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Cell-intrinsic C5a synergizes with Dectin-1 in macrophages to mediate fungal killing

Xaria X. Li[a], Jenny N. Fung[a], Richard J. Clark[a], John D. Lee[a], and Trent M. Woodruff[a,1]

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The complement factor C5a is a core effector product of complement activation. C5a, acting through its receptors C5aR1 and C5aR2, exerts pleiotropic immunomodulatory functions in myeloid cells, which is vital for host defense against pathogens. Pattern-recognition receptors (PRRs) are similarly expressed by immune cells as detectors of pathogen-associated molecular patterns. Although there is evidence of cross talk between complement and PRR signaling pathways, knowledge of the full potential for C5a–PRR interaction is limited. In this study, we comprehensively investigated how C5a signaling through C5a receptors can modulate diverse PRR-mediated cytokine responses in human primary monocyte-derived macrophages and observed a powerful, concentration-dependent bidirectional effect of C5a on PRR activities. Unexpectedly, C5a synergized with Dectin-1, Mincle, and STING in macrophages to a much greater extent than TLRs. Notably, we also identified that selective Dectin-1 activation using depleted zymosan triggered macrophages to generate cell-intrinsic C5a, which acted on intracellular and cell surface C5aR1, to help sustain mitochondrial ROS generation, up-regulate TNFα production, and enhance fungal killing. This study adds further evidence to the holistic functions of C5a as a central immunomodulator and important orchestrator of pathogen sensing and killing by phagocytes.

complement | macrophage | C5a | Dectin-1 | cytokine

Significance

Intercommunication between complement and pattern-recognition receptor (PRR) systems is required to effectively orchestrate host defense. C5a is the central inflammatory effector molecule of complement; however, how it may interact with the broad suite of PRRs expressed by immune cells remains unexplored. Here, we demonstrate that C5a effectively inhibits or amplifies PRR cytokine responses from human macrophages, dependent on the concentration of C5a. Dectin-1, Mincle, and STING were particularly responsive to C5a activity. Remarkably, Dectin-1 activation alone was capable of initiating cell-autonomous C5a activity, which interacted with intracellular C5aR1 to augment antifungal defense by human macrophages. This study therefore reveals a cell-intrinsic functional interaction between C5a and pattern-recognition systems that is essential for human macrophage immune defense.

The complement system is an essential component of the immune response, providing host defense toward a range of external and internal insults. Complement activation, through a series of cascading proteolytic events, cleaves the complement factor C5 to generate a major effector complement molecule, C5a (1).

Human C5a, a 74-amino acid glycoprotein, interacts with the seven-transmembrane-spanning receptors C5aR1 and C5aR2 (2), which are cooperatively expressed on broad immune and nonimmune cell types (3). C5aR1 signals through canonical G-protein-coupled signaling pathways such as the downregulation of cAMP/PKA signaling (4, 5), activation of the kinase signaling cascade Raf-MEK1-ERK1/2 (6–9), and intracellular calcium mobilization and β-arrestin recruitment (10–12). By contrast, C5aR2, although structurally homologous to C5aR1, is commonly recognized as incapable of G-protein coupling (13–17) but can signal through β-arrestins (17–22). C5a has pleiotropic functions both within and outside of the immune system and powerfully modulates multiple aspects of immune cell functions, which are paramount for host defense against microbial invasion and maintaining tissue homeostasis (3, 23, 24).

The complement system detects pathogen and danger signals and has critical roles in immune surveillance. For instance, the pattern-recognition molecule C1q detects distinct structures directly on microbial and apoptotic cells and activates the complement system via the classical pathway. Mannose-binding lectin and ficolins recognize carbohydrate patterns which in turn activate the lectin pathway (25). Other than the complement-dependent, humoral-based pattern recognition and defense, innate immune recognition is also achieved by the sentinel sensors, the pattern-recognition receptors (PRR). PRRs recognize conserved molecules or structural motifs, namely pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (26). The main families of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) (26). Similar to the C5a receptors, PRRs are widely expressed on diverse immune cells, particularly professional antigen-presenting cells, such as macrophages and dendritic cells (26).

The complement system and various PRRs detect and provide critical frontline defense against invading pathogens and internal tissue injury. The presence of intricate regulatory networks and cross talk between the two systems are therefore imperative for an optimized and efficient immune response (27). Prior studies have explored this potential cross talk

Author affiliations: [a]School of Biomedical Sciences, Faculty of Medicine, The University of Queensland, St Lucia, QLD 4072, Australia

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[1]To whom correspondence may be addressed. Email: t.woodruff@uq.edu.au.

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between C5a receptors and PRRs, with TLR4, typically activated by bacterial components such as LPS, being the most extensively studied (27, 28). C5a dampens LPS-induced TNFα and IL-6 and up-regulates G-CSF and IL-10 secretion by human macrophages (29, 30). C5a also synergizes with TLR2, elevating cAMP and causing macrophage immunosuppression (31). Besides TLRs, C5a, through C5aR1, down-regulates c-di-AMP-induced STING (a cytosolic DNA sensor) expression and TBK1 and p38 MAPK phosphorylation in murine dendritic cells (32). Further, in human T lymphocytes, both C5a receptors regulate NLRP3 inflammasome signaling, which is critically involved for T helper 1 differentiation and survival (17, 33, 34). Recently, a seminal study published by Desai et al. (35) reports both hepatocyte- and phagocyte-derived C5a, acts through C5aR1, to enhance Dectin-1 expression and promote fungal clearance in human phagocytes. Considering the wide implications of C5a-C5aR signaling in human diseases and the increasing usage of anti-C5a/C5aR1 therapies in clinical settings, there is an ever-growing interest in the field to understand the mechanisms and the physiological and pathological relevance of C5a–PRR cross talk.

Nevertheless, for the vast majority of PRRs, how C5a receptors may influence their functions remains to be explored. Mature macrophages harbor diverse phagocytic, complement and pattern recognition receptors, and are critical for fighting infection, maintaining tissue homeostasis, and regulating inflammatory responses (36, 37). As such, in this study, we examined how C5a, acting through either or both C5a receptors, may modulate these PRR-mediated responses in primary human macrophages, and observed a significant bidirectional effect. While a low concentration of C5a dampened PRR-mediated cytokine responses, a high concentration of C5a significantly up-regulated responses. Unexpectedly, we identified that C5aR1 synergizes with the non-Toll Dectin-1, Mincle, and STING pathways in macrophages to a much greater extent than TLRs, amplifying specific cytokine production by up to 40-fold. For Dectin-1, this synergistic activation also occurred in a cell-autonomous manner whereby Dectin-1 activation leads to increased cell C5a generation and C5aR1 activation, partly driven through Syk, ROS, and NfkB signaling. Finally, this cell-intrinsic synergy is necessary for effective fungal killing, as pharmacological blockade of C5aR1 inhibited the ability of macrophages to kill *Candida albicans.* This study therefore reveals crosstalk between C5a and the pattern-recognition system that is critical for the optimum defense functions of human macrophages.

Results

C5a Broadly Modulates Pattern Recognition–Mediated Cytokine Responses in Human Monocyte-Derived Macrophages. Prior studies have shown that complement C5a signaling powerfully modulates cytokine secretion profiles from macrophages, particularly in synergy with TLR4 activation (via LPS) (27, 28, 38–41); however, the activity of C5a toward the multitude of PRRs expressed by macrophages is largely unknown. Here, we undertook a comprehensive examination of how endogenous C5a, acting on both C5aR1 and C5aR2, may affect PRR-mediated cytokine responses in human macrophages. Eight PRRs were chosen, encompassing TLRs (TLR1/2, TLR3, TLR4, and TLR7), CLRs (Dectin-1, Dectin-2, and Mincle), and the cytosolic DNA sensor STING. We treated primary human monocyte-derived macrophages (HMDMs) with specific agonists targeting the respective PRRs and compared the cytokine output in the copresence or absence of C5a.

