EMR2 Receptor Ligation Modulates Cytokine Secretion Profiles and Cell Survival of Lipopolysaccharide-treated Neutrophils

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Background: Epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2 (EMR2) is an adhesion G protein-coupled receptor previously shown to potentiate neutrophil responses to a number of inflammatory stimuli. EMR2 activation promotes neutrophil adhesion and migration, and augments production of reactive oxygen species and degranulation. In this study, we examined the effect of EMR2 ligation by its specific antibody on the cytokine expression profile and cell survival of lipopolysaccharide (LPS)-treated neutrophils.

Methods: Neutrophils were treated with LPS in the absence or presence of the anti-EMR2 mAb, 2A1. Cell apoptosis was determined by flow cytometry analysis using annexin-V and propidium iodide staining. Cell supernatants were collected for the detection of cytokine secretion by enzyme-linked immunosorbent assay.

Results: We confirmed the specific priming effect of EMR2 on the response of neutrophils to formyl-Met-Leu-Phe by measuring the production of reactive oxygen species. Furthermore, we showed that EMR2 ligation suppresses LPS-induced neutrophil survival. In addition, we demonstrated that ligation of EMR2 changes the secretion profiles of multiple cytokines, including interleukin (IL)-6, IL-8, and monocyte chemotactic protein-1. Finally, higher levels of EMR2 were detected on neutrophils of liver cirrhosis patients and were correlated to a pro-apoptotic phenotype.

Conclusion: Collectively, the present data indicate a functional role for EMR2 in the modulation of neutrophil activation during inflammation.
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Key words: G protein-coupled receptor, apoptosis, neutrophil, cytokine, inflammation

Neutrophils are the principal effector cells during the early phase of the innate immune response. Although normally circulating in the blood, neutrophils are rapidly recruited to sites of infection to engulf and kill invading microorganisms. Neutrophils are terminally differentiated cells with a half life of 12 hours due to constitutive apoptosis.[1] Nevertheless, concomitant cell activation within

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inflamed tissues delays neutrophil apoptosis, prolonging its microbicidal activity.[2,3] It is now well-recognized that activation of neutrophils is tightly regulated, and is usually achieved via multiple mechanisms, including different stages of cellular activation, the requirement for either high stimulus concentrations or multiple stimuli for a full activation, and a tight control in apoptosis. Defects in these regulatory mechanisms often result in sub-optimal or overpowering cell activation, leading to chronic and persistent infection, tissue damage, systemic inflammation, or life-threatening organ failure.[4,5]

Previously we have identified the epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2 (EMR2) receptor as a new regulatory molecule of neutrophil activation.[6] EMR2 is a member of the human adhesion G protein-coupled receptor (GPCR) family characterized by the composition of two distinct protein domains, namely the long extracellular cell-adhesion domain (ECD) and the seven-span transmembrane (7TM) domain.[7,8] It was generally thought that ligand binding to the ECD may somehow induce the 7TM subunit to transmit intracellular signal(s). EMR2 is up-regulated during the differentiation and maturation of macrophages, but is down-regulated during dendritic cell maturation. In inflamed tissues, strong EMR2 expression is found in sub-populations of macrophages and neutrophils.[9] Patients with systemic inflammatory response syndrome (SIRS) display a much higher EMR2 level in their neutrophils than control subjects.[6] More recently, it was shown that foamy macrophages in atherosclerotic vessels and Gaucher cells in the spleen also express high levels of EMR2.[10]

In a previous report, we employed the 2A1 monoclonal antibody (mAb) that recognizes the ECD of EMR2 to investigate its role in neutrophil function.[6] We found that ligation of EMR2 by 2A1 specifically enhances neutrophil response to a number of inflammatory stimuli including formyl-Met-Leu-Phe (fMLP), platelet activating factor and phorbol 12-myristate 13-acetate. In the presence of these stimuli, 2A1 treatment increased neutrophil adhesion and migration, and augmented superoxide production and degranulation. Surprisingly, in the absence of an inflammatory stimulus, 2A1 treatment alone did not show specific effects on neutrophil activation.[6] These results strongly suggest that activation of EMR2 receptors by 2A1 binding leads to intracellular signal(s) that by itself is not strong enough to activate neutrophils, but is able to prime/potentiate the outputs of inflammatory stimuli leading to a much stronger activation.

