C5a and Bradykinin Receptor Cross-Talk Regulates Innate and Adaptive Immunity in Trypanosoma cruzi Infection

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Complement and the kallikrein–kinin cascade system are both activated in injured tissues. Little is known about their partnership in the immunopathogenesis of Chagas disease, the chronic infection caused by the intracellular protozoan \textit{Trypanosoma cruzi}. In this study, we show that pharmacological targeting of the C5a receptor (C5aR) or the bradykinin B2 receptor (B2R) inhibited plasma leakage in hamster cheek pouch topically exposed to tissue culture trypomastigotes (TCTs). Further, angiotensin-converting enzyme inhibitors potentiated TCT-evoked paw edema in BALB/c, C57BL/6, and C5-deficient A/J mice through activation of joint pathways between C5aR/B2R or C3aR/B2R. In addition to generation of C5a and kinins via parasite-derived cuzipain, we demonstrate that macrophages internalize TCTs more efficiently through joint activation of C5aR/B2R. Furthermore, we found that C5aR targeting markedly reduces NO production and intracellular parasitism in macrophages. We then studied the impact of C5aR/B2R cross-talk in TCT infection on the development of adaptive immunity. We found that IL-12p40/70 expression was blunted in splenic dendritic cells by blocking either C5aR or B2R, suggesting that codominant signaling via C5aR and B2R fuels production of the Th1-polarizing cytokine. Finally, we assessed the impact of kinins and C5a liberated in parasite-laden tissues on Th cell differentiation. As predicted, BALB/c mice pretreated with angiotensin-converting enzyme inhibitors potentiated IFN-γ production by Ag-specific T cells via C5aR/B2R cross-talk. Interestingly, we found that B2R targeting upregulated IL-10 secretion, whereas C5aR blockade vigorously stimulated IL-4 production. In summary, we describe a novel pathway by which C5aR/B2R cross-talk couples transendothelial leakage of plasma proteins to the cytokine circuitry that coordinates antiparasite immunity. The Journal of Immunology, 2014, 193: 000–000.

The complement system is an important effector arm of the innate and adaptive defense against invading pathogens (1). It can be activated by three pathways: the classical pathway that is activated when IgG or IgM molecules bind to the pathogen surface, allowing the assembly of the C1 complex (C1qrs2), which cleaves C2 and C4 to form the C3 convertase (C4b2a); the lectin pathway, activated when mannann-binding lectin or ficolins bind to the pathogen surface and associate with mannan-binding lectin–associated serine protease-2 to cleave C2 and C4, generating the C3 convertase C4b2a as in the classical pathway; and finally the alternative pathway, activated when activated C3 [H2O] binds to the pathogen surface and associates with factor B to form the C3 convertase C3bBb (1).

During complement activation, native C3 and C5 undergo limited proteolysis, releasing the C3a and C5a anaphylatoxins. C5a and C3a exert their biological functions mainly by activating cells from the myeloid lineage (2). C5a can bind to two distinct C5a receptors (C5aR): C5aR1 (CD88), which is a G-protein–coupled receptor (GPCR) mainly coupling to Gq, and C5aR2 (C5L2), which also belongs to the family of seven transmembrane receptors, but is uncoupled from G-proteins (3). The C5aR1 is highly expressed on innate immune cells of the myeloid lineage and, in lower numbers, on lymphocytes, epithelial, and endothelial cells (5–7). More recently, anaphylatoxin receptors expressed by dendritic cells (DCs) and CD4+ T cells were implicated in CD4+ Th activation and their differentiation into effector T or regulatory T cells (8–15).

In the current study, we explored the possibility that inflammation induced by infection with the etiologic agent of human Chagas disease, the parasitic protozoan \textit{Trypanosoma cruzi}, is intensified as result of convergent activation of complement and kinin proteolytic cascades. Afflicting millions of people, Chagas disease is a major cause of chronic myocardiopathy (CM) in South America (16). Transmitted to humans via blood-sucking triatomine bugs, the infective stages of \textit{T. cruzi} (i.e., metacyclic trypomastigotes) initiate human infection by invading skin tissues exposed upon insect proboscis (17). Alternatively, humans are infected accidentally after drinking natural fruit juices contaminated with the insect-borne parasites (16, 18). Irrespective of the types of host cells invaded by metacyclic trypomastigotes (mac-
rrophages, epithelial cells, and fibroblasts), the parasites rapidly escape from the parasitophorous vacuole and reach the host cell cytoplasm, where they transform into the oval-shaped amastigotes. After several rounds of binary division, the amastigotes transform into mammalian-stage trypomastigotes. Upon host cell rupture, these highly motile flagellated parasites reach the draining lymph nodes (DLNs) and/or fall in the bloodstream, from where the infection disseminates to the liver and spleen, before reaching heart and/or gastrointestinal tissues. Lasting a few months, the blood parasitemia and the acute inflammatory sequel gradually subside owing to the onset of antiparasite immunity. Despite the robust selective pressure imparted by complement-fixing (lytic) IgG Abs (19, 20), innate immunity (21), and IFN-γ-producing effector CD8+ T cells (22), sterile immunity is not achieved. Instead, chagasic patients develop a long lasting asymptomatic infection associated with subpatent blood parasitemia. In the south cone of the Americas, ~30% of the chronically infected patients develop a progressive form of CM that may lead to death due to congestive heart failure and/or arrhythmia (23). Although the pathological features of CM are heterogeneous, severe manifestations are characterized by presence of myocarditis, fibrosis, thromboembolic injuries, and cardiomegaly (23). Pathological studies in patients and experimental models support the concept that parasite persistence (low-grade) in the myocardial tissues is the primary determinant of CM (24). Genetic studies in CM cohorts suggest that patients exhibiting lower frequencies of IL-10–producing monocytes (25), IL-10–producing double-negative Y6 T cells (26), FoxP3+ CD25+ regulatory T cells (27) and IL-17–producing T cells (28) are more susceptible to chronic myocarditis, most likely reflecting inefficient regulation of heart-infiltrating T cells (26), FoxP3+ CD25+ regulatory T cells (27) and IL-17–producing T cells (28) (29, 30).