We observed significant modulatory effects of C5a toward multiple PRR-induced cytokines. In general, while low concentrations of C5a dampened cytokine output of most PRRs, higher concentrations of C5a substantially amplified cytokine release (*SI Appendix*, Fig. S1). For comparison among PRRs and C5a concentrations, we generated heatmaps for the average fold-change in TNFα and IL-6 responses (Fig. 1 *A* and *B*). Relative to the PRR ligand–only induced responses, cotreating cells with a high concentration of C5a (100 nM) markedly up-regulated the TNFα response mediated by TLR3 (8.7-fold), Dectin-1 (4.1-fold), Mincle (2.5-fold), and STING (41-fold). A clear dose-dependent C5a-mediated TNFα response was seen for Dectin-1 and STING, with 10 nM C5a also demonstrating a clear enhancing effect on TNFα secretion (Fig. 1 *C* and *D*). Consistent with our previous observations (42), rhC5a (100 nM) by itself triggered a modest increase in IL-6, without significantly affecting TNFα or IL-10 (*SI Appendix*, Fig. S1 *A*, *F*, and *K*). For IL-6 secretion, the most profound C5a-driven increase was observed for Dectin-1, Mincle, and STING (Fig. 1*B* and *SI Appendix*, Fig. S2). Our data also confirm prior studies (30, 43), whereby C5a administration at all doses tested suppressed TLR4-mediated cytokine production (Fig. 1 *A* and *B* and *SI Appendix*, Figs. S1 and S2). Finally, for TLR1/2 and TLR7, a low concentration of C5a (1 nM) dampened TNFα and IL-6 secretion by ~30-50%, however, the effect dissipated at higher C5a concentrations. Our findings reveal a distinctive synergy between C5a and PRR ligands in human macrophages, wherein the direction of cytokine output is dependent both on the type of PRR and the concentration of C5a.

C5a-Mediated Cytokine Modulation Is Not Observed with C5a-desArg. Among the panel of PRR ligands tested, the Dectin-1 agonist D.zymosan and the STING agonist cAIM[PS]2 Difluor [Rp/Sp] demonstrated the most consistent and potent upregulation of cytokine production in the copresence of C5a. We next explored whether a similar modulatory effect could also be present for C5a-desArg. After complement activation and C5-convertase-mediated C5a generation, the C-terminal arginine residue of C5a is rapidly removed by serum and cell-surface carboxypeptidases to form C5a-desArg. This protein represents the predominant form of C5a found in human plasma (3), and arguably possesses similar binding and functional activity as C5a toward both C5a receptors (44, 45). Unexpectedly, when we compared the effects of Dectin-1 costimulation with a high concentration (100 nM) of C5a versus C5a-desArg, we observed that C5a-desArg did not induce TNFα production (Fig. 2*A*). C5a-desArg was also largely inactive toward other PRR-mediated responses (*SI Appendix*, Fig. S3). This indicates that only intact full-length C5a, but not C5a-desArg, is capable of synergizing with PRRs in HMDMs. We also examined whether the copresence of C5a-desArg may alter the C5a-Dectin-1 synergistic response. Although C5a-desArg dampened C5a-mediated upregulation of TNFα by ~20%, this did not reach statistical significance (*SI Appendix*, Fig. S4). As C5a receptor signaling has recently been linked to fungal defense (35), we focused further on Dectin-1. We first analyzed a total of 13 inflammatory markers using a multiplex assay cytometric bead array (*SI Appendix*, Fig. S5), and observed that in addition to TNFα, IL-6, and IL-10 that was observed in prior experiments, C5a (100 nM) also significantly enhanced Dectin-1-mediated secretion of IL-1β, IL-12p70, and IL-23, albeit to a lesser extent relative to TNFα. We also confirmed that plasma-purified C5a similarly amplifies Dectin-1 TNFα responses (Fig. 2*B*, 67% versus 57% increase for rhC5a and phC5a, respectively), ruling out any nonspecific effect associated with recombinant C5a (42).

C5a Enhances Dectin-1-Mediated TNFα Release Specifically through C5aR1. C5a binds equally to two C5a receptors, C5aR1 and C5aR2, which both have been linked to PRR-mediated

