S5a binds to death receptor-6 to induce THP-1 monocytes to differentiate through the activation of the NF-κB pathway

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ABSTRACT

Analyses of supernatants from apoptotic cells have helped in the identification of many signals that modulate the states of cell activation and differentiation. However, the current knowledge of the soluble factors that are released during apoptosis is rather limited. Previous studies have shown that S5a and angiocidin (both encoded by PSMD4) induce human acute monocytic leukemia cells (THP-1 cells) to differentiate into macrophages, but the cell-surface receptor of S5a has not been identified. In this study, we show that apoptotic THP-1 cells release endogenous S5a that binds to death receptor-6 (DR6, also known as TNFRSF1), which was identified as an orphan receptor, to induce THP-1 cells to differentiate. Furthermore, we found that the NF-κB pathway is activated, and that the transcription factors WT1 (Wilms’ tumor 1) and c-myc mediate S5a-induced THP-1 differentiation. We also show that differentiation is blocked by anti-DR6 antibody, DR6 siRNA, DR6-Fc, NF-κB inhibitor or WT1 siRNA treatment. Our findings indicate that the interaction between cells can determine their differentiation, and we provide evidence for a functional interaction between S5a and DR6, which provides a novel potential mechanism to induce the differentiation of cancer cells, especially during biotherapy for leukemia.

KEY WORDS: S5a, DR6, Differentiation, NF-κB, THP-1 cells

INTRODUCTION

Apoptosis is the process of programmed cell death that occurs in multicellular organisms. The effective clearance of apoptotic cells requires direct contact between apoptotic cells and phagocytes, along with specific recognition, engulfment and degradation of apoptotic cells by phagocytic cells (D’Mello and Birge, 2010; Bialik et al., 2010). Recent studies on the clearance of apoptotic cells have led to the identification of specific molecules and signaling pathways that are responsible for this clearance (Erwig and Henson, 2008; Lauber et al., 2004; Ravichandran and Lorenz, 2007; Panaretakis et al., 2009). Cells undergoing apoptosis release soluble factors or ‘find-me’ signals, which attract professional phagocytes (Peter et al., 2010; Elliott and Ravichandran, 2010). Subsequently, dying cells expose ‘eat-me’ signals that are recognized by the phagocytes that engulf them (Nagata et al., 2010; Zitvogel et al., 2010; Poon et al., 2010). Apoptotic-cell-derived find-me signals contribute to the process of cell corpse clearance and modulate the state of activation and differentiation of phagocytic cells to influence the consecutive immune responses (Gregory and Pound, 2011; Gregory and Pound, 2010). Analyses of apoptotic cell supernatants have allowed the identification of the following chemotactic or find-me signals – lysophosphatidylcholine (LPC), sphingosine-1-phosphate (SIP), CX3CL1 (also known as fractalkine) and thrombospondin-1 (TSP-1) and its heparin-binding domain (HBD) (Gude et al., 2008; Truman et al., 2008; Krispin et al., 2006; Muñoz et al., 2010). However, the current knowledge of the soluble factors that are released during apoptosis is rather limited.