Beyond immunoregulatory dysfunctions, it is still unclear why patients with severe CM present conspicuous microvascular derangement in the myocardium (31). Studies in chronically infected mice have linked adverse cardiac remodeling to upregulated expression of the potent vasoconstrictor endothelin-1 in paralyzed cardiomyocytes (32). Research on the kallikrein–kinin system also linked pathogenic outcome to infection-associated microvascular responses (33). Early studies showed that T. cruzi trypomastigotes proteolytically liberate kinins from an internal moiety of high-m.w. kininogens (HKs) through the activity of cruzipain (34–38), a lysosomal cysteine protease that was initially characterized as a therapeutic target of Chagas disease (39). Once released in parasite-laden tissues, the short-lived kinins (and their metabolites) activate adjacent microvascular beds, inducing plasma extravasation through the activation of distinct subtypes of G-protein–coupled bradykinin (BK) receptors expressed at the endothelium: BK B2 receptor (B2R; constitutively expressed) and BK B1 receptor (B1R; upregulated in injured/inflamed tissues) (37, 38).

The concept that activation of the kinin system may translate into mutual benefits to the host/parasite relationship (33) is supported by several lines of evidences. Briefly, we have previously shown that kinins, acting cooperatively with endothelins, stimulate parasite invasion of a broad range of nonphagocytic cells, including cardiomyocytes, through the cross-talk between B2R/endothelin receptors (36, 37, 40). Beyond contributing to parasite virulence, BK acts as a Th1-directing adjuvant of endogenous origin by stimulating IL-12 production by DCs via B2-R (41, 42). Consistent with this view, studies in B2R-deficient mice revealed that these transgenic animals succumbed to acute T. cruzi infection, the susceptible phenotype being ascribed to DC dysfunction and impaired T cell (type 1) effector development (42).

In this study, we report evidence that C5a and kinins are released in peripheral sites of T. cruzi infection. Forging an interface with the microvascular system, the interstitial edema driven via the C5aR/B2-R pathway integrates the proinflammatory complement and kinin cascades to the cytokine circuitry that coordinates innate/adaptive immunity in T. cruzi–infected mice.

**Materials and Methods**

**Mice**

Experiments were conducted with male BALB/c, A/J, C57Bl/6 wild-type (WT; B2 R−/−) and C57Bl/6 B2 R−/− mice (43), weighing 25–30 g. The experiments reported in this study were made in accordance with the guidelines of the Brazilian College of Animal Experimentation, strictly followed the Brazilian law for Procedures for the Scientific Use of Animals (11.794/2008), and were approved by ethics committee of the Federal University of Rio de Janeiro (License IBCCF 101).

**Parasites**

The Dm28c strain of T. cruzi was used in all experiments. Epimastigotes were cultured at 28°C in standard liver infusion tryptose medium containing 10% heat-inactivated FBS. Metacyclogenesis was done by incubating stationary-phase epimastigotes in Grace's medium (pH 5.5) for 7 d at 28°C. Tissue culture trypomastigotes (TCTs) were harvested from the supernatants of infected monkey kidney fibroblast cell line LLC-MK2 in DMEM supplemented with 2% FBS (HyClone, Ogden, UT). Suspension of freshly released parasites were washed twice with excess of PBS before being tested either in vivo or in vitro.

**C5a release assays**

We measured the concentrations of C5a in reaction mixtures of human C5 and cruzipain, or TCT suspensions, by ELISA using mAb561 as the capture mAb and biotinylated mAb557 for detection, as described (44). Recombinant human C5a (Sigma-Aldrich, St. Louis, MO) was used as standard. After activating stock solutions of Dm28c-purified cruzipain (5 μM) with 2.5 mM DTT, the mixture was incubated with C5 (15 μM) in 250 μl reaction buffer containing 50 mM Na2HPO4 (pH 6.5), 200 mM NaCl, and 0.25 mM EDTA and C5a release was quantified as indicated above. Experiments were done with enzyme preincubated with 30 μM E-64 (1-trans-epoxysuccinyl- l-leucylamido-(4-guanidino) butane]; Sigma-Aldrich). Controls were performed with enzyme preincubated with 30 μM E-64. The C5a-releasing activity of trypomastigotes was measured after incubating 15 μM C5 with TCTs (1 × 10⁷ parasites) in 250 μl Ham’s F12, 12.5 mM HEPES, and 1 mg/ml BSA (pH 6.5), for 30 min at 37°C. As a control, TCTs were preincubated with 30 μM E-64 for 30 min at 37°C before adding the C5. The reaction was stopped by adding 30 μM E-64 (final concentration of 30 μM) to the cell suspension. Parasites were centrifuged at 3000 rpm for 10 min, the supernatants were filtered through 0.2-mm Millipore filters, and C5a release was quantified as indicated above. Experiments were done in triplicates.