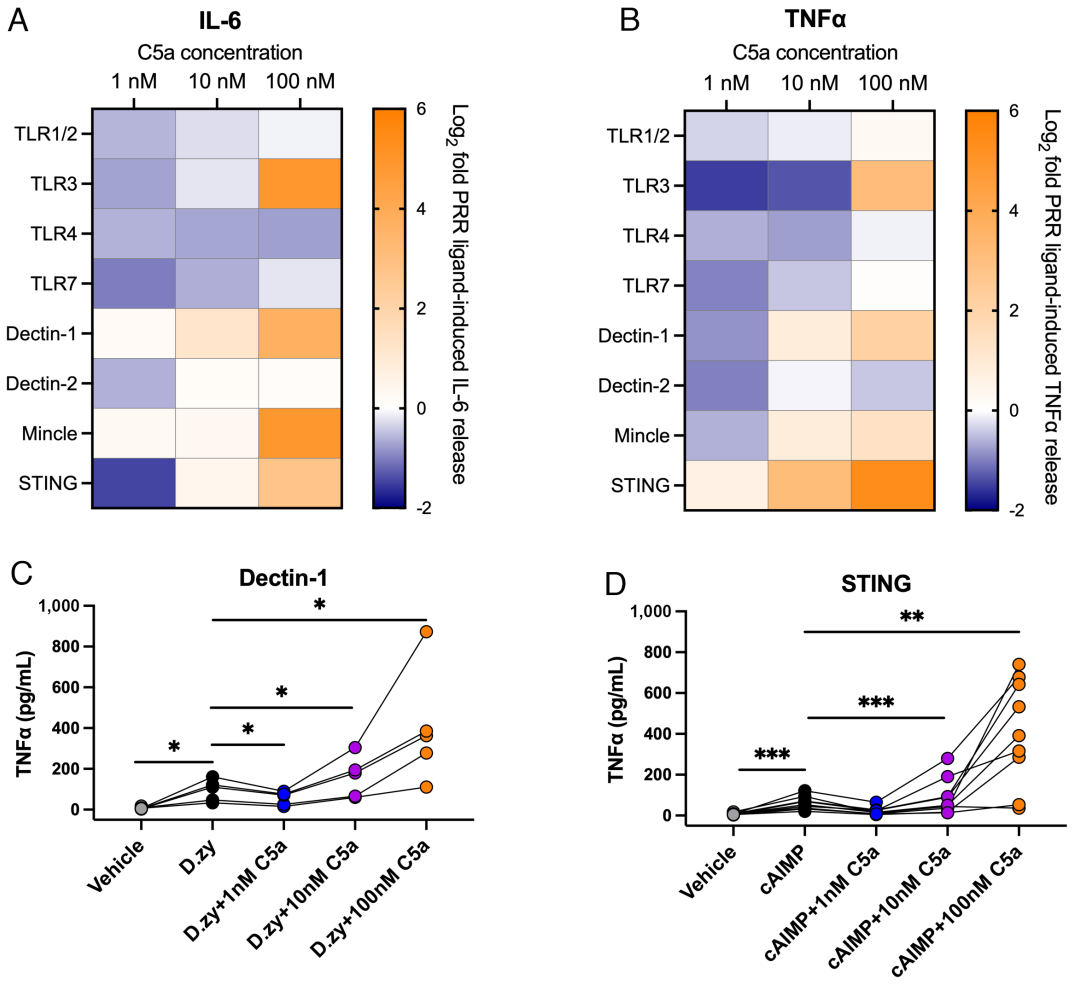

Fig. 1. C5a modulates pattern recognition–mediated cytokine responses in HMDMs. HMDMs (100,000/well) were stimulated with respective PRR ligands, Pam3CSK4 (100 ng/mL, TLR1/2), poly (A:U) (20 μg/mL, TLR3), LPS (10 ng/mL, TLR4), Gardiquimod (10 μg/mL, TLR7), depleted zymosan (D.zymosan, 100 μg/mL, Dectin-1), Furfurman (50 μg/mL, Dectin-2), GlcC14C18 (10 μg/mL, Mincle), or cAIM(PS)2 Difluor (Rp/Sp) (cAIMP, 5 μg/mL, STING) in the copresence of 1, 10, or 100 nM of recombinant human C5a. The supernatant content of TNFα and IL-6 after 24-h stimulation was quantified using ELISA. (*A* and *B*) The mean of triplicate measurements from each of five to nine independent donors (n = 5 to 9) was normalized to the PRR ligand alone–induced responses and then combined. The graph represents a heat map of the $\log_2$ fold increase in (*A*) TNFα and (*B*) IL-6 release in the copresence of C5a relative to PRR ligand–only levels (blue: reduction in baseline PRR response; orange: increase in baseline PRR response). Raw TNFα measurements for (*C*) Dectin-1 and (*D*) STING are shown (mean ± S.E.M.). Two-tailed paired *t* test; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; PRR ligand and C5a cotreated versus PRR ligand–only treated donor-matched cells.

cytokine modulation (41, 46). As there are no selective inhibitors for C5aR2, we interrogated C5aR1 by inhibiting this receptor selectively using the noncompetitive inhibitor PMX53 (47). We first confirmed that the inhibitor was able to block C5a-C5aR1 signaling at the concentration used by measuring the inhibitory effect of the ligand toward C5a-induced and C5a+D.zy-induced ERK signaling. C5aR1 inhibition with PMX53 demonstrated near-complete inhibition of C5a alone-induced ERK1/2 phosphorylation, confirming the functionality of the ligand even in the presence of a high concentration of C5a (Fig. 2*C*). PMX53 also mildly dampened, but did not abrogate, D.zy+C5a-induced ERK signaling, to a level similar to that mediated by Dectin-1 activation alone. This suggests that inhibiting surface C5aR1 using PMX53 does not affect Dectin-1-mediated ERK signaling. Notably, when we used PMX53 in the PRR assay, a near-complete suppression of D.zymosan and C5a-induced TNFα response was observed (Fig. 2*D*). Considering PMX53 has limited cell permeability and thus unable to engage intracellular C5aR1 (48), we conclude that the synergistic effect between C5a and D.zymosan on HMDM TNFα production is driven predominantly through surface C5aR1.

Both C5a Priming and Costimulation Enhance Macrophage Dectin-1 TNFα Production. We next investigated whether simultaneous activation of C5aR1 and Dectin-1 was mandatory for the C5a-mediated synergistic effect to occur. We used an alternative priming treatment scheme, where HMDMs were pretreated with 100 nM C5a for 3 h to allow sufficient time for potential transcription activation and de novo protein synthesis (49). The C5a was then extensively washed off, followed by the addition of D.zymosan. We found that C5a priming also significantly up-regulated Dectin-1-mediated TNFα production by 45%, but, at a much lower level relative to the cotreatment scheme (135%) (Fig. 2*E*). Therefore, although acute priming of C5a alone can enhance macrophage cytokine output in response to Dectin-1 activation, the response is stronger for direct coactivation.

The Synergistic Cytokine Production under Costimulation Requires Syk, ROS, and NFκB Activity. We next aimed to decipher the signaling mechanisms underlying this C5a-mediated synergistic effect. Several signaling molecules known to participate in Dectin-1-mediated cell signaling were examined (50). We adopted the following pharmacological inhibitors: R406 (Syk inhibitor, 10