In 1993, Tuszynski and co-workers isolated a novel TSP-1-binding protein, angiocidin, which was overexpressed in tumor stroma and widely implicated in mechanisms of tumor progression (Tuszynski et al., 1993). Angiocidin is a potent inhibitor of angiogenesis and tumor growth and might inhibit angiogenesis by binding to collagen, as well as to the collagen receptor α2β1 (Sabherwal et al., 2006). Additionally, angiocidin induces monocyte-to-macrophage differentiation in a monocytic leukemia cell line, THP-1, which was isolated by Tsuchiya and colleagues from a boy suffering from acute monocytic leukemia (Auwerx, 1991). This differentiation is concurrent with dramatic alterations in both cell morphology and in the expression of macrophage markers (Araujo et al., 2004; Gaurnier-Hausser et al., 2008). It was also found that angiocidin treatment increases the ability of monocytes to present antigen to T lymphocytes and that functional macrophages could directly or indirectly elicit tumor cell destruction (Gaurnier-Hausser and Tuszynski, 2009). A BLAST search of the full-length cDNA of angiocidin revealed that the protein sequence has a high degree of similarity to that of S5a (supplementary material Fig. S1). In fact, both S5a and angiocidin are encoded by the same gene, PSMD4, but angiocidin contains an additional three amino acids in its C-terminus, along with a change to a single amino acid residue; however, these differences are unlikely to cause differences in the overall structure between the two proteins. S5a is an internal polyubiquitin-recognition subunit of the 26S proteasome enzyme complex, which binds to polyubiquitin, a signal that targets proteins for destruction (Wang et al., 2005). We postulated that S5a might perform the same function as angiocidin, inducing THP-1 adhesion and differentiation. However, the cell-surface receptor of S5a remains unknown.
In 1998, researchers identified a new family member of the tumor necrosis factor receptor (TNFR) superfamily, named death receptor-6 (DR6 or TNFRSF21), by searching the EST database using the protein sequence of the extracellular cysteine-rich ligand-binding domain of TNFR2 (Pan et al., 1998). Human DR6 is a type I transmembrane protein consisting of 655 amino acids. DR6 regulates CD4+ T cell proliferation and Th cell differentiation and is also expressed on resting B cells but is downregulated upon activation of the latter (Liu et al., 2001; Schmidt et al., 2002). Studies on DR6-deficient (DR6−/−) mice have shown the crucial regulatory role played by this receptor in the development of the adaptive immune response (Liu et al., 2001; Schmidt et al., 2005). High expression of DR6 in human cancer cell lines and tumor samples seems to be regulated by the TNF-α-induced NF-κB activation pathway, the activity of which correlates with a constitutive high basal level of NF-κB activation (Benschop et al., 2009). Interactions between the β amyloid precursor protein, APP, and DR6 have been reported to activate the caspase enzyme, which then contributes to caspase-mediated nerve cell apoptosis (Nikolaev et al., 2009). However, this claim has not been verified in other tissues and cells. There have been no studies reporting typical ligands for DR6. Therefore, DR6 remains an orphan member of the TNF-receptor superfamily.

In this study, the research focused on investigating the differentiation effect of exogenous S5a but resulted in the unexpected discovery of a new phenomenon – namely, that THP-1 cell cultures have a tendency to spontaneously differentiate. Researchers have been able to detect angiocidin in tumor-conditioned medium and in the serum of cancer patients (Sabherwal et al., 2007). As the protein sequence of angiocidin has a high degree of similarity to that of S5a, we hypothesized that these proteins could share a similar function, inducing THP-1 adhesion and/or differentiation. We collected the concentrated culture supernatant of THP-1 cells after 96 h of cell culture. S5a protein was detected by western blotting in the supernatants of cultures that contained apoptotic THP-1 cells (Fig. 1B). According to our data and previous research, we concluded that apoptotic THP-1 cells released endogenous S5a during the apoptotic process.

**RESULTS**

**S5a is present in the culture supernatant of cultures containing apoptotic THP-1 cells**

Interestingly, we found that THP-1 cells, a monocytic leukemic cell line grown in suspension, underwent differentiation, with the cells gradually aging and dying during the period of culture (Fig. 1A). During their differentiation, the cells became flattened, elongated and firmly attached to the tissue culture plastic. Earlier reports have indicated the presence of angiocidin in tumor-conditioned medium and in the serum of cancer patients (Sabherwal et al., 2007). Because of the high sequence similarity between angiocidin and S5a, we postulated that these proteins could share a similar function, inducing THP-1 adhesion and/or differentiation. We collected the concentrated culture supernatant of THP-1 cells after 96 h of cell culture. S5a protein was detected by western blotting in the supernatants of cultures that contained apoptotic THP-1 cells (Fig. 1B). According to our data and previous research, we concluded that apoptotic THP-1 cells released endogenous S5a during the apoptotic process.

**Recombinant S5a induces THP-1 differentiation**

THP-1 is a model system for studying the differentiation of monocyes (Auwerx, 1991; Tsuchiya et al., 1980). We cloned full-length S5a cDNA into a maltose binding protein (MBP)-tag expression vector, expressed it in *Escherichia coli*, and purified MBP-S5a using a MBP purification protocol as described in Materials and Methods. We treated THP-1 cells with 10 µg/ml MBP-S5a for 12, 24 or 48 h and, subsequently, the treated cells became flattened and elongated. Flow cytometric analysis showed that recombinant S5a could activate the NF-κB pathway.

These experimental data fully demonstrate that S5a binds to DR6 to induce THP-1 monocytes to differentiate by activation of the NF-κB pathway. From the results of this study, we have developed a model whereby dying cells or those slipping into senescence send out signals, such as S5a, that influence the fate of neighbors and their ability to adapt to the environment. We define S5a as the ‘change-me’ signal in the process of cell death. Above all, these results provide a novel potential method to induce the differentiation of cancer cells, which will be especially useful in the biotherapy of leukemia.
that the number of apoptotic cells increased at 24 h and decreased significantly at 48 h (Fig. 2A,B). Additionally, the expression of the macrophage markers CD14 and CD36 (Huh et al., 1996; Park et al., 2007) on MBP–S5a-treated THP-1 cells was detected at 48, 72 and 96 h by flow cytometry, and their expression increased both over time in culture and following treatment with MBP–S5a (Fig. 2D,E). Concurrently, a significant alteration in cell morphology and increased adherence of these cells to the culture plastic was observed (Fig. 2C). According to our data and previous research, we concluded that S5a could induce the differentiation of THP-1 cells.

