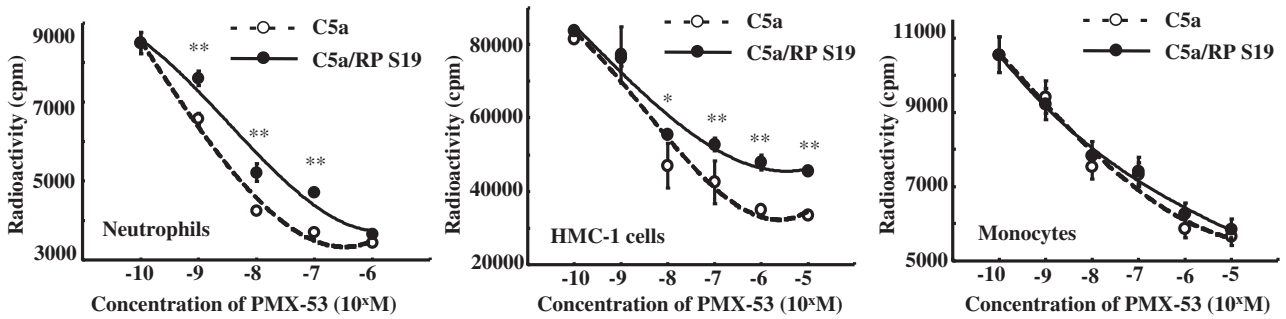

Fig. 6 Different binding affinities of C5a and C5a/RP S19 to the C5aR among leucocytes. Neutrophils, HMC-1 cells and monocytes pretreated with anti-C5L2 goat IgG were mixed with ^{125}I-labelled C5a (dotted line with open circles) or C5a/RP S19 (solid line with closed circles) in the presence of various concentrations of a C5aR antagonistic peptide, PMX-53 ($n = 4$).

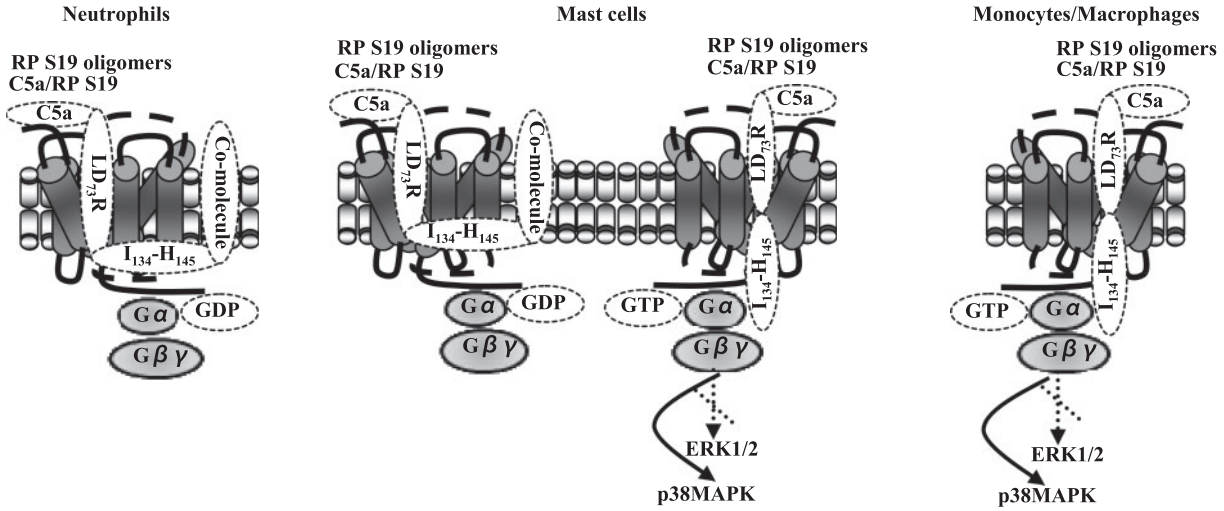

Fig. 7 Proposed models for two types of C5aR with or without a receptor co-molecule. Upon binding to the co-molecule, the C-terminus (I_{134}–H_{145}) of RP S19 oligomers interferes with the C5aR–Gi protein interaction in neutrophils. The free I_{134}–H_{145} interferes with the classical ERK1/2 pathway, resulting in the activation of the alternative p38MAPK pathway in monocytes/macrophages. Both types of C5aR are present in mast cells.

developed a model of the C5aR in different leucocytes shown in Fig. 7. The neutrophil type C5aR is associated with the C5aR co-molecule, and the monocyte type C5aR is free; however, the C5aR is present in both states on HMC-1 cells due to an insufficient amount of the C5aR co-molecule. The expression ratio of these C5aR states among the different types of leucocyte explains the different C5aR-mediated responses against C5a/RP S19.

Mast cells have been long viewed to be effector cells in acquired immunity, including type I allergy. However, it has been shown that mast cells are capable of engulfing bacteria, migrating to lymph nodes, and presenting antigen information to T cells (*21*, *22*). Mast cells, therefore, should be recognized as a kind of phagocytic leucocyte that possesses macrophage-like functions. The different chemotactic capacities against C5a/RP S19 among the different types of leucocytes somehow seem consistent with the stratification of the macrophage-like functions among the phagocytic leucocytes.

Pathophysiologically, the opposing C5aR-mediated effects on chemotaxis in monocytes/macrophages and neutrophils cause the predominant infiltration of monocytes/macrophages in apoptotic lesions and are seen in rheumatoid arthritis synovium (*23*) and atheromatous aorta (*24*), where RP S19 oligomers are generated.

Apoptotic cell-derived chemoattractants, such as thrombosposin 1, lysophosphatidylcholine and ATP/UTP, upon their respective binding to GPCRs, attract monocytes/macrophages (*25*–*27*). However, these receptors are not only expressed on monocytes/macrophages but also on neutrophils. Therefore, our future experiments will be important to delineate whether there is an identifying signal in macrophages, which will be used to search for apoptotic cells.

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Conflict of interest

None declared.

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