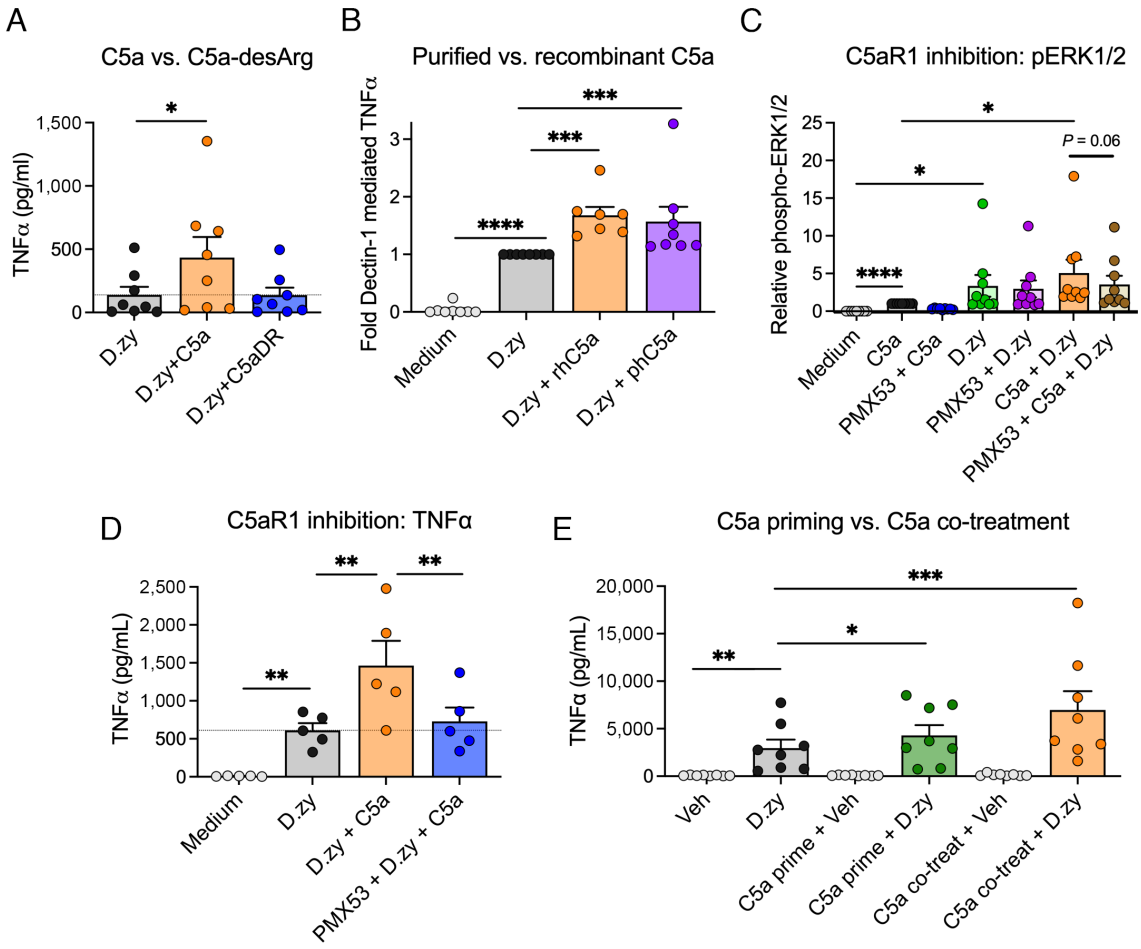

Fig. 2. C5a enhances Dectin-1-induced TNFα through C5aR1 in HMDMs. (*A*) HMDMs were stimulated with depleted zymosan (D.zy, 100 μg/mL) in the copresence of recombinant human C5a (100 nM) or purified human C5a-desArg (C5aDR, 100 nM) for 24 h. (*B*) HMDMs were stimulated with D.zy (100 μg/mL) in the copresence of recombinant human C5a (rhC5a, 100 nM) or purified human C5a (phC5a, 100 nM) for 24 h. The supernatant content of TNFα was measured using ELISA and expressed as fold D.zy-only induced responses. (*C* and *D*) HMDMs were pretreated with C5aR1 inhibitor PMX53 (10 μM) for 40 min and then costimulated with D.zy (100 μg/mL) and/or rhC5a (100 nM). For (*C*), the phospho-ERK1/2 content in cell lysate was detected using AlphaLISA, normalized to the C5a alone–triggered response, and expressed as relative phospho-ERK1/2 level. For (*D*), the supernatant content of TNFα was measured using ELISA. (*E*) HMDMs were primed with rhC5a (100 nM, designated as *C5a prime*) for 3 h, washed, and incubated with the respective ligands (vehicle-*Veh* or 100 μg/m D.zy). For comparison, HMDMs were also cotreated with the D.zy and C5a (100 nM) (designated as *C5a cotreat*). Supernatant TNFα after 24-h stimulation was quantified using ELISA. Each data point represents the mean of triplicate measurements from each of five to nine independent donors (n = 5 to 9). Bars represent the mean ± S.E.M. Statistical analyses were performed as follows: (*A*, *B*, *D*, and *E*) one-way ANOVA with Sidak's post hoc analysis; (*C*) two-way ANOVA with Sidak's post hoc analysis. *P < 0.05, **P < 0.01, ***P < 0. 001, and ****P < 0.0001.

μM), diphenyleneiodonium chloride (DPI, NADPH oxidase inhibitor, 10 μM,), BAY11-7082 (dual inhibitor of NFκB and NLRP3, 10 μM) (51), and CA-074 Me (cathepsin B inhibitor, 20 μM). Inhibition of Syk kinase, reactive oxygen species (ROS) and the NFκB/NLRP3 pathways all significantly reduced the D.zymosan alone-induced TNFα response (Fig. 3 *A*–*C*), and the IL-1β response to a milder extent (*SI Appendix*, Fig. S6 *A*–*C*). Notably, inhibition of these pathways also diminished the synergistic cytokine response (both TNFα and IL-1β) under C5a-D.zymosan cotreatment (Fig. 3 *A*–*C* and *SI Appendix*, Fig. S6 *A*–*C*). However, the cysteine protease, cathepsin B, a mediator of Dectin-1-mediated cytokine production in macrophages (52, 53), did not significantly alter the D.zymosan, or D.zymosan + C5a induced TNFα or IL-1β production (Fig. 3*D* and *SI Appendix*, Fig. S6*D*). Thus, Syk kinase, ROS, and the NFκB/NLRP3 pathways are necessary for the observed C5a and Dectin-1 synergistic cytokine effect in macrophages.

Dectin-1-Mediated TNFα Production Requires Cell-Intrinsic C5a-C5aR1. An emerging paradigm in complement research is the appreciation that immune cells can synthesize complement components that act functionally in the absence of liver/serum-derived complement (48, 54). A recent report identified that human macrophages constitutively express C5 and generate C5a via formation of an intracellular C5 convertase (48). We hypothesized that macrophages may respond to PRR ligands like Dectin-1 by generating C5a and enabling a cell-intrinsic synergy axis. To test this, we treated HMDMs with the C5aR1 antagonist PMX53 prior to stimulation with D.zymosan. Remarkably, the presence of PMX53 significantly attenuated TNFα production, even without the addition of an external source of C5a (Fig. 4*A*), indicating the involvement of endogenously produced C5a in the intrinsic cytokine response of Dectin-1. To confirm this, we measured the total cell C5a expression in HMDMs under different treatment conditions (Fig. 4*B*). As expected, stimulating cells with C5a (100 nM) alone enhanced the total C5a detected (by 16%), likely caused by residual amount of external C5a that remain tethered to cell membrane-expressed receptors, or internalized but remaining undegraded (13, 55). Activating Dectin-1 with D.zymosan alone also significantly increased total C5a expression by 46%, confirming the presence of intrinsic C5a derived from cells. We further measured cell surface and total C5aR1 expression

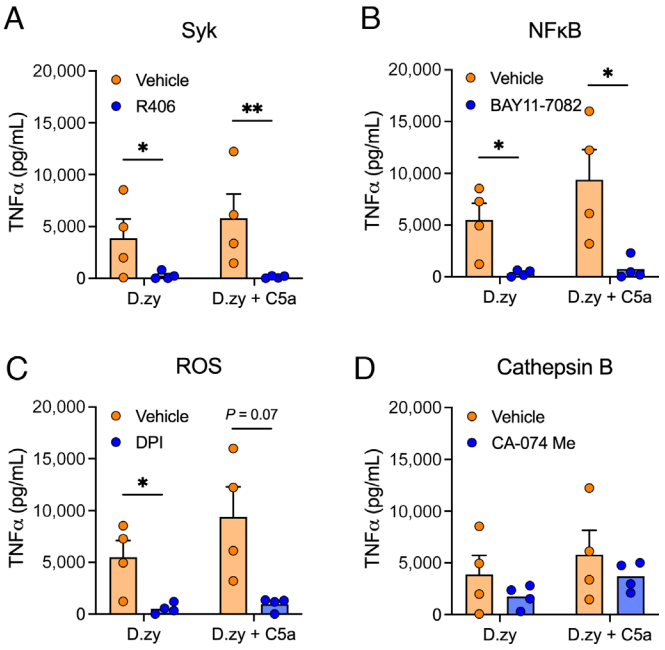

Fig. 3. C5a-Dectin-1 synergy requires Syk, ROS, and NFκB activity. HMDMs were pretreated with respective inhibitors (*A*) R406 (5 μM), (*B*) diphenyleneiodonium chloride (DPI, 10 μM), (*C*) BAY11-7082 (10 μM), and (*D*) CA-074 Me (20 μM) for 40 min before being stimulated with depleted zymosan (D.zymosan, 100 μg/mL) alone or in the presence of C5a (100 nM). The supernatant content of TNFα after 24-h stimulation was quantified using ELISA. Each data point represents the mean of triplicate measurements from each of the 4 independent donors ($n = 4$). Bars represent the mean ± S.E.M. Two-tailed paired *t* test was performed (*$P < 0.05$ and **$P < 0.01$, inhibitor-treated versus vehicle-treated donor-matched cells under the same stimulation condition).

upon Dectin-1 activation using in-cell Western assays. Total C5aR1 expression (both surface and intracellular) was significantly up-regulated following Dectin-1 stimulation (Fig. 4*C*), while minimal change was observed for C5aR1 expression at the cell surface (Fig. 4*D*), which was also supported by surface staining for C5aR1 using immunocytochemistry (Fig. 4*E*), suggesting that the majority of C5aR1 upregulation observed at 24 h post stimulation takes place intracellularly, possibly through de novo protein synthesis. Correspondingly, treating cells with D.zymosan also markedly enhanced C5aR1 phosphorylation (Fig. 4*F*), supporting a heightened C5aR1 activation and signaling environment upon Dectin-1 activation. These findings demonstrate the presence of a cell-intrinsic C5a-C5aR1 system in macrophages that is triggered upon Dectin-1 activation.