**S5a physically interacts with DR6**

To determine the mechanism by which S5a induced the differentiation of THP-1 cells, plasma membrane proteins were extracted from THP-1 cells and pulldown experiments were performed to identify proteins that were associated with MBP–S5a. MBP–S5a-associated proteins were resolved by SDS-PAGE. The most prominent protein bands were cut out and subjected to mass spectrometry. Analysis of the mass spectrometry results revealed several proteins that were specifically associated with MBP–S5a, and one of these proteins, DR6, caught our attention (supplementary material Fig. S3). We performed pulldown followed by western blot analyses to confirm the presence of DR6, and the MBP-bound sample was used as a control. After probing with an anti-DR6 antibody, 90-kDa and 110-kDa bands were visualized in the MBP–S5a-bound sample but not in the MBP-bound sample (Fig. 3A). This result is consistent with results obtained by Klíma and co-workers, who showed that DR6 is expressed as two forms, with molecular masses of 90 kDa and 110 kDa, rather than as a protein with the predicted molecular mass of 70 kDa (Klíma et al., 2009). DR6 has been shown to be an extensively post-translationally modified transmembrane protein, and N- and O-glycosylations of amino acids located in its extracellular domain have been found to be mainly responsible for its ~40-kDa mobility shift in SDS polyacrylamide gels (Klíma et al., 2009). The above data confirm that the 90-kDa and 110-kDa proteins were the major functional forms of DR6 in THP-1 cells. The S5a–DR6 interaction was further tested using the far-western method (Fig. 3B). The distribution of S5a and DR6 on intact THP-1 membranes was visualized by immunofluorescence confocal microscopy (Fig. 3C). To confirm the interaction between DR6 and S5a, we used an ELISA to show that purified DR6–Fc bound to purified recombinant S5a–biotin. The interaction detected by ELISA was of high affinity (EC$_{50}$=145±13 nM; ±s.d.), and this binding was blocked by the addition of an anti-DR6 antibody (Fig. 3D). The interaction of DR6–Fc with MBP–S5a was also of high affinity (EC$_{50}$=285±15 nM) and anti-DR6 antibody also blocked this interaction (Fig. 3E). These results indicate that S5a is a functional DR6 ligand.
DR6 mediates the differentiation of THP-1 cells

To confirm the role of DR6 in the differentiation of THP-1 cells, we used DR6 siRNA to knock down the expression of DR6 in THP-1 cells (supplementary material Fig. S6), as well as using anti-DR6 antibody and DR6–Fc to block the interaction of S5a and DR6. Following these treatments, we observed morphological alterations of the cells and a decrease in expression of the macrophage markers CD14⁺ and CD36⁺, compared with cells that were untreated or treated with a scrambled control siRNA (Fig. 4A–C). Although anti-DR6 antibody inhibited S5a-induced THP-1 differentiation, it had no effect on the THP-1 differentiation induced by phorbol-12-myristate-13-acetate (PMA, supplementary material Fig. S4). At the same time, we investigated whether DR6-siRNA-treated THP-1 cells displayed a decrease in the S5a-induced phagocytic activity towards fluorescent microparticles that were designed specifically for this purpose. S5a-treated cells displayed an increase in phagocytic activity, with >95% of the cells internalizing these fluorescent microparticles, whereas S5a could not induce DR6-siRNA-treated THP-1 cells to phagocytose microparticles as efficiently (Fig. 4D,E). These findings suggest that S5a induces the differentiation of THP-1 cells by a mechanism that is dependent on binding to DR6.