In the present study, we show that EMR2 activation induces changes in the secretion profiles of multiple cytokines and suppresses the delayed apoptosis of lipopolysaccharide (LPS)-treated neutrophils. Patients with liver cirrhosis were found to have higher levels of EMR2 on their neutrophils, which displayed a pro-apoptotic phenotype. Taken together, the present data expand the role of EMR2 as a regulatory molecule of neutrophil activation and function.

METHODS

Materials

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified. LPS used in the study was from *E coli*. serotype O111:B4 (Sigma). Hank's balanced salts solution (HBSS) and Iscove's Modified Dulbecco's Media (IMDM) were obtained from Invitrogen. Ficoll-Paque was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). The following antibodies, Anti-CD97/1 (Clone: MEM-180), anti-EMR2 (Clone: 2A1); and D1.3, a mouse Ab directed against hen egg white lysozyme, were described previously;[6] Mouse anti-caspase 3 Ab (clone 3G2) was from Cell Signaling. TACS® Annexin V Kit and 7-amino-actinomycin D (7-AAD) were obtained from R & D Systems and Sigma, respectively.

Preparation of human neutrophils and cell culture

In total, 28 healthy volunteers and 28 patients with liver cirrhosis were enrolled in the study. All signed informed consents and the study was approved by the local ethics committee. The diagnosis of liver cirrhosis was either by abdominal sonography, computer tomography or clinical confirmation with endoscopic findings of esophageal/gastric varices. Neutrophils were isolated from fresh venous blood using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. In short, neutrophils were isolated using a standard

method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes as described previously.[6] Purified neutrophils that contained > 95% viable cells, as determined by trypan blue exclusion, were resuspended in HBSS or IMDM for subsequent use. When necessary, cells were cultured in IMDM supplemented with autologous serum or fetal calf serum (FCS) and 50 U/ml streptomycin and penicillin at 37°C.

Detection of reactive oxygen species (ROS)

Isolated neutrophils (2×10^6 cells/ml) were resuspended in HBSS supplemented with 0.2% bovine serum albumin, 5 mM glucose, and 2 mM NaN_3, and incubated with 2 μM dihydrorhodamine-123 (DHR123; Molecular Probes) for 25 min at room temperature. Cells were then incubated for 15 min with either 2A1 (5 μg/ml) or CD97/1 (5 μg/ml) before stimulation with f-MLP (1×10^{-7} M) for 30 min at 37°C. The accumulation of H_2O_2 was analyzed immediately by flow cytometry using Cell Quest Software (Becton Dickinson). For the assay of O_2- generation, the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c was measured using standard procedures described previously.[11] Briefly, after supplementation with 0.5 mg/ml ferricytochrome c and 1 mM Ca^{2+}, neutrophils were equilibrated at 37°C for 2 min and incubated with 2A1 (5 μg/ml) for 5 min. Cells were then activated with f-MLP (1×10^{-7} M) for 10 min.

Detection of neutrophil apoptosis

The binding of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide or 7-ADD was used to differentiate between early apoptosis and late apoptosis as described previously.[12] Neutrophils (2×10^6 cells/ml) were cultured in the presence of 10% autologous serum with or without LPS (100 ng/ml) and Abs (2A1 and D1.3, 5 μg/ml) before harvesting for analysis at 20 hrs. Specific binding of annexin V-FITC was performed according to the manufacturer's instructions (R & D Systems). After incubation, the cells were analyzed using a flow cytometer and Cell Quest Software.

Western blot analysis

Western blot analysis was performed as described previously.[13] Neutrophils were cultured at the same conditions as described above for the detection of cell apoptosis. Total cell lysates were collected at indicated time points and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were transferred to nitrocellulose membranes and incubated in blocking buffer (tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk) for 1 hour. Mouse anti-caspase 3 Ab (1:500) was used as the primary Ab. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000) was used as the secondary antibody, followed by electrochemiluminescence for detection. The intensities of specific protein bands were quantified using Gel-Pro Analyzer 3.1 (Media Cybernetics, Bethesda, MD, U.S.A.).