**Isolation and cultivation of peritoneal macrophages**

Mouse macrophages (Mφ) were harvested from peritoneal lavage and plated on 13-mm round coverslips. The adherent Mφ were obtained after 18 h of incubation of single-cell suspension in 24-well tissue-culture plates at 37°C in complete medium (RPMI 1640 + FBS [10%] plus 100 U/ml penicillin and 100 μg/ml streptomycin). The nonadherent cells were removed by washing with complete medium. Invasion assays were performed by adding the parasites to the wells in a final volume of 350 μl/well containing 10% fresh FBS in RPMI 1640/DMEM (1:1), containing 5% fresh FBS (35). The ratio (ratio of 2:1 parasite/host cell), the culture medium was supplemented, or not, with 100 nM C5aR/C5aR2 antagonist (C5aRA) A85787 (45) and/or 100 nM B2R antagonist (HOE-140). In separate wells, the RPMI 1640–5% FBS medium was either supplemented with the C5aR1-neutralizing Ab mAb 20/70 (Hyульт Biotechnology) or with rat IgG2b isotype control (BD Biosciences), used at a final concentration of 10 μg/ml. After incubating the TCT/Mφ cultures for 2 h at 37°C in a humidified chamber containing 5% CO2, the TCT were removed from the wells, and the monolayers were washed three times with HBSS before being fixed with Bouin and stained with Giemsa. For assessment of intracellular parasite growth/survival in Mφ, the monolayers were washed at the end of the 2-h interaction period, and the cultures were incubated with complete medium for another 24 h. Infection rate was quantified by counting the number of intracellular parasites (2 h versus 24 h) in a total of 100 Mφ/coverslip. Values represent means ± SD of three independent experiments, each done in triplicate under blinded conditions. Keratinocyte-derived chemokine (KC) determination were made in culture supernatants harvested (2 h) and stored
at −20°C. The levels of KC in the supernatant of MØ cultures were quantified using commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, MN), as specified by the manufacturer. All assays were done in triplicates. The detection limit of the assays was 15.6 pg/ml. Culture supernatants were harvested 24 h later and stored at −20°C for determinations of secretion levels of TGF-β and NO. The levels of TGF-β in the supernatant of MØ cultures were quantified using commercially available ELISA kits (Duoset; R&D Systems), as specified by the manufacturer. All assays were done in triplicates. Nitrite was determined in culture supernatants using Griess reagent with a standard curve of 0.8–100 mM NaNO₃ (46).

**Determination of edema**

Measurements of paw edema induced by s.c. injection of Dm28c TCT (1 × 10⁶) in BALB/c, C57BL/6, or A/J mice were done as previously described (37). Before parasite challenge, separate groups of mice were pretreated (−40 min) with a single dose i.p (4 mg/kg) of captopril (Sigma-Aldrich), an inhibitor of the angiotensin-converting enzyme (ACEi). The role of the kinin/B₂R pathway was investigated by injecting the specific antagonist HOE-140 (100 μg/kg) s.c. 1 h before injecting in the pathogen. In separate groups of mice, we investigated the influence of C5aR and C3aR by coinjecting the TCT in a PBS suspension containing 1 μM C5aRA or C3aRA (47). Edema responses were measured using a plethysnometer 3 h after the onset of infection. The differences between edema volumes (test versus contralateral paws injected with PBS) were measured as microliters.

**Intravitral digital microscopy in the hamster cheek pouch**

Hamsters were anesthetized by i.p. injection of sodium pentobarbital supplemented with i.v. α-chloralose (2.5% w/v, solution in saline) through a femoral vein catheter. A tracheal canula (PE 190) was inserted to facilitate spontaneous breathing, and the body temperature was maintained at 37°C by a heating pad monitored with a rectal thermometer. The hamster cheek pouch (HCP) was prepared for intravitral microscopy as previously reported (48, 49). The microcirculation of the HCP was observed using an Axioskop 40 microscope, objective 4× and oculars 10× (Carl Zeiss) equipped with appropriate filters for observations of fluorescence of FITC-dextran 150 kDa (TdB Consultancy, Uppsala, Sweden) as epiluminescence. A digital camera, AxioCam HRc, and a computer with the AxioVision 4.4 software program (Carl Zeiss) was used for image analysis of total fluorescence in a representative rectangular area (5 mm²) of the prepared HCP. The recorded fluorescence at 30 min after FITC-dextran injection in each experiment was adjusted to 2000 relative fluorescent units (RFU) for statistical reasons. Images were recorded at 5-min intervals during the entire experiment, and fluorescence of the observed area (5 mm²) was used to measure plasma leakage expressed as RFU. HCPs were topically exposed during microscopic observation to superfusion with either saline (controls) or TCT. One of the experimental groups was treated with C5aRA (1 μM) added to the superfusion solution 5 min prior to TCT application.

**In vitro activation of CD11c+ DCs**

Spleenic DCs were isolated with anti-CD11c magnetic beads (Miltenyi Biotec) as previously reported (42). Such DCs were then stimualted in vitro with TCT (3 × 10⁵/well) in DMEM/10% FBS for 18 h at 37°C in the presence of or absence of 25 μM lisinopril (Sigma-Aldrich), an inhibitor of the ACEi, and/or 25 μM HOE-140 or C5aRA, as indicated. Controls were done with 10 nM BK. For intracellular staining of IL-12, 10⁶ DCs were washed and preincubated with 2% of normal mouse serum (NMS) supplemented with anti-mouse CD16/CD32 (clone 2.4G2; BD Biosciences). The washed cells were stained with anti-mouse CD11c-FITC (BD Biosciences) in PBS/2% NMS. After washing (2× PBS), the cells were fixed in 2% paraformaldehyde, washed, and permeabilized with 0.05% saponin (Sigma-Aldrich). Staining with PE-labeled anti-IL-12 p40/p70 (BD Biosciences) was performed in PBS/2% NMS/0.5% saponin. Samples were acquired by flow cytometry (FACS Calibur; BD Biosciences), and data analyses were done with CellQuest software (BD Biosciences). After stimulation, culture supernatants were harvested 18 h later and stored at −20°C for TNF-α and IL-6 determination. The levels of these cytokines were quantified using commercially available ELISA kits (Duoset; R&D Systems), as specified by the manufacturer. All assays were done in triplicates.

**Cytokine production by T cells from infected mice**

Mouse DLNs were collected from infected or naive mice 30 d postinfection (p.i.), and single-cell suspensions were prepared from DLNs. Single-cell suspensions were suspended in DMEM with penicillin/streptomycin and 10 mM 2-ME. DLN cells (5 × 10⁵/well) were stimulated with 25 pg/ml boiled soluble T. cruzi epimastigote Ag. The culture supernatants were collected after 72 h, and the concentrations of IFN-γ, IL-4, and IL-10 were quantified by ELISA (R&D Systems).

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA). With regard to the small sample sizes, normal distribution was assumed. Comparisons of the means of the different groups were done by either unpaired t test or one-way ANOVA. When the mean values of the groups showed a significant difference, pairwise comparison was performed with the Tukey test. A p value <0.05 was considered significant.