Activation of the NF-κB pathway mediates the differentiation and growth inhibition of THP-1 cells after S5a treatment

Previous studies have shown that angiociadin can upregulate various components of the NF-κB signaling pathway at the mRNA level and that phosphorylation of these proteins in THP-1 cells was increased in response to angiociadin treatment (Gaurnier-Hausser et al., 2008). To confirm that recombinant S5a was able to activate NF-κB pathways, the phosphorylation of IκBα (also known as NFKBIA) and p65 (also known as RELA) was analyzed by western blotting. The phosphorylation level of IκBα significantly increased after MBP–S5a treatment compared with that observed in the MBP control group (Fig. 5A). MBP–S5a treatment also increased the phosphorylation level of p65 compared with that of the control group (Fig. 5B). The phosphorylation level of p65 protein in THP-1 cells also significantly increased after treatment with recombinant S5a–biotin compared with that of the negative control group (Fig. 5C). In addition, we pretreated THP-1 cells with the NF-κB inhibitor IKK Inhibitor VII (150 nM) for 24 h before treating cells with MBP–S5a for 48 h. Following this pretreatment, we observed a significant alteration in cell morphology, and the phagocytic ability of THP-1 cells was decreased in the group that was pretreated with IKK Inhibitor VII compared with that of the group induced by S5a in the absence of inhibition.
of the inhibitor (Fig. 5D,E, $P<0.05$). In addition, a cell proliferation assay was performed to assess the growth status of cells that were treated with S5a for 48 h. The results suggested that S5a inhibited the growth of cells and that the NF-κB pathway mediated this effect (Fig. 5F, $P<0.05$). According to our data and to previous research, we concluded that S5a can induce the activation of NF-κB pathway, which results in the differentiation and growth inhibition of THP-1 cells.
The NF-κB pathway activates the expression of WT1, which mediates the differentiation and growth inhibition of THP-1 cells treated with S5a

Upon further investigation, we observed the expression of the transcription factor WT1 (Wilms’ tumor 1) in THP-1 cells after treatment with S5a. We found that the mRNA level of WT1 significantly increased and reached a peak of 8-fold upregulation at 12 h (Fig. 6A, *P*<0.01), and the protein level of WT1 was also significantly upregulated in response to treatment with S5a (Fig. 6B). In addition, this upregulation can be blocked by the use of the NF-κB inhibitor (Fig. 6C,D). These data indicated that the upregulation of WT1 expression depended on the activation of NF-κB pathway. Next, we pretreated THP-1 cells with WT1 siRNA for 8 h and analyzed the expression of WT1. The results indicated that the upregulation of WT1 was successfully blocked by siRNA even if THP-1 cells were treated with S5a (supplementary material Fig. S8, *P*<0.01). In order to determine whether WT1 mediated the growth inhibition of THP-1 cells, a cell proliferation assay was performed. We found that the S5a-induced growth inhibition of THP-1 cells was attenuated after treatment with WT1 siRNA (Fig. 6E, *P*<0.01). To confirm that WT1 mediated the differentiation of THP-1 cells, we pretreated THP-1 cells with WT1 siRNA for 8 h, then treated cells with MBP–S5a for 48 h. We subsequently observed the morphology of these cells and detected their phagocytic ability by flow cytometric analysis. The data showed a significant alteration in cell morphology and decreased phagocytic ability of THP-1 cells from the WT1-siRNA-pretreated group compared with that of the group induced by S5a without pretreatment with WT1 siRNA (Fig. 6F,G, *P*<0.01). Collectively, these findings show that the NF-κB pathway activates the expression of WT1, which mediates the S5a-induced differentiation and growth inhibition of THP-1 cells.

WT1 suppresses the expression of c-myb, thus promoting the differentiation of THP-1 cells

Previous studies have shown that the expression of c-myb is downregulated during the process of PMA-induced THP-1 differentiation, and this downregulation promotes the differentiation of THP-1 cells by regulating the expression of...
many associated downstream molecules (Suzuki et al., 2009). To confirm the role of c-myb in THP-1 cell differentiation, we detected the expression of c-myb after treatment with S5a. At the mRNA level, c-myb expression showed a gradual downregulation during a 24-h treatment with S5a, leading to a 10-fold reduction at 24 h (Fig. 7A, \( P < 0.01 \)). c-myb expression also significantly decreased at the protein level after cells were treated with S5a for 32 h, as determined by western blotting (Fig. 7B). More importantly, this downregulation could be blocked or attenuated by treatment with either the NF-κB inhibitor or WT1 siRNA (Fig. 7C–E, \( P < 0.01 \)). In addition, according to bioinformatics analysis, the WT1 transcription factor can bind to the sequence 5'-CGCCCCCGC-3' in the c-myb promoter (supplementary material Fig. S2). The results of a chromatin immunoprecipitation (ChIP)-PCR assay also suggested that WT1 bound to the 5'-CGCCCCCGC-3' sequence in the c-myb promoter, suppressing the transcription of c-myb in our research model (Fig. 7F).
DISCUSSION

Given the results presented here, we believe that DR6 mediates the differentiation of THP-1 cells that have been treated with S5a. As a membrane receptor of S5a, DR6 transmits the signal after binding to S5a. This signal activates the NF-κB pathway, thus significantly increasing the expression of WT1 at both the mRNA and the protein level. WT1 subsequently suppresses the transcription of c-myb. We speculate that the downregulation of c-myb promotes the differentiation of THP-1 cells, based on some previous reports. The above mechanism is illustrated in the schematic presented in supplementary material Fig. S5.