Measurement of cytokines by sandwich ELISA

Neutrophils (1×10^7 cells/ml) were cultured in IMDM containing 10% FCS and treated as indicated for 6 and 20 hours. Cell-free supernatants were collected for sandwich enzyme-linked immunosorbent assay (ELISA) analysis to determine the concentration of interleukin (IL)-6 (#DY206), IL-8 (#DY208) and chemokine (C-C motif) ligand 2 / monocyte chemotactic protein-1 (CCL2/MCP-1) (#DY279) using the Duo Set ELISA Development System (R & D Systems) according to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. Results are shown as the mean ± SD. Statistical analysis of multiple experimental groups was compared by one-way ANOVA, followed by Dunnett post-test analysis using SPSS 17.0 (SPSS, Chicago, IL, U.S.A.). A probability (*p*) of ≤ 0.05 was considered significant.

RESULTS

Ligation of EMR2 potentiates ROS production in neutrophils

To confirm the specific effect of 2A1 in potentiating the production of anti-microbial mediators by neutrophils, we determined the augmentation of ROS production in neutrophils using two independent methods. First, DHR123-loaded neutrophils were pre-treated with either of two EMR2 specific antibodies, 2A1 or CD97/1, followed by stimulation

with or without fMLP. The respiratory burst was measured by flow cytometry assay (Fig. 1A). Incubation of neutrophils with 2A1 alone showed a minimal increase in fluorescence, while a control Ab CD97/1 had no effect (Fig. 1A and data not shown). Pretreatment of neutrophils with 2A1 prior to fMLP stimulation greatly enhanced H_2O_2 generation, producing the strongest fluorescence in the different groups. In contrast, CD97/1 pre-incubation did not have a similar augmentation effect, suggesting a specific effect for 2A1 (Fig. 1A).

Second, the O_2•− generation by neutrophils was measured by SOD-inhibitable cytochrome *c* reduction (Fig. 1B). When neutrophils were incubated with 2A1 alone, the O2•− generation was increased slightly compared with untreated controls. In contrast, a significant increase was seen in fMLP-stimulated cells, and 2A1 treatment prior to fMLP stimulation led to the highest O2•− generation (Fig. 1B) in the differrent groups. Incubation with control IgG (mIgG) had no effect on O2•− generation either alone or in combination with fMLP (data not shown). These results indicate that 2A1 is a potent agonist for EMR2 and the ligation of EMR2 in neutrophils by 2A1 leads to intracellular signaling events that augment the output of inflammatory stimuli.

Activation of EMR2 by 2A1 attenuates LPS-induced neutrophil survival

Neutrophils are short-lived cells that undergo constitutive spontaneous apoptosis in resting conditions.[3,14] Nevertheless, neutrophil survival can be prolonged by various means such as exposure to cytokines and inflammatory stimuli as well as changes in the microenvironment, including oxygen tension and extracellular matrix composition.[2,12,15] Our findings on the priming effects of EMR2 ligation prompted us to study the consequences of 2A1 binding in neutrophil survival. We first evaluated the effect of LPS on spontaneous apoptosis of neutrophils. As expected, neutrophil apoptosis was clearly delayed by LPS treatment (Fig. 2A, B). To investigate the effect of EMR2 ligation on LPS-induced neutrophil survival, cells were treated with 2A1 or an isotype control D1.3 mAb in the presence or absence of LPS. As shown in Fig. 2B, 2A1 alone had no obvious effect on neutrophil spontaneous apoptosis, however the extent of apoptosis was sig-