**Results**

**Functional interplay between C5aR and B₂R in T. cruzi–induced inflammation**

In previous studies, we demonstrated that TCT (Dm28 strain) induce inflammatory edema in mice through the sequential activation of TLR2, CXCR2, B₂R, and ETA/EETR (38, 40, 48). Using intravitral microscopy, we now asked whether C5aRA could inhibit plasma leakage otherwise evoked by the topical application of TCT to the stroma of the HCP. As previously reported (38, 40), we found that TCT evoked significant plasma leakage through postcapillary venules, peaking 10–15 min after topical administration to the HCP (Fig. 1A). In contrast, we found that C5aRA treatment virtually abolished TCT-evoked plasma extravasation (p < 0.05). Next, we studied the functional interplay between C5aR and B₂R in the mouse model of s.c. T. cruzi infection. As predicted, we found (Fig. 1B) that TCT induced a vigorous paw edema response in BALB/c mice (3 h p.i.), and the inflammation was reverted in animals pretreated with the B₂R antagonist HOE-140 (Fig. 1B). Consistent with the observations made in the HCP, we found that C5aRA treatment (local application) almost completely abolished the inflammatory edema elicited by TCT (Fig. 1B). We then tested whether C5aRA or HOE-140 treatment has an impact on T. cruzi–induced edema in mice pretreated with captopril, an inhibitor of kinin degradation by ACEi. As we previously reported (48), experiments performed in ACEi-treated BALB/c mice (Fig. 1B) showed that HOE-140 reverted the TCT-induced swelling. Intriguingly, C5aRA treatment resulted in an inhibitory effect that was even stronger than that mediated by HOE-140 (Fig. 1B). Because the infection-associated swelling response is weaker in the C57BL/6 mouse strain (38), the effects of HOE-140 (internal control) and C5aRA were studied in ACEi-treated C57BL/6 mice. Under these conditions, the inflammatory edema was significantly reduced by both antagonists (Fig. 1C). Next, we examined the outcome of TCT injection in the footpad of C5-deficient A/J mice (50). Interestingly, TCT failed to evoke discernible paw edema in A/J mice in the absence of captopril (Fig. 1D). Although we have not investigated the role of TLR2 as sensors of TCT in A/J mice, these results suggest that innate immunity (TLR2/CXCR2-driven pathway) may not efficiently couple to the kinin proteolytic cascade system (38, 48) in the absence of functionally intact C5. Noteworthy, although the microvascular beds of A/J mice are less sensitive to TCT as compared with BALB/c, the microvascular reactivity was rescued upon systemic administration of ACEi (Fig. 1D). Similar to the effects observed in other mouse strains, we found that HOE-140 blocked the paw edema in infected A/J mice (Fig. 1D), implying that vasoactive kinins (B₂R agonists) are also formed independently of activation of the C5a/C5aR axes. Of note, C5aRA blunted the footpad edema induced in ACEi-treated A/J mice (Fig. 1D), suggesting that complement activation in peripheral sites of infection induces edema in the C5-deficient A/J mice through the local release of proinflammatory C5a and kinins.
Collectively, these studies suggest that C5a or C3a (A/J mice), acting cooperatively with kinins, propagate the inflammatory edema elicited by Dm28 TCT through joint engagement of C5aR, C3aR, and B2R.

C5a generation from human C5 released by cruzipain and T. cruzi trypomastigotes

Previously, we have reported that T. cruzi trypomastigotes liberate lysyl-BK from HK bound to cell-surface via heparan sulfate (36). Studies in the s.c. model of infection in C57BL/6 mice revealed that TCT evoke neutrophil-driven influx of plasma (including HK and complement components) into peripheral tissues through the activation of innate sentinel cells via the TLR2/CXCR2 pathway (38, 48). In this study, we addressed the possibility that TCT may rely on cruzipain to generate C5a in inflammatory exudates. To this end, we added native C5 to a suspension of TCT and measured the levels of immunoreactive C5a by ELISA. Our results (Fig. 2A) showed that TCT released significant amounts of C5a at 30 min (Fig. 2A). However, there was no appreciable release of C5a before 15 min, suggesting that the N-terminal excision of the C5a polypeptide may depend on additional processing of native C5 by parasite proteases. As the incubation proceeded, the levels of C5a sharply decreased, suggesting that the liberated C5a polypeptide is further degraded. We then added the irreversible cysteine protease inhibitor E-64 to the TCT suspension and found that it abolished the C5a-generating activity (Fig. 2A). Next, we checked whether purified cruzipain could directly release C5a from native C5. Indeed, we found that cruzipain released immunoreactive C5a from native C5 (Fig. 2B) and the reaction was inhibited by E-64 (Fig. 2B). Together, these in vitro studies suggest that TCT (Dm28 strain) exploit the enzymatic versatility of cruzipain to release vasoactive kinins and proinflammatory C5a peptides in inflammatory exudates.

Macrophage susceptibility to T. cruzi infection is modulated via activation of C5aR1 and B2R