Endogenous S5a is a crucial protein for monocyte self-regulation

Previous studies have shown that the treatment of THP-1 cells and freshly isolated human peripheral blood mononuclear cells (PBMCs) with PMA increases the secretion of angiocidin (Gaurnier-Hausser and Tuszynski, 2009). Angiocidin was also detected in tumor-conditioned medium and in serum from cancer patients (Araujo et al., 2004; Gaurnier-Hausser et al., 2008). In this paper, S5a that had been released into culture supernatants of THP-1 cells was isolated and identified by western blot analysis. It was found that the number of aging and dying THP-1 cells increased gradually with increasing time in culture. On the basis of this evidence, we deduced that apoptotic THP-1 cells released endogenous S5a, which sent a signal that induced companion and neighboring cells to undergo differentiation. Here, we define S5a as the ‘change me’ signal in the process of cell death. Previous reports have suggested that differentiated cells and undifferentiated cells showed different sensitivities to pro-apoptotic stimuli (Daigneault et al., 2010). THP-1 cells that have been treated with S5a struggle against environmental factors in the first 48 h. During this time, neighboring cells show resistance to apoptotic stimuli and maintain levels of differentiation.
Investigation of S5a, a new ligand of the orphan receptor DR6, reveals a new function of DR6 in cell death and differentiation

Intracellular S5a is a receptor for ubiquitylated proteins (Haririnia et al., 2007). Recent studies have reported that S5a/angiocidin inhibits endothelial cell proliferation and induces endothelial cell apoptosis and antitumor activities that are related to the activity of polyubiquitin conjugates (Saeki and Tanaka, 2008). Restructuring angiocidin offers a variety of mechanisms for antitumor activity in vitro and in vivo (Sabherwal et al., 2007; L’Heureux et al., 2010; Liebig et al., 2009; Kremlev et al., 2008; Yang et al., 2006). In this study, we focused on another aspect of the antitumor activity of S5a, by inducing the differentiation of THP-1 cells into macrophages. Using pull-down assays, we found a protein called DR6 that interacts with S5a. Recent reports have indicated that the glycosylation of DR6 can potentially regulate its interaction with the recently described ligands APP or APLP2, thereby activating caspase enzymes and contributing to caspase-mediated nerve cell apoptosis (Nikolaev et al., 2009). However, this claim has not been verified in other tissues and cells. Researchers have shown that DR6 is an extensively post-translationally modified transmembrane protein that mainly exists in N- and O-glycosylated forms, although potentially other modifications of its extracellular region also occur (Klima et al., 2009). However, the role of post-translational modifications in the interaction between DR6 and S5a remains to be explored. To this end, we used a computational workflow (Rosetta Docking) to predict and analyze the DR6–S5a interactive region of DR6 was located in its cysteine-rich domains (CRDs), which contain DR6-specific surface patches responsible for the exclusive recognition of its ligand. The predicted interactive region of S5a was located in its ubiquitin-interacting motifs (UIMs), indicating that the interaction might have some relation to ubiquitylation. These data provide strong support to the idea that S5a binds to DR6 with high affinity.

The proposed mechanisms of differentiation induced by S5a through DR6 in THP-1 cells

According to our data and to previous research, we conclude that S5a induces the differentiation of THP-1 cells. Further experimental data fully demonstrate the functional interactions between S5a and DR6. We have also shown that S5a treatment results in an increase in the phosphorylation of IkBz and p65 in the NF-κB pathway. In timecourse experiments, we observed a slight phosphorylation of p65 in the MBP control group; however, this phosphorylation level was weak and transient compared with that observed in the MBP–S5a treatment group. We speculate that the slight phosphorylation of p65 occurred because of a transient stimulus to cells caused by MBP treatment. However, this transient phosphorylation was insignificant, and S5a increased the phosphorylation level of p65 in THP-1 cells more strongly and continuously. Thus, it seems likely that the constitutive activation of the NF-κB signaling pathway has a profound impact on the differentiation of THP-1 cells. From our results, combined with those of other reports (Gaurnier-Hausser et al., 2008), we conclude that S5a induces monocytes to undergo differentiation through a mechanism that is mediated by activation of the NF-κB pathway. As a transcription factor that is closely related to hematopoietic differentiation (Greig et al., 2008), c-myb also plays an important role during THP-1 differentiation (Suzuki et al., 2009). It is a key molecule that mediates THP-1 differentiation after PMA treatment, and its downregulation promotes THP-1 differentiation by regulating the expression of many downstream molecules (Suzuki et al., 2009).