Cell-Intrinsic C5aR1 Signaling Modulates Dectin-1-Mediated ROS Production. Dectin-1 activation, through Syk, triggers ROS production (56). In accordance, inhibiting ROS with DPI significantly dampened TNFα secretion (Fig. 3*D*). To examine this further, we measured how exogenous, or cell-derived C5a may affect Dectin-1-mediated ROS generation in HMDMs, using the cell-permeable CellRox Deep Red reagent. D.zymosan triggered significant ROS production in HMDMs [increase by 100 % from the baseline (Fig. 4*G*)]. C5a addition, by itself, did not cause ROS production, nor did it alter the Dectin-1-mediated effect. However interestingly, pretreating cells with the C5aR1 inhibitors PMX53 and PMX205, in the absence of external C5a, significantly enhanced Dectin-1-mediated ROS generation (Fig. 4*H*). Therefore, inhibition of cell-derived C5a-C5aR1 signaling, but not the addition of exogenous C5a, enhances Dectin-1-induced ROS production in HMDMs.

Cell-intrinsic C5a-C5aR1 Signaling Is Required for *C. albicans* Killing by HMDMs. Our findings suggest that Dectin-1 activation initiates cell-intrinsic C5a-C5aR1 signaling, which in turn modulates the Dectin-1-mediated cell responses. As such, we next explored whether this autonomous C5a system may translate into any functional outcome by measuring fungal killing by HMDMs. Specifically, we established a coculture system of HMDMs and *C. albicans* and measured fungal survival upon modulating C5aR1 or Dectin-1. Treating cells with increasing concentrations of C5a, or the surface C5aR1 inhibitor PMX53 (48), did not have any effect (Fig. 4 *I* and *J*). We further examined an alternative C5aR1 inhibitor, PMX205, which is more lipophilic (57, 58), thus allowing better inhibition of intracellular C5aR1. By contrast to PMX53, PMX205 enhanced fungal survival (78% for PMX205 versus 50% for vehicle), suggesting a significant involvement of intracellular C5aR1 in the antifungal response of HMDMs (Fig. 4*K*). The lack of effect from addition of external C5a provides further support for the presence and requirement of cell-intrinsic C5a signaling in HMDM antifungal defense. Interestingly, addition of D.zymosan markedly dampened the killing response (Fig. 4*L*), possibly because D.zymosan by binding to Dectin-1, impedes the *C. albicans* from being recognized by Dectin-1 receptors and initiating the antifungal response. Based on these data, we can conclude that activation of C5aR1 by cell-intrinsic C5a, as well as fungal recognition by Dectin-1, are both required for efficient fungal killing by human macrophages.

Discussion

Both the complement system and diverse PRRs collectively detect foreign antigens, forming a critical frontline defense against invading pathogens and internal tissue injury. The intricate interplay and regulatory networks between the complement and pattern-recognition systems are orchestrated to ensure an optimized and highly efficient response (27). Although much prior focus has surrounded complement and TLR interactions, a recent study reported a novel role for C5a in regulating Dectin-1 expression and fungal clearance (35). Extending from those findings, this study characterized the modulatory activities of C5a on diverse PRR-mediated responses in human primary macrophages and reports a concentration-dependent, bidirectional action of C5a, with a low concentration of C5a dampening, and a high concentration of C5a markedly up-regulating cytokine responses mediated by several non-TLR PRRs, such as Dectin-1, Mincle, and STING. Focusing on Dectin-1, we confirmed a cell-intrinsic interaction with C5aR1 necessary for antifungal defense by human macrophages. Based on our findings, we propose an "inside-out" mechanism of C5a-Dectin-1 cross talk whereby Dectin-1 activation by fungal components triggers the generation of cell-derived C5a, which, acting through both intracellular and surface C5aR1, up-regulates proinflammatory cytokine production and ROS generation, thereby augmenting antifungal defense by macrophages. This study contributes additional evidence to underscore the comprehensive roles of C5a, functioning as a pivotal immunomodulator and a crucial coordinator of pathogen detection and elimination by phagocytes.

We observed broad bidirectional modulatory actions of C5a on diverse PRR-mediated cytokine responses in human macrophages. C5a, at a low nanomolar concentration, exhibited a generally suppressive effect, significantly dampening the release of the acute response cytokine IL-6 and the proinflammatory TNFα for multiple PRRs. For TLR4, our observations agree with previous findings for both human and mouse macrophages (29, 30, 38, 59). Notably, higher concentrations of C5a markedly enhanced =IL-6