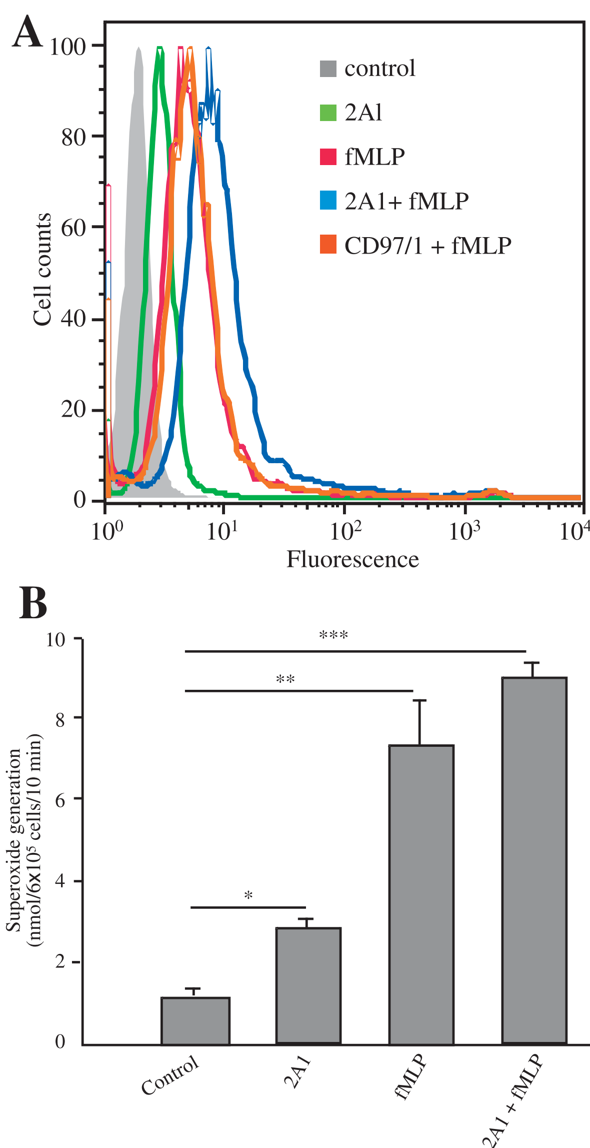

Fig. 1 Ligation of EMR2 potentiates the ROS production by neutrophils. (A) Isolated peripheral blood neutrophils loaded with DHR123 were preincubated with either 2A1 or CD97/1 (5 μg/ml). Cells were then incubated with or without fMLP (1×10^{-7} M) for 20 minutes and ROS production was measured by flow cytometry. (B) Neutrophils were incubated with or without 2A1 or control mouse IgG (5 μg/ml) for 5 min, and then activated by fMLP. O_2•− generation was measured using SOD-inhibitable cytochrome c reduction. Results represent mean ± SD ($n = 3$). ** $p < 0.01$; ***$p < 0.001$ compared with the control. * $p < 0.05$ between two groups. Abbreviations used: EMR2: epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2; ROS: reactive oxygen species; fMLP: formyl-Met-Leu-Phe; DHR123: dihydrorhodamine 123; SOD: superoxide dismutase.

nificantly enhanced with 2A1 plus LPS compared with LPS-treated cells. This effect is specific as D1.3 failed to show similar attenuation on cell survival.

These results strongly indicate that EMR2 activation suppresses LPS-induced neutrophil survival. To provide a possible mechanistic explanation, we further compared the integrity of pro-caspase 3, whose proteolysis-induced activation plays a critical role in the execution of apoptosis. As expected, the level of pro-caspase 3 was the weakest in control aged neutrophils but highest in LPS-treated cells, representing strong and reduced caspase 3 activation, respectively (Fig. 2C, D). Simultaneous treatment with 2A1 and LPS however did not reduce the pro-caspase 3 level significantly compared with LPS-treated cells (Fig. 2C, D). This result suggests that the inhibitory effect of EMR2 activation on LPS-induced neutrophil survival might not be fully dependent on caspase 3 activation.[16]

Activation of EMR2 by 2A1 changes cytokine profiles in LPS-treated neutrophils

It is well known that neutrophil apoptosis can be modulated by various cytokines.[1,15,16] To examine