Previous research also suggests that c-myb can maintain the stability of cells – its downregulation is necessary for the differentiation of some cells (Ramsay and Gonda, 2008), and its high expression blocks monocyte differentiation (Knopfova and Smarda, 2008). In our research, the expression of c-myb was also significantly downregulated in THP-1 cells following treatment with S5a. We hypothesize that S5a induces THP-1 cells to differentiate by decreasing the expression of c-myb. A previous study has shown that the activation of NF-κB pathway can increase the expression of WT1 (Dewji et al., 1998), and that WT1 can bind to the c-myb promoter and suppress the expression of c-myb in T and B cell lines (McCann et al., 1995; Rauscher et al., 1990). Our results indicate that WT1 suppresses the transcription of c-myb, thereby promoting the differentiation of THP-1 cells after S5a treatment. The WT1 gene was first identified as a tumor suppressor gene in Wilms’ tumor (Lee and Haber, 2001). It is located on chromosome 11p13, and it encodes a zinc finger protein that functions as a transcription factor. For a long time, WT1 was considered to be a tumor suppressor (Scharnhorst et al., 2001), but some research has indicated that it is highly expressed in leukemia cells, playing a cancer-promoting role (Miwa et al., 1992). However, WT1 functioned as a tumor suppressor after S5a treatment of THP-1 cells. To explain these contrasting results, we think that WT1 can function as either an oncprotein or a tumor suppressor, depending on the specific conditions, such as the expression level of WT1 or the specific cell state.

In summary, our study illustrates a crucial intrinsic role for S5a and DR6 in the differentiation of THP-1 cells, and it indicates that the interaction between cells or even cancer cells can determine their cell fate. On the basis of the results presented in this study, we believe that S5a and DR6 might have great therapeutic potential in the treatment of hematologic cancer. By contrast, in some pathologic situations, monocyes differentiate to macrophages and release large amounts of inflammatory cytokines, amplifying the inflammatory response in a process that is harmful to the body. The results of our study might contribute to an understanding of ways in which it would be possible to restrict inflammation and the processes of autoimmune disease.

MATERIALS AND METHODS

Antibodies and reagents

Anti-MBP antiserum was purchased from New England Biolabs, and a mouse monoclonal antibody (mAb) against the proteasome 19S S5a protein and a rat mAb against DR6 were purchased from Abcam. Dylight-649-conjugated goat anti-rat-IgG was purchased from Jacksonimmuno, and Rhodamine-labeled antibody against mouse IgG was purchased from KPL. A rat mAb against WT1 protein and a rat mAb against c-myb were purchased from Epitomics. A rat mAb against GAPDH was purchased from BioWorld. The control siRNA and siRNA for DR6 were from Qiagen, and DR6–Fc was from Sino Biological. S5a–biontin was from Boston Biochem. Anti-phospho-p65 (Ser536) rabbit mAb, anti-p65 rabbit mAb, anti-IkBa (L35A5) mouse mAb and anti-f-actin were all purchased from Cell Signaling Technology. The S5a polyclonal antibody was purchased from ProteinTech Group. PE-conjugated anti-human-CD36 and FITC-conjugated anti-human-CD14 were purchased from BioLegend, and protease inhibitor cocktail (R1321) and the cyttoplasmic and nuclear protein extraction kits were purchased from Fermentas. The NF-κB inhibitor IKK Inhibitor VII was from Merck. The microparticles were from Bangslabs. The cyttoplasmic and
nuclear protein extraction kits were purchased from Beyotime, along with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Phosphatase inhibitor cocktail tablets were purchased from Roche. 3’-[3-cholamidopropyl]-dimethylammonio)1-propanesulfonate (CHAPS) was purchased from Boehringer Mannheim, and Super ECL plus detection reagent was purchased from Pierce.

**Construction, expression and purification of MBP-tagged recombinant S5a**

Two primers were designed from the GenBank human S5a coding sequence (NM_002810) and the plasmid pMAL-c2 reading frame. EcoRI restriction sites were introduced into the upstream 5’ end and HindIII restriction sites were introduced into the downstream 5’ end. The sequence for the upstream primer (P1) was 5’-GGGAATTCATGGTGGTGGAAAGCAGT-3’ and the sequence for the downstream primer (P2) was 5’-GCCAAGTTCTACCTTGGTCTCTC-3’. Restriction enzyme sites are underlined. The S5a coding sequence which was observed by mass spectrometry.