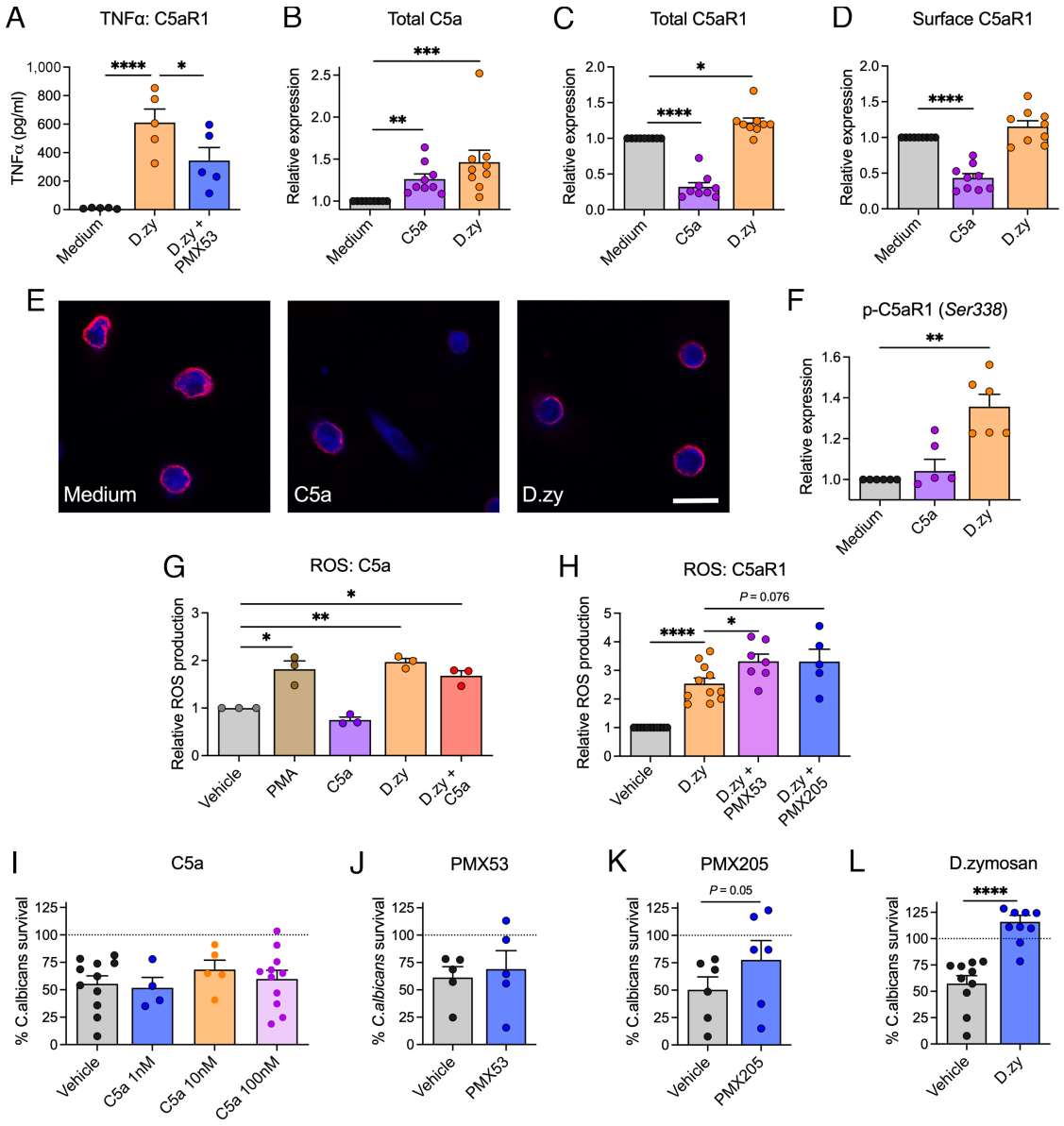

Fig. 4. Cell-intrinsic C5a-C5aR1 signaling modulates Dectin-1-mediated antifungal response in HMDMs. (*A*) HMDMs were pretreated with C5aR1 inhibitor PMX53 (10 μM) for 40 min and then costimulated with depleted zymosan (D.zy, 100 μg/mL). The supernatant content of TNFα after 24-h stimulation was quantified using ELISA. (*B–E*) HMDMs were stimulated with respective ligands (D.zy, 100 μg/mL; C5a, 100 nM) for 24 h, and the expression of the following markers measured using in-cell western assays and expressed as a fold-change of the medium only-treated levels: (*B*) total (cell surface and intracellular) C5a, (*C*) total C5aR1 and (*D*) cell surface C5aR1. (*E*) HMDMs treated with medium, D.zy (100 μg/ml), and C5a (100 nM) were imaged using immunocytochemistry for surface C5aR1 staining (Scale bar, 20 μm). (*F*) Phosphorylated C5aR1 (Ser338) in C5a or D.zy -stimulated HMDMs were measured using in-cell western assays as per (*B–E*). (*G* and *H*) HMDMs were stained with CellROX Deep Red reagent (3 μM) and underwent the following treatements: (*G*) stimulated with D.zy (100 μg/mL), rhC5a (100 nM), individually or combined, and PMA (100 nM); (*H*) pretreated with vehicle, PMX53 or PMX205 (10 μM) for 20 min before being stimulated with D.zy (100 μg/mL). The fluorescence signal (Ex/Em: 644/665 nm) at 60 min after ligand addition was measured and expressed as fold of the vehicle-induced response. (*I–L*) HMDMs (100,000/well) were pretreated with C5a (1, 10, or 100 nM), PMX53 (10 μM), PMX205 (10 μM), or D.zy (100 μg/mL) for 1 h and then added to *C. albicans* (5,000/well). After 24 h, HMDMs were lysed using cold water. Surviving *C. albicans* were quantified using XTT assay and expressed as a percentage of the *C. albicans* number in the absence of HMDMs. Each data point represents the mean of triplicate measurements from each of three to nine independent donors ($n = 3$ to 9). The bar shows the mean ± S.E.M. For *G* and *H*, one-way ANOVA with Sidak's post hoc analysis was conducted. For *I–L*, two-tailed paired *t* test was performed between vehicle- or ligand-treated donor-matched cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

secretion induced by STING, and TNFα production caused by Dectin-1, TLR3 and Mincle by up to 40-fold. Although several studies have observed significant modulatory roles for C5a on PRR-induced cytokine responses (30, 60, 61), this report further demonstrates the presence of a profound increase in human primary macrophages. Biologically, 1 nM of C5a can be found in the circulation under physiological conditions or during mild inflammation, whereas higher C5a concentrations (e.g., 100 nM) are generally only found within tissues during acute infection/inflammation (3, 62). The dynamic, bidirectional action of C5a identified in this study supports a role for C5a in maintaining homeostasis and limiting inflammation under basal conditions, while enabling macrophages to mount an efficient, robust local immune response when under threat.

Interestingly, C5a-desArg, which is the predominant form of C5a in circulation and traditionally recognized as an "inactive" form of C5a (3, 45), did not display significant modulation toward any PRR-mediated cytokine responses. The biological roles of C5a-desArg remain to be understood, with several recent reports demonstrating C5a-desArg to be equipotent as C5a at triggering

C5aR2 signaling and capable of inducing significant levels of cell activation at physiological concentrations (21, 45). Considering that most of the C5a in circulation exists in the form of C5a-desArg, the lack of activity of C5a-desArg provides further evidence for the spatial control of C5aR1 immune processes.

Dectin-1 is the primary receptor of fungal β-glucan and plays an imperative role in fungal recognition and antifungal defense. The pleiotropic roles of Dectin-1 are increasingly recognized which further encompass protection against a variety of other pathogens and self-recognition of T cells and apoptotic cells through unknown ligands (63). We observed a marked enhancement in Dectin-1-mediated cytokine production by HMDMs in the copresence of C5a. As a Group V C-type lectin receptor, Dectin-1 contains a single extracellular C-type lectin domain which permits calcium-independent ligand interactions (50). Dectin-1-mediated signaling is primarily Syk-dependent, which, through the NFκB-inducing kinase, or the PLC/PKC-mediated CARD9-Bcl10-Malt1 pathway, activates the IKK kinase complex (50). The IKK complex phosphorylates IκB kinase, leading to NFκB activation and the subsequent transcription of cytokines such as IL-1β, IL-6, TNFα, IL-10, and IL-23 (50, 64). Dectin-1/Syk signaling, additionally generates ROS and activates the lysosomal cathepsin B protease, which subsequently induces NLRP3 inflammasome activation and IL-1β secretion (52). ROS can also directly mediate NFκB-dependent cytokine production (65). Accordantly, in the current study, inhibiting Syk, ROS, and the NFκB pathway markedly reduced D.zymosan-induced TNFα and IL-1β production. The C5a-mediated enhancement effect was also abolished, suggesting that the observed synergistic production of TNFα and IL-1β primarily resulted from C5a-dependent upregulation of a Dectin-1-mediated response, rather than vice versa. Cathepsin B blockade did not alter the TNFα response, suggesting the molecule to be exclusively involved in other pathways (52). Although the ROS inhibitor DPI dampened both D.zymosan alone and D.zymosan + C5a-induced cytokine responses, subsequent measurement of intracellular ROS in HMDMs did not detect any significant C5a-driven change. This suggests that Dectin-1 elicits cytokine release through a ROS-dependent pathway, and C5a likely acts downstream of ROS to enhance cytokine production.

Recently, a fundamental role for the C5-C5aR1 axis in yeast cell uptake by neutrophils and intracellular nonoxidative killing by macrophages was identified (35). In that study, C5aR1-deficient murine renal macrophages or blockade of C5aR1 using CCX168 (avacopan) in human HMDMs and neutrophils, significantly reduced *Candida* killing. Consistent with this, in our study, we also observed significant reduction in both cytokine production and fungal killing by macrophages upon pharmacological inhibition of C5aR1. Importantly, these effects were observed in the absence of any exogenous C5a being added, thus supporting the hypothesis that macrophages are capable of initiating an intrinsic C5a system when activated by fungal stimuli. In this study, we looked deeper into the changes in cell-intrinsic complement components upon fungal stimulation. Using an in-cell western assay directly detecting cell-expressed C5a, C5aR1, and phosphorylated (activated) C5aR1 in macrophages, we confirmed that Dectin-1 activation triggers the generation of cell-intrinsic C5a and simultaneously up-regulates the expression of intracellular C5aR1, allowing for enhanced C5a-C5aR1 activation. The ability of macrophages to initiate and sustain an intrinsic C5a-C5aR1 system may account for our observed lack of additive effect from externally added C5a on Dectin-1-mediated ROS production and *C. albicans* killing.