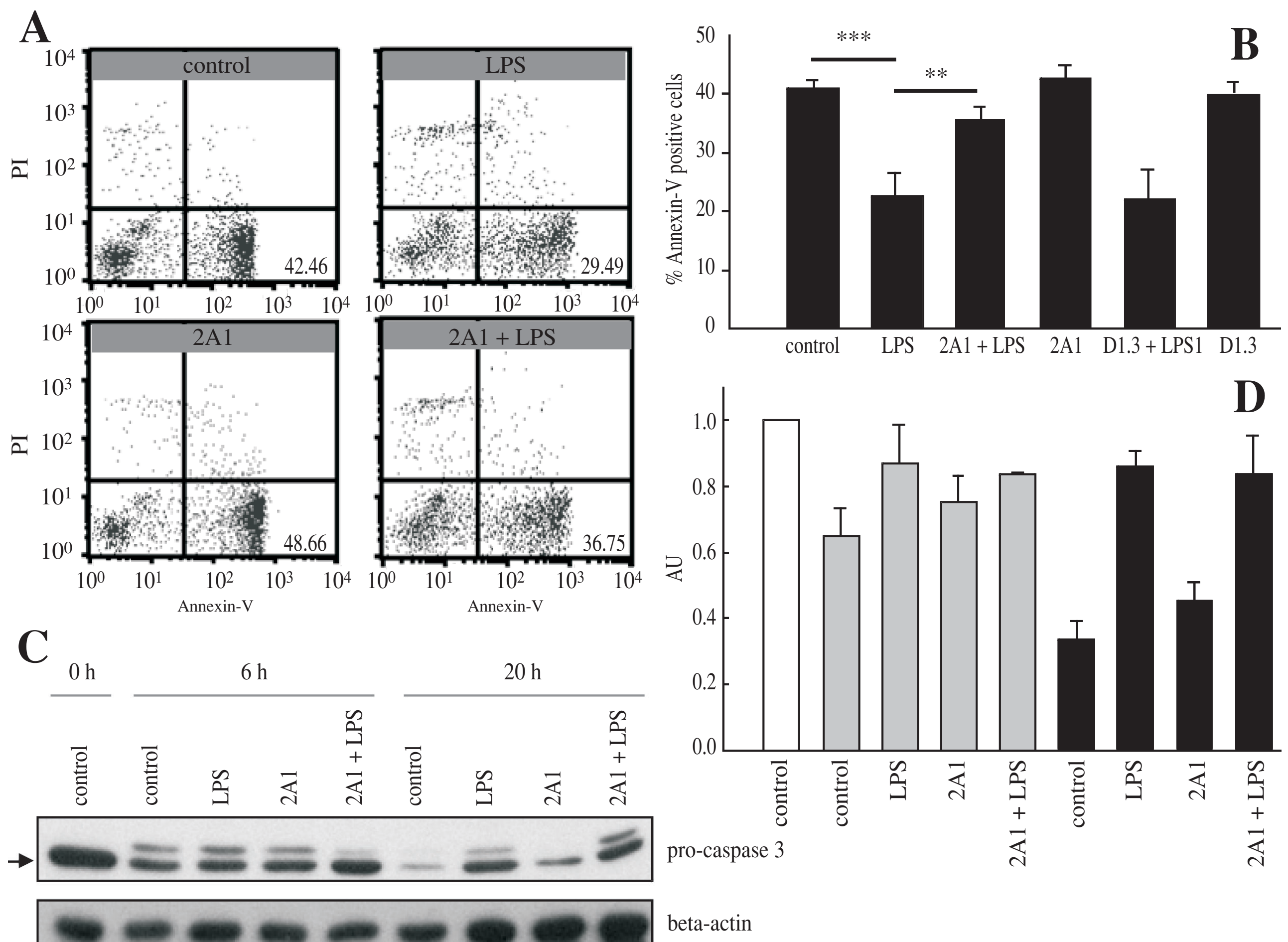

Fig. 2 EMR2 ligation attenuates LPS-induced suppression of neutrophil apoptosis. (A, B) Neutrophils were cultured in the presence or absence of LPS (100 ng/ml) and 2A1 (5 μg/ml) for 20 hrs. Neutrophil apoptosis was determined by annexin-V/PI staining. Results represent mean ± SD ($n = 3$); ** $p < 0.01$, *** $p < 0.001$. (C) Total cell lysates of neutrophils under the same treatment for 6 hrs and 20 hrs were analyzed for the integrity of pro-caspase 3 (arrow) by western blotting. (D) Comparison of the relative intensity of pro-caspase 3 in different treatment groups. The AU (arbitrary unit) of the untreated cell control group was set at 1.0. Grey bar: samples with 6 hr incubation. Dark bar: samples with 20 hr incubation. Results represent mean ± SD ($n = 3$). Abbreviations used: EMR2: epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2; LPS: lipopolysaccharide; PI: propidium iodide.