Turning to in vitro cellular systems, we then asked whether C5aR1 and B2R were involved in the phagocytic uptake of TCT by resident (peritoneal) M$\phi$. To this end, we first added C5aRA to serum-containing medium and monitored the extent of TCT internalization after 2 h incubation with WT M$\phi$ (C57BL/6). Interestingly, we found that C5aRA treatment markedly reduced TCT uptake by WT (B2R$^{+/+}$) M$\phi$ (Fig. 3A; $\sim$70% inhibition; $p < 0.01$). Moreover, we found that HOE-140 also markedly reduced the pathogen uptake by WT (B2R$^{+/+}$) M$\phi$ (Fig. 3A). Consistent with these pharmacological data, we found that phagocytosis was also hampered in B2R$^{-/-}$ M$\phi$ (Fig. 3A; filled bars; 72%; $p < 0.01$). In contrast to the C5aRA-mediated inhibition of phagocytic uptake observed in cultures of WT B2R$^{+/+}$ M$\phi$ (Fig. 3A), addition of this GPCR blocker did not reduce the baseline levels of parasite uptake by B2R$^{-/-}$ M$\phi$ (Fig. 3C). These results suggest that resident M$\phi$ deficient in B2R cannot efficiently internalize TCT via activation...
Interestingly, however, we found that WT M \( \phi \) revealed that the parasite burden was 2-fold higher (Fig. 3B) than that were initially infected in the presence of C5aRA or C5aR1-∅ cultures (2-fold increase; Fig. 3B versus 3A). HOE-140, which was previously mentioned, B2R\(^{-/-}\) M \( \phi \) showed reduced phagocytic uptake of TCT (Fig. 3C). Similarly to the permissive phenotype of HOE-140-treated WT (B2R\(^{-/-}\) M \( \phi \)) (3.9-fold increase, Fig. 3A, 3B), we found that the intracellular amastigote outgrowth increased (3.3-fold; Fig. 3C versus 3D) in B2R\(^{-/-}\) M \( \phi \) (24 h), further indicating that B2R signaling limits intracellular parasitism. Noteworthy, unlike the phenotype of C5aRA-treated WT M \( \phi \) (Fig. 3B versus 3A), the addition of C5aRA to B2R to B2R\(^{-/-}\) M \( \phi \) during the initial 2-h long interaction with TCT did not change the outcome of intracellular infection (Fig. 3D versus 3C). Thus, these observations suggest that C5aR1 was not able to revert the permissive phenotype of B2R-deficient M \( \phi \).

To further investigate the impact of C5aR blockade on the outcome of M \( \phi \) infection, we then measured the levels of NO and TGF-\( \beta \) in the supernatants of these cultures. Consistent with the permissive phenotype of C5aRA or HOE-140–treated WT M \( \phi \), we found that NO production was almost completely abolished in response to C5aRA or HOE-140 treatment (Fig. 3E). Conversely, both C5aRA and HOE-140 rescued production of TGF-\( \beta \) (Fig. 3F), a regulatory cytokine for which secretion was lowered in M \( \phi \) cultures exposed to TCT (51). Combined, these studies indicate that functional engagement of C5aR and B2R at an early stage of TCT interaction upregulates NO production while downmodulating TGF-\( \beta \) secretion by resident M \( \phi \).

Chemokines such as KC are potent chemoattractants of innate immune cells. In previous studies, we showed that Dm28 TCTs induce KC secretion via TLR2. Interestingly, we have previously shown that C5aR1 regulates TLR2-driven cytokine production from spleen-derived DCs (12). To investigate whether C5a might induce KC secretion via TLR2/C5aR1, we incubated TCT with BALB/c elicited M \( \phi \) during 2 h in the presence or absence of C5aRA. The results showed that KC responses remained high in the presence of C5aRA, implying that C5a is not required for KC upregulation in the in vitro settings (Fig. 3G). Along similar lines, KC production remained high in A/J-stimulated M \( \phi \), indicating that endogenous production of C5 is dispensable for KC induction (Fig. 3G). Taken together, these results suggest that C5aR and B2R, acting cooperatively, promote the coupling of at least two essential innate effector functions of M \( \phi \) in T. cruzi infection: phagocytosis and intracellular activation of trypanocidal mechanisms.

C5aR plays a role on DC cell function and T cell effector development in T. cruzi–infected mice

In a previous study, we demonstrated that TCT (Dm28 strain) rely on the kinin-releasing activity of cruzipain to activate immature CD11c\(^{+}\) DCs (splenic origin) via B2R (42). In this study, we sought to determine whether the C5a/C5aR axes were likewise engaged in the DC activation response. To this end, splenic CD11c\(^{+}\) DCs (B6 mice) were cocultured with TCT (Fig. 4A). Control experiments showed that BK (10 nM) induced IL-12p40/70 (Fig. 4A, bottom panel). TCT induced similar IL-12p40/70 responses in DCs irrespective of ACEi treatment. Of note, IL-6 and TNF-\( \alpha \) production were not affected by C5aRA treatment (Fig. 4B). In light of our findings of the C5a/C5aR axes. Collectively, these initial studies suggested that C5a and kinins, presumably released pericellularly, potentiate the phagocytic internalization of TCT by resident WT M \( \phi \) via cross-talk between C5aR and B2R. Considering that C5aRA acts as a potent antagonist of both C5aR1 and C5aR2 (45), we then checked whether C5aR1 played a role in the phagocytic uptake of TCT by adding a C5aR1-specific neutralizing Ab to the M \( \phi \) cultures. Our results (Fig. 3A) revealed that the C5aR1-neutralizing mAb blocked TCT internalization by resident M \( \phi \) to the same extent as the pharmacological treatment with C5aRA.

Next, we investigated whether the functional blockade of C5aR1 or B2R at the onset of WT M \( \phi \) infection (2-h interaction) could influence macrophage susceptibility to intracellular amastigote outgrowth. After 24-h incubation, the inspection of the number of intracellular parasites in control WT (B2R\(^{-/-}\) M \( \phi \)) cultures revealed that the parasite burden was 2-fold higher (Fig. 3B) than the number of TCT initially (2 h) internalized by these phagocytes (Fig. 3A). Interestingly, however, we found that WT M \( \phi \) cultures that were initially infected in the presence of C5aRA or C5aR1-specific neutralizing Ab (2 h) became far more permissive to intracellular parasite outgrowth (in both cases, ~5.5-fold increase; Fig. 3B versus 3A) as compared with control infected WT M \( \phi \) cultures (2-fold increase; Fig. 3B versus 3A). HOE-140, which also reduced the phagocytic uptake in the first 2 h (Fig. 3A), similarly favored the outgrowth of intracellular amastigotes at 24 h p.i. (3.9-fold increase; Fig. 3A versus 3B). Given indications that C5aR1/B2R engagement at early stages of TCT/M \( \phi \) interaction increased host resistance to infection, we next asked whether the innate effector functions of C5aR1 were preserved, or not, in B2R-deficient M \( \phi \). As previously mentioned, B2R\(^{-/-}\) M \( \phi \) outgrowth. After 24-h incubation, the inspection of the number of TCT initially (2 h) internalized by these phagocytes revealed that the parasite burden was 2-fold higher (Fig. 3B) than that were initially infected in the presence of C5aRA or C5aR1-∅ cultures (2-fold increase, Fig. 3A, 3B), we found that the intracellular amastigote outgrowth increased (3.3-fold; Fig. 3C versus 3D) in B2R\(^{-/-}\) M \( \phi \) (24 h), further indicating that B2R signaling limits intracellular parasitism. Noteworthy, unlike the phenotype of C5aRA-treated WT M \( \phi \) (Fig. 3B versus 3A), the addition of C5aRA to B2R to B2R\(^{-/-}\) M \( \phi \) during the initial 2-h long interaction with TCT did not change the outcome of intracellular infection (Fig. 3D versus 3C). Thus, these observations suggest that C5aR1 was not able to revert the permissive phenotype of B2R-deficient M \( \phi \).