**Preparation of THP-1 plasma membrane proteins**

The THP-1 cell pellet was washed twice with 10 volumes of pH 6.5 buffer containing 0.102 M NaCl, 3.9 mM KH2PO4, 3.9 mM Na2HPO4, 22 mM NaH2PO4 and 5.5 mM glucose. The pellet was then suspended in 15 mM Tris-HCl pH 7.6, 0.14 M NaCl, and 5 mM glucose [Tris-buffered saline with glucose (TBSG)] at 5×106 cells/ml. The washed pellet was directly lysed in lysis buffer (250 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM EDTA, 2 mg/ml aprotinin, 100 mg/ml PMSF and 1% CHAPS). After treatment for 20 minutes at 4°C, the samples were centrifuged at 13,000 g for 15 min at 4°C in a microcentrifuge. Both membrane protein and crude extract were used immediately for further analysis or stored at −20°C for up to a month before use.

**Pull-down and western blot analysis**

Purified MBP–S5a was incubated with amylase-resin at 4°C, and the column was washed with 10 volumes of 20 mM Tris-HCl pH 7.4, 0.2 M NaCl, 10 mM β-mercaptoethanol and 1 mM EDTA. After washing to remove unbound proteins, plasma membrane protein crude extracts were added to the MBP–S5a-bound resin, or to the MBP-bound resin (control). The binding reaction was performed at room temperature for 2 h, and the resin was eluted using the above buffer plus 10 mM maltose. The purity and size of MBP–S5a were checked by SDS-PAGE analysis, and the purified protein contained <10 EU endotoxin/mg of protein.

**Culture of THP-1 cells**

Human monocytic leukemia cells (THP-1) were purchased from Shanghai Cell Bank, Chinese Academy of Sciences and maintained in RPMI-1640 (Gibco, A10491) supplemented with 10% fetal bovine serum (HyClone). All cells were maintained at a temperature of 37°C in a humidified growth chamber under 5% CO2.

**Flow cytometric analysis of cell surface proteins and apoptosis**

THP-1 cells were plated for 6 h and then treated with MBP–S5a or MBP for 48 h, 72 h or 96 h, and then, without fixing (to allow the detection of CD14 and CD36 at the cell surface), the cells were subjected to flow cytometric analysis using PE-conjugated anti-CD36 antibody or FITC-conjugated anti-CD14 antibody. To detect apoptosis, the cells were stained with Annexin V and propidium iodide, and were subsequently analyzed by FACS.

**Western blot analysis**

The proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with the various antibodies at 4°C overnight. The immunocomplexes were visualized using a HRP-conjugated antibody followed by a chemiluminescence reagent (Millipore, Billerica, MA) and detected using ChemiDoc™ XRS+ purchased from BioRad.

**Inhibition of DR6 by anti-DR6, DR6 siRNA and DR6–Fc**

THP-1 cells were pretreated with anti-DR6 antibody (1:1000) or DR6–Fc (50 μg/ml) at 37°C for 1 h. siRNA transfections were performed with DR6 siRNA (100 nM) and HiPerFect Transfection Reagent (Qiagen). Quantitative (Q)RT-PCR, western blotting and flow cytometric analysis were performed to confirm the knockdown effect after DR6 siRNA treatment for 48 h.

**Inhibition of WT1 by siRNA**

The siRNAs against human WT1 were chemically synthesized by Shanghai GenePharma. The siRNA sequences were as follows: siRNA-WT1, 5’-GAGACAUACAGGUGUAAATT-3’ (forward) and 5’-UUUCACUCCGUAUUGUCCTT-3’ (reverse). The negative control dsRNA sequences were as follows: 5’-UUUCUCCGACUGUACUCGU- TT-3’ (forward) and 5’-ACGGUGACACGUGGAGAAAT-3’ (reverse). The transfection of WT1 siRNAs was achieved by using LipoFectamine 2000 (Invitrogen, Gaithersburg, MD). Cells were incubated at 37°C in a CO2 incubator and the medium was changed after 8 h.