whether the cytokine profile of neutrophils was changed by EMR2 ligation, we compared the secretion of IL-6, IL-8 and MCP-1, all of which have been shown to modulate neutrophil apoptosis.[17-21] Indeed, a clear change in the cytokine profile was identified in neutrophils under various treatment conditions (Fig. 3). Specifically, it was shown that while cells treated with 2A1 alone showed cytokine levels comparable with the control group, secretion of all three cytokines was enhanced in LPS-treated neutrophils. Interestingly, the IL-8 level was significantly higher in the 2A1 plus LPS treatment group compared with cells treated with LPS alone (Fig. 3B). In contrast, the levels of IL-6 and MCP-1 were reduced significantly in this group of cells in comparison with LPS-treated cells (Fig. 3A, C). Moreover, the MCP-1 level in the cells treated with 2A1 and LPS was the lowest among the different groups of cells. Hence, 2A1 binding of EMR2 either synergized or interfered with LPS in cytokine secretion. It is thought that the combination of these cytokines, and probably others, is responsible in part for the suppressive effect on LPS-induced neutrophil survival.

Neutrophils of liver cirrhosis patients express significantly higher levels of EMR2 and are pro-apoptotic

Our previous study has found that patients with SIRS have elevated EMR2 levels on their neutrophils.[6] To investigate whether the upregulation of EMR2 on neutrophils is a common phenomenon during systemic inflammation, EMR2 expression levels on neutrophils of healthy individuals and patients with liver cirrhosis, a disease of chronic endotoxemia/inflammation,[22] were compared. We again found significantly higher EMR2 expression (~1.6 fold) on the neutrophils of cirrhotic patients than those of healthy controls (Fig. 4A). Furthermore, neutrophils from cirrhotic patients displayed a much stronger tendency to undergo spontaneous apoptosis than normal neutrophils (Fig. 4B). These results suggest that EMR2 upregulation on neutrophils is indeed associated with systemic inflammation and is linked to important neutrophil phenotypes such as activation and apoptosis.