To further investigate the impact of C5aR blockade on the outcome of M \( \phi \) infection, we then measured the levels of NO and TGF-\( \beta \) in the supernatants of these cultures. Consistent with the permissive phenotype of C5aRA or HOE-140–treated WT M \( \phi \), we found that NO production was almost completely abolished in response to C5aRA or HOE-140 treatment (Fig. 3E). Conversely, both C5aRA and HOE-140 rescued production of TGF-\( \beta \) (Fig. 3F), a regulatory cytokine for which secretion was lowered in M \( \phi \) cultures exposed to TCT (51). Combined, these studies indicate that functional engagement of C5aR and B2R at an early stage of TCT interaction upregulates NO production while downmodulating TGF-\( \beta \) secretion by resident M \( \phi \).

Chemokines such as KC are potent chemoattractants of innate immune cells. In previous studies, we showed that Dm28 TCTs induce KC secretion via TLR2. Interestingly, we have previously shown that C5aR1 regulates TLR2-driven cytokine production from spleen-derived DCs (12). To investigate whether C5a might induce KC secretion via TLR2/C5aR1, we incubated TCT with BALB/c elicited M \( \phi \) during 2 h in the presence or absence of C5aRA. The results showed that KC responses remained high in the presence of C5aRA, implying that C5a is not required for KC upregulation in the in vitro settings (Fig. 3G). Along similar lines, KC production remained high in A/J-stimulated M \( \phi \), indicating that endogenous production of C5 is dispensable for KC induction (Fig. 3G). Taken together, these results suggest that C5aR and B2R, acting cooperatively, promote the coupling of at least two essential innate effector functions of M \( \phi \) in T. cruzi infection: phagocytosis and intracellular activation of trypanocidal mechanisms.

C5aR plays a role on DC cell function and T cell effector development in T. cruzi–infected mice

In a previous study, we demonstrated that TCT (Dm28 strain) rely on the kinin-releasing activity of cruzipain to activate immature CD11c\(^{+}\) DCs (splenic origin) via B2R (42). In this study, we sought to determine whether the C5a/C5aR axes were likewise engaged in the DC activation response. To this end, splenic CD11c\(^{+}\) DCs (B6 mice) were cocultured with TCT (Fig. 4A). Control experiments showed that BK (10 nM) induced IL-12p40/70 (Fig. 4A, bottom panel). TCT induced similar IL-12p40/70 responses in DCs irrespective of ACEi treatment. Of note, IL-6 and TNF-\( \alpha \) production were not affected by C5aRA treatment (Fig. 4B). In light of our findings...
FIGURE 3. Complementary roles of C5aR1 and B2R in macrophage infection in vitro. Parasite invasion was performed with resident MØ from C57BL6 B2R<sup>+/+</sup> (A) or B2R<sup>−/−</sup> (C) mice in medium containing 5% of fresh FBS in the presence or absence of 100 nM of C5aRA, 10 µg/ml C5aR1-specific mAb (clone 20/70), or an isotype-matched control rat IgG or 100 nM HOE-140. TCT were added at a ratio of 2:1 (parasite versus cell), and the interaction proceeded for 2 h at 37˚C. (B and D) For assessment of intracellular survival of <i>T. cruzi</i>, MØ were infected with TCT and washed thoroughly after 2 h to remove extracellular parasites and the GPCR blockers before being incubated in complete medium for 24 h. After fixing and staining with Giemsa, the number of intracellular amastigotes in the coverslips was counted. Data (means ± SD) represent the number of parasites internalized in 100 host cells; assays were run in triplicates and represent four independent experiments. (E) NO production in supernatants measured at 24 h p.i. (F) TGF-β secretion in culture supernatants (24 h p.i.). Data represent the means ± SD of triplicates and represent at least three different experiments. (Figure legend continues)
suggesting that TCT activate CD11c+ DCs via C5aR and/or B2R, we evaluated whether treatment with their respective antagonists interferes with effector T cell development in vivo. Briefly, we injected Dm28c TCT in the footpad of BALB/c mice pretreated or not with HOE-140 or C5aRA. Analysis of recall responses by LN cells of chagasic mice (30 d p.i.) demonstrated that IFN-γ levels were low, albeit significantly higher as compared with noninfected animals (Fig. 5A). In contrast, the IFN-γ response of LN cells was essentially abolished in BALB/c mice coinjected (s.c.) with TCT and C5aRA (Fig. 5A). Conversely, the injection of C5aRA strongly upregulated IL-4 responses in T. cruzi-infected mice (Fig. 5B). Interestingly, HOE-140 also reduced IFN-γ production by experienced T cells, but unlike C5aRA, the B2R antagonist did not upregulate IL-4 responses (Fig. 5A, 5B). IL-17 responses were below detection range in all experimental settings (data not shown). We then checked whether C5aR/B2R modulated IL-10 responses in T. cruzi-infected mice. ELISA measurements revealed that IL-10 secretion was substantially reduced in infected mice pretreated with HOE-140, but not with C5aRA (Fig. 5C).