In our findings, Dectin-1 activation significantly up-regulated intracellular C5aR1 expression. It is thus plausible that intracellularly localized C5aR1 plays a major role in cell antifungal defense. Indeed, the concept of intracellular C5aR1 is now attracting significant attention, highlighted by research findings encompassing multiple immune cell types, including T lymphocytes, monocytes, and macrophages (33, 48). This cell-intrinsic C5a system can be readily triggered by danger signals such as cholesterol crystals, which in turn directs cell inflammatory output (48). A similar machinery may also be initiated by Dectin-1. However, the sole involvement of intracellular C5aR1 does not reconcile with the data obtained for PMX53. PMX53 has low cell permeability and limited ability to inhibit intracellular C5aR1 (48). In our study, PMX53 nearly completely blocked (exogenous) C5a-mediated upregulation of Dectin-1 TNFα secretion, and significantly dampened Dectin-1 alone-induced TNFα release. As such, it is logical that at least part of the cell-intrinsic C5a activity was driven through surface C5aR1. Intriguingly, blocking C5aR1 with PMX53 did not alter the fungal killing response by HMDMs, whilst the more lipophilic and membrane-permeable PMX205 (57, 58) promoted fungal survival (reduced killing). This supports a more predominant role of intracellular C5aR1 in HMDM-mediated fungal killing.

Finally, to our surprise, while adding external C5a did not significantly alter D.zy-induced ROS, blocking C5aR1 using PMX53 or PMX205 up-regulated it. Considering both PMX53 (cell impermeable) and PMX205 (more cell permeable) enhanced ROS to a similar extent, this suggests that Dectin-1-mediated ROS production requires the generation of cell-intrinsic C5a acting on surface C5aR1. One limitation to our findings however is the reliance on pharmacological inhibitors to understand the role of C5a–PRR responses in primary macrophages, which may potentially impart off-target activities (66). Future support of our findings using genetic or nonpharmacological approaches will help validate our results and further decipher roles for intracellular C5aR1 and possible contributions of C5aR2 in human primary cells.

Based on our findings, we propose a paradigm whereby Dectin-1 activation triggers macrophages to generate cell-intrinsic C5a, which can (i) act intracellularly by binding to mitochondrial C5aR1 and (ii) be excreted and activate surface-expressed C5aR1 in an autocrine or paracrine manner. This signaling between C5a and C5aR1, operating through both autocrine and paracrine pathways, contributes to the sustained generation of mitochondrial ROS, the elevation of TNFα production, and the reinforcement of fungal eradication. We note that the findings derived in this study are limited to differentiated primary human macrophages. However other myeloid cell phagocytes are also primary responders in antifungal defense (67, 68). Indeed, a recent study by Desai et al. (35) highlighted differential roles for C5aR1 in antifungal defense in murine monocytes, neutrophils, and macrophages. Future studies could therefore investigate whether the regulatory mechanisms via intracellular C5a identified in our study, also hold for other human immune cells. Interrogation of the intracellular localizations of C5aR1 following Dectin-1 and other PRR stimulation in these human primary immune cells would also be an interesting avenue for further study.

In conclusion, this study characterized how C5a acting through C5a receptors modulates pattern recognition–mediated responses in primary human macrophages. We observed a significant synergy between C5a-C5aR1 and non-Toll receptors, namely Dectin-1, Mincle, and STING that facilitated cytokine responses. Notably, Dectin-1 activation alone initiated cell-autonomous C5a activity, facilitated by both intracellular and cell surface C5aR1, thereby augmenting antifungal defense. Overall, this study reveals a cell-intrinsic functional interaction between C5a and the pattern-recognition system within human macrophages.

Materials and Methods

Ligands and Materials. Recombinant human C5a (rhC5a) was purchased from Sino Biological (Beijing, China; rhC5a-S; purity, >94 %; endotoxin, <1 EU/μg) or from alternative suppliers: rhC5a-A (Abcam, Melbourne, Australia; purity, >95 %; endotoxin, <0.1 EU/μg) and rhC5a-R (R&D Systems, Minneapolis, USA; purity, >95 %; endotoxin, <0.1 EU/μg). All the in vitro assays in this study were performed using rhC5a from Sino Biological unless otherwise stated. Purified, plasma-derived human C5a (phC5a) was purchased from Complement Technologies (Tyler, USA; purity, >97 %; endotoxin, <0.1 EU/μg). The C5aR1 antagonists PMX53 and PMX205 were synthesized in-house (43). The signaling inhibitors were obtained as follows: R406 (InvivoGen), BAY11-7082, CA-074 Me, and diphenyleneiodonium chloride (DPI) (Merck). All pattern-recognition receptor ligands were obtained from InvivoGen. Phorbol myristate acetate (PMA) and bovine serum albumin (BSA) were purchased from Merck. For cell culture, Iscove's Modified Dulbecco's Medium (IMDM) and penicillin–streptomycin were purchased from Thermo Fisher Scientific. Dulbecco's phosphate-buffered saline was purchased from Lonza.

Cell Culture. HMDMs were generated and cultured as previously described (41–43). Briefly, human buffy coat blood from anonymous healthy donors was obtained through the Australian Red Cross Blood Service. Human CD14+ monocytes were isolated from blood using Lymphoprep density centrifugation (STEMCELL) followed by CD14+ MACS magnetic bead separation (Miltenyi Biotec). The isolated monocytes were differentiated for 6 d in IMDM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 15 ng/mL recombinant human macrophage colony stimulating factor (BioLegend) on 10-mm square dishes (Thermo Fisher Scientific). Nonadherent cells were removed by washing with DPBS, and the adherent differentiated HMDMs were harvested by gentle scraping.

Cytokine Release Assays. The cytokine-inducing activity of C5a was assessed in HMDMs as previously described (42, 44). All treatment ligands were prepared in serum-free IMDM containing 0.1% BSA. The following PRRs were examined (respective ligands used are indicated in the brackets): TLR1/2 (Pam3CSK4, 100 ng/mL), TLR3 (Poly[A:U], 20 μg/mL), TLR4 (LPS, 10 ng/mL), TLR7 (Gardiquimod, 10 μg/mL), Dectin-1 (depleted zymosan, D.zymosan, 100 μg/mL), Dectin-2 (Furfurman, 50 μg/mL), Mincle (GlcC14C18, 10 μg/mL), and STING (cAIM[PS]2 Difluor [Rp/Sp], 5 μg/mL). HMDMs (100,000 /well), seeded in 96-well tissue-culture plates (Corning), were treated with various PRR ligands in the absence or presence of different concentrations of C5a for 24 h (37 °C, 5 % CO_2). For inhibition studies, HMDMs were pretreated with PMX53 (10 μM), BAY11-7082 (10 μM), R406 (5 μM), diphenyleneiodonium chloride (DPI, 10 μM), or CA-074 Me (20 μM) for 40 min, before the addition of PRR ligands with/without C5a. Cell culture supernatant was collected and stored at −20 °C till use. The supernatant content of TNF-α, IL-6, and IL-10 was quantified using respective enzyme-linked immunosorbent assay (ELISA) kits (BD) as per the manufacturer's protocols. IL-1β content was measured using ELISA kits from R&D Systems.

Phospho-ERK1/2 Assays. Ligand-induced ERK1/2 phosphorylation was assessed using the AlphaLISA *Surefire Ultra* p-ERK1/2 (Thr202/Tyr204) kit (PerkinElmer) following the manufacturer's protocol. HMDMs were treated as described in the above section. Upon supernatant collection, cells were immediately lysed using AlphaLISA lysis buffer on a microplate shaker (450 rpm, 10 min). For the detection of phospho-ERK1/2 content, cell lysate (5 μL/well) was transferred to a 384-well ProxiPlate (PerkinElmer) and added to the donor and acceptor reaction mix (2.5 μL/well, respectively), followed by a 2-h incubation at room temperature in the dark. On a Tecan Spark 20M (Tecan), the plate was measured using standard AlphaLISA settings.