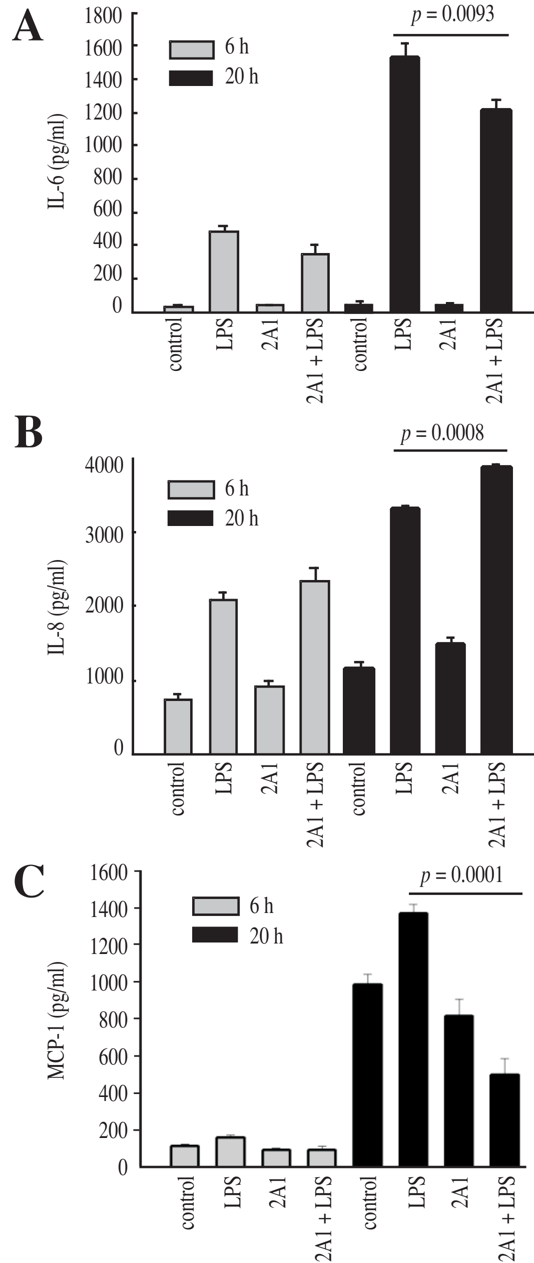

Fig. 3 EMR2 ligation changes cytokine profiles in LPS-treated neutrophils. Neutrophils were cultured in the presence or absence of LPS (100 ng/ml) and 2A1 (5 μg/ml). Cell supernatants were collected and analyzed for the production of IL-6 (A), IL-8 (B), and MCP-1 (C) at 6 hrs (grey bar) and 20 hrs (dark bar) by ELISA. Results represent mean ± SD (n = 4-5). Abbreviations used: EMR2: epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2; LPS: lipopolysaccharide; IL: interleukin; MCP-1: monocyte chemotactic protein-1; ELISA: enzyme-linked immunosorbent assay.

DISCUSSION

Our previous findings have shown that EMR2

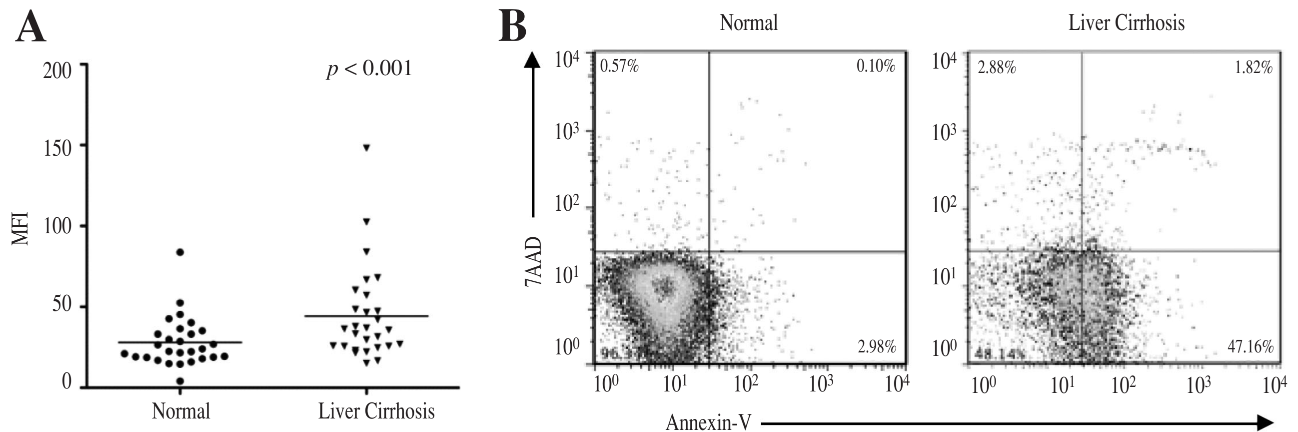

Fig. 4 Neutrophils from patients with liver cirrhosis show significantly higher cell surface EMR2 expression levels and a pro-apoptotic phenotype compared with those from healthy volunteers. (A) Neutrophils from healthy volunteers and patients with liver cirrhosis were evaluated for cell surface EMR2 expression by flow cytometry. EMR2 expression is significantly higher in cirrhotic patients (n = 28, MFI = 44.24 ± 5.48) than in healthy volunteers (n = 28, MFI = 28.10 ± 2.88); $p < 0.001$. (B) Spontaneous apoptosis of neutrophils from healthy volunteers and cirrhotic patients were evaluated within 1 hr after isolation by flow cytometry using annexin-V and 7-AAD staining. Representative FACS profiles of neutrophils from a healthy volunteer (left panel) and a patient with cirrhosis (right panel) are shown. Abbreviations used: MFI: mean fluorescence intensity; EMR2: epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2; 7-AAD: 7-amino-actinomycin D; FACS: fluorescence-activated cell sorting.

ligation can potentiate neutrophils at the "early stage" of inflammation, including changes in cell adhesion molecules and cell morphology, enhanced chemotaxis, production of anti-microbial mediators and degranulation.[6] These events are critical for the rapid and efficient elimination of pathogens by neutrophils during the early phase of infection. The results of this study not only confirm the specific priming effect of EMR2 activation, but also indicate that EMR2 is likely involved in the "late stage" of neutrophil activation, namely the resolution phase. Interestingly, EMR2 ligation seems to suppress the effect of LPS in delaying neutrophil apoptosis, leading to a shorter survival time for the cells. This would imply a more rapid resolution of inflammation and hence, minimization of tissue damage.