**Discussion**

LNs are secondary lymphoid tissues comprising DCs specialized in sampling and immune discrimination of pathogens while being at the same time exposed to lymphatic drainage of fluids and cells accumulating in injured/inflamed tissues. In the current work, we have demonstrated that neutrophil-driven plasma leakage, a peripheral microvascular response elicited by T. cruzi trypomastigotes via the TLR2/CXCR2 pathway (38, 48), is further amplified by C5a and BK, which are two major split products derived from the proteolytic activation of the complement and kinin cascades. Transduced by their cognate GPCRs (i.e., C5aR/B2R), the activation signals relayed by C5a and BK provide a bridge between the microvascular system and innate/adaptive immunity. In previous studies, we have dissected the activation pathways underlying activation of the kinin system in peripheral tissues exposed to Dm28c TCT. Based on multiple evidences, we demonstrated that the generation of kinins was preceded by neutrophil-dependent influx of plasma-borne proteins, including kininogens, into parasitemladen tissues. Analysis of the roles of neutrophils/macrophages

![FIGURE 4. C5aR signaling is required for TCT-induced IL-12p40/70 responses of CD11c+ DCs. Cytokine production was determined after isolation of CD11c+ DCs from the spleen of C57BL/6 mice. Splenic DCs were cultured in medium containing 10% of FBS in the presence or absence of 100 nM of C5aRA or HOE-140. TCTs were added at a ratio of 5:1 (parasite versus cell), and the interaction proceeded for 18 h at 37°C. Numbers in the top right quadrants indicate the percentage of CD11c+IL-12p40/70+ cells. (A) After administration of the stimulus, cells were double stained by surface CD11c FITC and intracellular IL-12p40/70 PE. The results shown in each panel are representative of two experiments. Secretion of TNF-α (B) and IL-6 (C) were measured in the supernatants of cell cultures. Data represent the means ± SD of triplicates and three independent experiments. *p < 0.05 (ANOVA).](http://www.jimmunol.org/)
in the upstream end of the inflammatory cascade elicited by TCT has linked microvascular leakage to the upregulated secretion (TLR2-dependent) of CXC chemokines (KC/MIP-2) by the infected macrophages (48). Rapidly expanded via secretion of CXC chemokines, the CXCR2-dependent transendothelial leakage leads to the rapid accumulation of plasma-borne kininogens and intact complement components in parasite-laden tissues. Once released from kininogens, the primary kinins (B2R agonists) and their metabolites (B1R agonists) further increase the interstitial edema in parasite-laden tissues by sequentially activating the constitutively expressed endothelial B2R and the inducible B1R (33, 37). In the current study, we provided evidence that the proteolytic phase of the inflammatory edema evoked by TCT depends on the joint activation of G-protein coupled C5aR, C3aR and B2R by their respective ligands (i.e., complement anaphylatoxins [C3a and C5a]) and vasoactive kinins. Experiments performed in s.c.-infected mice revealed that ACEi (captopril) potentiated the footpad edema via the B2R/C5aR pathway. These results imply that C3a, acting cooperatively with the released kinins, might fuel the infection-associated inflammatory response, hence compensating for the deficient production of C5a anaphylatoxins in A/J mice. It remains to be determined whether the hyperreactivity of the microvasculature of infected A/J mice pretreated with captopril only reflects upregulated ACE activity and/or results from enhanced activation of the kinin/endothelin pathway (40, 52) in the C5-deficient strain.

Given that the inflammatory edema was measured shortly after TCT injection in naive mice, we may surmise that generation of C5a and C3a resulted from complement activation by Ab-independent mechanisms. Although T. cruzi calreticulin is able to circumvent C1q-dependent activation of the complement system (53), it is plausible that soluble complement-sensing molecules such as mannan-binding lectin, the ficolins, or properdin (54, 55) might overcome this subversion strategy through the binding of conserved microbial motifs displayed by Dm28c TCT, such as the highly polymorphic GPI-linked surface mucins and/or trans-sialidase Ags (56). Alternatively, it is possible that plasma kallikrein–dependent activation of plasmin may couple the kallikrein–kinin pathway to plasmin-driven activation of C3, ultimately generating C3a or C5a at the downstream end of the inflammatory cascade (57, 58). Although these fluid-phase processes may account for the bulk of anaphylatoxin and BK production during the ascending phase of inflammation, it must be pointed out that these

![FIGURE 5. C5aR and B2R activation drives Th1 responses in mice s.c. infected with T. cruzi. BALB/C mice were inoculated with T. cruzi. TCT and DLN cells were recovered 30 d.p.i. IFN-γ (A), IL-4 (B), and IL-10 (C) production by bulk DLN cells was determined 72 h after restimulation with T. cruzi Ag. The values of recall responses are represented as means ± SD of the cytokine concentrations in culture supernatants from three mice per group. Data are representative of three independent experiments. *p < 0.05, **p < 0.01 (ANOVA).]
short-lived complement peptides can also be generated by cellular mechanisms. For example, activated alveolar macrophages rely on a serine protease to cleave C5 into C5a (59). Similarly, neutrophils and lymphocytes exert this task via secretion of the serine protease granzyme B (60) and cathepsin L (61). Beyond the role of host serine proteinases, the list of C5-cleaving enzymes expressed by pathogens is rapidly growing, including serine proteinases from the house dust mite Dermatophagoides farinae (62), gingipains (cysteine protease) from the periodontal bacteria Porphyromonas gingivalis (63, 64), and a serine proteinase from Aeromonas sobria that produces C5a at the site of infection or in the circulation (65).

Our in vitro studies showed that TCT liberate C5a from native C5 in a cruzipain-dependent manner. Although the kinetics of the processing reaction was rather slow, our previous studies of cruzipain-mediated processing of HK offered an interesting precedent of a GPCR agonist (lysyl-BK) that is efficiently released by cruzipain in vivo, but only when HK is bound to cell-surface heparan sulfates (35, 36).