In-Cell Western Assays to Measure C5a and C5aR1 Expression. In-cell western assays were performed following the technical guidelines provided by LI-COR Biosciences as previously described (69). Briefly, HMDMs were seeded (80,000/well) in poly D-lysine-coated (Merck) black-wall clear-bottom tissue culture 96-well plates (Corning) for 24 h. All ligands are prepared in serum-free IMDM containing 0.1% BSA (Merck). Cells were treated for 24 h with D.zymosan (100 μg/mL) and/or rhC5a (100 nM) (24 h, 37 °C, 5 % CO_2). Following treatment, the cells were fixed using 4% paraformaldehyde (Alfa Aesar) (10 min, room temperature). Upon gentle washing with DPBS, the cells were permeabilized using 0.3% Triton X-100 (Merck) in DPBS and then blocked using Odyssey Blocking Buffer in PBS (LI-COR Biosciences) (1.5 h, room temperature). No permeabilization step was performed for cell surface–only stained cells. The cells were then incubated with the rabbit anti-human C5a antiserum (1:200, Complement Technologies, Tyler, USA), Rabbit Anti-Human CD88 antibody (1:250, Clone C85-2506, BD Biosciences), or rabbit Anti-phospho-CD88/C5aR ($pSer^{338}$) antibody (1:200, Merck) at 4 °C overnight. Upon further washing with DPBS containing 0.5 % Tween-20, the cells were stained with IRDye 680RD donkey anti-rabbit secondary antibody (1:1,000, LI-COR Biosciences) and DAPI (1:3,000, Thermo Fisher Scientific) for 1.5 h at room temperature. The plate was then washed with DPBS containing 0.5% Tween-20 and blotted dry. For fluorescence quantification, the plate was read (Ex/Em: 667/707 nm and 342/505 nm for IRDye 680DW and DAPI, respectively) on a Tecan Spark 20M microplate reader (Tecan). All fluorescence measurements were corrected to cell number by division using DAPI values.

Immunocytochemistry. HMDMs were seeded (150,000/well) onto poly-D-lysine-coated coverslips in a 24-well tissue culture plate (Corning) for 24 h (37 °C, 5 % CO_2). Cells were treated with medium, 100 nM rhC5a or 100 μg/mL D.zymosan for 24 h (37 °C, 5 % CO_2). Fluorescence immunocytochemistry was performed to show surface C5aR1 expression on HMDM in response to C5a and D. zymosan. Briefly, cells were washed with PBS (pH 7.4) and then blocked in PBS containing 3% goat serum for 5 min at 4 °C. Cells were incubated for 30 min at 4 °C with monoclonal C5aR1 antibody in 1× PBS (S5/1 clone, 1:250, Thermo Fisher Scientific). Cells were washed 3× 5 min with PBS prior to incubation with an Alexa 555 goat anti-mouse IgG (1:1,000, Thermo Fisher Scientific) for 1 h at 4 °C. Following 2× 5 min washes in PBS, the cells were incubated for 5 min in DAPI (1:25,000, Thermo Fisher Scientific). All cells were mounted with Prolong Gold Anti-Fade medium (Thermo Fisher Scientific). Mouse IgG isotype control and secondary antibody only staining were performed to give a measure of non-specific background staining.

Fungal Killing Assays. *C. albicans* killing by HMDMs was assessed using a colorimetric method as per previously described (70). Briefly, HMDMs (50,000/well) were seeded in clear tissue culture 96-well plates (Corning) overnight for adherence. On the assay day, HMDMs were treated with respective ligands (as indicated in the figure legend) prepared in serum-free IMDM/0.1% BSA for 1 h. Live *C. albicans* (Strain SC5314, ATCC #MYA-2876) were prepared in SF IMDM and added to the cells at a cell-to-target ratio of 100:1 (~5,000 yeasts per well). After 24-h incubation (37 °C, 5 % CO_2), all media were removed, and HMDMs were lysed using 200 μL/well cold water (30 min, 450 rpm on an orbital shaker). The plate was centrifuged (400 rpm, 5 min), and then, the cold water was removed and replaced with 100 μL/well of SF IMDM. The number of *C. albicans* in each well was determined using the CyQUANT™ XTT Cell Viability Assay kit (Thermo Fisher Scientific). The XTT reagent was prepared by mixing Components A and B following the kit instructions. The reagent was then added to the cell plate (50 μL/well). Following 2-h incubation at 37 °C, the absorbance at 450 nm was measured using a Tecan Spark 20M microplate reader with background correction at 660 nm. The *C. albicans* survival was computed as a percentage of fungal cell number in wells without HMDMs.

Reactive Oxygen Species Measurement. ROS generation by HMDMs was determined using the cell-permeant dye CellROX Deep Red Reagent following the manufacturer's instructions (ThermoFisher). Briefly, HMDMs (80,000/well) were seeded in black clear-bottom 96-well tissue culture plates (Corning). Cells were first stained with 3 μM of CellROX Deep Red Reagent (1X HBSS, 20 mM HEPES) for 30 min (37 °C, 5 % CO_2). Respective ligands were prepared in HBSS buffer containing 0.5 % BSA. Inhibitor (PMX53-10 μM) pretreatment commenced at 20 min prior to agonist addition. On a Tecan Spark 20M (Tecan), upon the addition of respective agonists (PMA-100 nM, D.zymosan-100 μg/mL in the absence or presence of rhC5a-100 nM), the fluorescence signals (Ex/Em: 644/665 nm) were continuously monitored at 3 min intervals with the final reading taken at 70 min. Data were normalized and expressed as a fold of the vehicle-only readings.

Cytometric Bead Array. HMDMs were treated as per the "Cytokine release assays" described above. After 24-h treatment, the supernatant was collected and stored at −80 °C till use. The CBA assay was performed using the

BioLegend® LEGENDplexTM CBA Human Inflammation panel 13-Plex kit (BioLegend) following the manufacturer's protocol. The kit measures a total of 13 analytes: interferon-α (IFNα), IFNγ, TNF-α, Monocyte chemoattractant protein 1 (MCP-1; CCL2), IL-1β, IL-6, chemokine (C-X-C motif) ligand 8 (CXCL8 or IL-8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Briefly, cell supernatant samples were defrosted on ice and centrifuged for 5 min at 450*g* to remove any debris that might be present. A standard calibration curve was established for each kit. All samples were measured in duplicate. On the assay plate, each well was filled with 25 μL of bead solution, 25 μL of assay buffer, and 25 μL of standard or 25 μL of undiluted samples (total volume of 75 μL). The plate was incubated at room temperature under agitation (500 rpm) for 2 h. Then, 25 μL of Phycoerythrin (PE)–labeled detection antibodies were added consecutively to each sample well, followed by further incubation (500 rpm, 1 h, room temperature). To reveal the secondary antibody, 25 μL of streptavidin–PE was added (30 min, room temperature). To obtain the median fluorescence intensities (MFI), bead fluorescence readings were recorded by a flow cytometry (BD™ LSR II) apparatus. FACS data were processed using LEGENDplex Gognit software (BioLegend).

Data Collection, Processing, and Analysis. All experiments were conducted in triplicate using HMDMs derived from different human donors. Data were analyzed using GraphPad software (Prism 10.0.3) and expressed as mean ± S.E.M. unless otherwise stated. The original measurements from each donor are combined where indicated. Statistical analysis was performed through one-way ANOVA or two-tailed paired *t* tests as detailed in the figure legends. Donor-matched statistical comparisons were made whenever possible so that each individual donor served as their own control for the experiment to account for variability in responses. Differences were deemed significant when $P < 0.05$.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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