In the clinical setting, it is of interest to correlate the observation of higher levels of EMR2 expression and pro-apoptotic phenotypes in neutrophils of liver cirrhosis patients. This observation echoes our previous finding that showed a ~ 3-fold increase of EMR2 expression in neutrophils of SIRS patients.[6] Similarly, neutrophils with strong EMR2 staining were also identified previously in several other inflamed tissues such as liver abscesses, lung abscesses and acute suppurative appendicitis.[9] It is possible that EMR2 is a general marker of activated neutrophils in systemic inflammation. Alternatively, enhanced EMR2 expression and activation might contribute to the activation of neutrophils and the severity of systemic inflammation. Whatever the possibilities, a clear link between enhanced EMR2 expression and neutrophil activation is evident. In fact, the function of EMR2 in fine-tuning the neutrophil response identified here is reminiscent of those described for members of the triggering receptor expressed on myeloid cells (TREM) family. TREM receptors are immunoglobulin superfamily proteins that modulate the activation and differentiation of various myeloid cells.[23,24] By amplifying or dampening the activity of major "decision-making" receptors such as Toll-like receptors, TREM receptors play a crucial role in regulating the outcome of inflammatory responses. Thus, TREM receptors have been found to be critically involved in the control of systemic inflammatory diseases such as sepsis or autoimmune diseases such as multiple sclerosis.[23,24]

GPCR activation is known to regulate many cellular functions of human neutrophils, including chemotaxis and degranulation.[25,26] The present report adds EMR2 as a new GPCR modulator of neutrophil

function. Interestingly, a closely related adhesion-GPCR, CD97, was also shown to be involved in the cellular migration and homeostasis of neutrophils in various animal models using either specific mAbs or knock-out mice.[27-29] Therefore, these myeloid-restricted adhesion-GPCRs clearly play a regulatory role in the innate immune responses of granulocytes.

Acknowledgements

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EMR2 受體集結調控嗜中性白血球的細胞激素分泌型態及其存活率

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背 景：EMR2 是一種附著性 G 蛋白偶聯受體，經由 EMR2 的作用，除了可以更進一步加強嗜中性白血球對各種發炎性刺激所產生的反應之外，透過 EMR2 活化的途徑，嗜中性白血球的附著與遷移的能力也會明顯的增加。這其中還伴隨著嗜中性白血球的顆粒消失 (degranulation) 與含氧活化物的產生 (reactive oxgen species)。本實驗的目的在於：檢測 EMR2 和其專一的抗體結合後，是否會造成脂多醣類處理過的嗜中性白血球改變其細胞激素的表現和分泌，及影響此類嗜中性白血球的存活率。

方 法：將 EMR2 專一的抗體 2A1 加入經由脂多醣類處理過後的嗜中性白血球後，以流式細胞儀分析其細胞中膜聯蛋白 5 的表現，同時細胞也以丙啶碘染色，然後用流式細胞儀檢測其染色程度，以分析此類嗜中性白血球存活率，ELISA 用以分析細胞上清液中細胞激素的分泌。

結 果：實驗結果顯示，EMR2 與其專一的抗體結合後，會降低脂多醣類對嗜中性白血球生存率的提升。此外，EMR2 與其專一抗體的結合也會造成脂多醣類處理後嗜中性白血球分泌介白質 6、介白質 8 和 MCP-1 情況的改變。同時臨床上的研究也顯示肝硬化病患中的嗜中性白血球會大量表現 EMR2，此現象與此細胞自我凋亡的能力有密切的關係。

結 論：本實驗證實，在發炎的環境中，EMR2 調控嗜中性白血球的活化過程，其中調控機制包括了介白質 6、介白質 8 和 MCP-1 分泌的改變，以及對嗜中性白血球存活率的影響。

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關鍵詞：G 蛋白偶聯受體，細胞凋亡，嗜中性白血球，細胞激素，發炎反應

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