**Determination of receptor binding by ELISA**

Nunc MaxiSorp plates were coated for 3 h with DR6–Fc (10 ng per well) in 0.1 M sodium carbonate/bicarbonate buffer (pH 8.6) at 37°C, and the remaining binding places were subsequently blocked with 2% BSA for
2 h. Anti-DR6 (1:1000) antibody or PBS was added, and the plates were incubated for 2 h at room temperature. After being washed six times with Tris-buffered saline containing 0.5% Tween-20 (TBST, pH 7.5), serial dilutions of soluble SSA–biotin or MBP–SSA (0–500 nM per well) were added to the wells and incubated for 2 h at room temperature. After being washed six times with TBST, Avidin–HRP was added and incubated with SSA–biotin for 2 h at room temperature. For MBP–SSA, a 1:1000 dilution of anti-MBP antibody (New England Biolabs) was added and incubated for 2 h at room temperature and, after being washed six times with TBST, was subsequently incubated with a 1:10,000 dilution of an HRP-conjugated goat anti-rabbit-IgG antibody. After being washed six times with TBST, 100 µl of 1-step Turbo TM solution (Pierce) was added and, after 20 min, the reaction was quenched with 100 µl of 1 M sulfuric acid. The absorbance was measured at 450 nM on a microplate reader (Thermo Scientific).

Phagocytosis assay
A total of 1 µl of fluorescent green 0.5-µm microparticles (Bangs labs, 10^6 particles/ml) was added to 100 µl of granulocytes (10^6 cells/ml in PBS) in a polypropylene tube and incubated with gentle shaking for 1 h at 37°C. Phagocytosis was stopped by the addition of 1 ml of ice-cold PBS at the end of the 1 h incubation. Samples were mixed and the cells were washed five times with ice-cold PBS. The cells were resuspended in 300 µl of cold PBS, kept at 4°C and analyzed by fluorescence microscopy or flow cytometry.

Cell proliferation assay
The THP-1 cells were either subjected to a variety of treatments – including MBP–SSA (40 µg/ml), MBP (20 µg/ml), MBP–SSA with WT1 siRNA (pretreating for 8 h), MBP–SSA with scrambled siRNA (pretreating for 8 h), MBP–SSA with NF-kB inhibitor (pretreating for 24 h) and NF-kB inhibitor only (pretreating for 24 h) – or were left untreated. After treatment with MBP–SSA for 48 h, the proliferation of THP-1 cells was analyzed and compared with that of all parallel groups. The cell growth was determined by using the Cell Counting Kit-8 assay (R&D Biotechnology, Shanghai) according to the manufacturer’s protocol. Experiments were performed in triplicate.

ChIP-PCR
The ChIP assay kit and protocol were provided by Beyotime. THP-1 cells were crosslinked with 37% formaldehyde in culture medium for 10 min at 37°C, then treated with glycine solution (10x) for 10 min and centrifuged. The supernatant was removed and cells were washed with cold PBS containing 1 mM PMSF. All cell samples were divided to 10^6 cells/tube. Cells were treated with SDS lysis buffer containing 1 mM PMSF for 10 min on ice and sonicated three times at 10 s intervals. Samples were then subjected to centrifugation for 5 min at 14,000 g at 4°C, and the supernatants were diluted with ChIP dilution buffer. To reduce non-specific background signal, samples were precleared with salmon sperm DNA and Protein-A+G-agarose for 30 min at 4°C with agitation. Primary antibody was added to the samples, which were then incubated overnight at 4°C. The Protein-A+G-agarose slurry was added to each sample, which was then incubated for an additional hour. The protein-A+G-antibody–DNA complexes were washed and eluted according to the manufacturer’s protocol and then reverse cross-linked by heating at 65°C for 4 h. DNA fragments were purified by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. Purified DNA from immunoprecipitations and DNA inputs was used for PCR amplification, using oligonucleotide primers specific for the c-Myb promoter (forward, 5’-TTCAATGGGCTTAGTCCCTACACTGAGCA-3 ; reverse, 5’-TTCCCTGAGACCAACCCCGCTCC-3 ; 272-bp product). The PCR conditions were as follows: the DNA template was denatured at 94°C for 1 min, annealed at 59°C for 50 s and extended at 72°C for 2 min per cycle for 35 cycles. The PCR product was visualized on a 1.0% agarose gel.

Statistical analysis
All data are expressed as the mean±s.d. The two-tailed Student’s t-test was used for comparisons between the two groups, and differences were considered to be significant when P<0.05.

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Competing interests
The authors declare no competing interests.

Author contributions
L.-H.W. and B.-H.J. conceived of the project. Experiments were designed by Z.W., C.F., H.-F.Z., L.-H.W. and B.-H.J. Experiments were carried out by Z.W., C.F. and H.-F.Z. The manuscript was written by Z.W., C.F., H.-F.Z., L.-H.W. and B.-H.J. J.-S.L., M.-J.S., J.-W.S., Y.L. and H.-D.J. provided valuable help for this research.

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