In keeping with the notion that the expression of B1R is upregulated in injured/inflamed tissues (66), we have previously documented that the infection-associated edema in B1R-deficient mice is resolved earlier than WT chagasic mice (37). More recently, we observed that heart tissues of chronically infected B1R-deficient mice are virtually free from inflammatory infiltrates and the associated fibrosis, thus linking activation of the B1R pathway to the development of chronic chagasic myocardopathy (D. Andrade, A.C.S. Oliveira, C. Nascimento, E. Svenjo¨, and J. Scharfstein, unpublished observations). It remains to be determined whether des-Arg-kinins may drive cardiac tissue remodeling in Chagas disease (67) via cross-talk between B1R and C5aR1 (D. Andrade, A.C.S. Oliveira, C. Nascimento, E. Svenjo¨, and J. Scharfstein, unpublished observations).

In previous studies, we demonstrated that T. cruzi exploits the kinin-releasing activity of cruzipain to potentiate the invasion of nonphagocytic host cells (human smooth muscle cells, endothelial cells, and cardiomyocytes) that naturally overexpress B1R (36) and/or B2R (37). Extending these studies to macrophages, in this study, we showed evidence that activation of C5aR1/B2R at early stages of TCT/M∅ interaction profoundly influences the phagocytic response as well as the subsequent outcome of intracellular parasitism. Whether using resident M∅ from genetically deficient mice (B1R−/−) or employing pharmacological tools (HOE-140, C5aRA, or a neutralizing Ab to C5aR1), our studies revealed that the phagocytic uptake of TCT by WT-M∅ is strongly potentiated as result of the activation of the C5aR1/B2R pathway.

Remarkably, although the WT M∅ were only briefly exposed to C5aRA (strictly during the short-term period of TCT interaction), we found that the C5aR blockade: 1) virtually turned off NO production by the infected M∅; 2) reciprocally increased TGF-β production; and 3) upregulated the outgrowth of intracellular parasites (amastigotes). The demonstration that activation of the C5aR/B2R pathways at early stages of T. cruzi/M∅ interaction (including during phagocytosis) may suppress TGF-β production, whereas reciprocally upregulating NO synthesis offers new clues to investigate the well-established functional connection between NO upregulation in M∅ to innate resistance against T. cruzi infection (68). Although speculative, it is possible that sustained C5aR-driven NO production by M∅ might fuel microbicidal responses by rescuing the transcriptional factor NF-κB from inactivation by cruzipain (69, 70).

It is well established that C5aR1 signaling can modulate innate and adaptive immune response both at the APC and T cell levels (61, 71). It has previously been shown that C5a negatively modulates synthesis of IL-12, IL-23, and IL-27 induced by activation of TLR4 on M∅ (72, 73). In this case, C5aR1-mediated signaling results in anti-inflammatory pathways, which are in apparent contrast to the many proinflammatory properties of C5a. In line with such findings, C5a decreased the synthesis of IL-12 induced by LPS or Staphylococcus aureus strain Cowan-1 in human monocytes but not in human DCs derived from monocytes (74, 75). In contrast, C5 deficiency in murine M∅ decreases the production of IL-12 in response to stimulation with S. aureus strain Cowan-1 plus IFN-γ, suggesting that C5a can increase the production of IL-12 (76).

This data indicate that the role of C5a in the regulation of IL-12 production varies depending on the cell type studied and the stimulus that drives IL-12 production. Recent reports have highlighted the participation of complement components during early stages of effector T cell differentiation in response to DC activation. In this context, it has been demonstrated that both DCs and T cells produce complement components including C3, C5, and factors B and D as wells C3aR and C5aR1 (7, 11). Further, Weaver et al. (12) showed that C5aR1-deficient DCs express lower cell-surface levels of MHC class II and costimulatory (CD40 and CD86) molecules as compared with WT DCs. In addition to pathogen-derived danger molecules, DCs can sense danger signals via receptors for endogenous proinflammatory mediators, such as ATP, uric acid (77), and BK (41, 42). During cognate interaction of DC and T cells, the mature APCs deliver Th-polarizing cytokines, such as IL-12p70, to the T cell, which is critical for Th lineage commitment. In the current work, we showed that C5aRA inhibited the production of IL-12p40/70 by DCs stimulated by TCTs. In line with previous data, HOE-140 also blocked IL-12p40/70 production by splenic DCs, recapitulating results obtained with immature B1R−/− (splenic) DCs exposed to TCT (42). Using the same pharmacological tools, studies with bone marrow–derived CD11c+ DCs confirmed that Dm28c TCT induce IL-12p40/70 in a C5aR-dependent manner (V. Schmitz, J. Köhl, and J. Scharfstein, unpublished observations).

However, we have consistently failed to detect B1R-dependent activation responses under the same conditions, suggesting that there is no coordinate expression/function of these GPCRs, at least so in bone marrow–derived DCs.

In the past years, the role of complement component in adaptive immunity has been systematically dissected. There is evidence that the lack of signaling via C3aR and C5aR1 decreases T cell survival and costimulatory signals required for effector T cell response (7, 78). It is also known that the engagement of C3aR, C5aR1, and C5aR2 is necessary for the differentiation of Th1 and Th17 cells through the production of IL-12, IL-6, and IL-23 by DCs and through the expression of IL-12R by CD4+ T cells (12). Some studies have demonstrated that signaling via the C3aR and/or C5aR1 is essential to DCs (79, 80), whereas others demonstrated that both DCs and T cells require the engagement of C3aR or C5aR1 (7, 78).

Finally, it was reported that the simultaneous absence C3aR and the C5aR1 signaling pathway favors production of regulatory T cells (14, 15). In a previous study, we found prominent (B1R-dependent) IFN-γ production by Ag-stimulated LN cells from ACEi-treated infected (footpad) mice (38). Correlating with the blunted edema, we found that HOE-140 reverted Th1 induction in ACEi mice. In the current study, we found that in the absence of ACEi, Ag-specific LN cells from infected BALB/c mice only produced mild IFN-γ responses. Interestingly, however, the recall assays revealed that these LN T cells concomitantly produced IL-10. Pharmacological targeting of either the C5aR or
the BßR at the onset of T. cruzi infection resulted in distinct phenotypic outcomes. C5aR targeting led to IL-4 production by DLN cells. However, blockade of BßR signaling strongly inhibited IL-10 production. This is a significant finding as it suggests that the integration of C5aR and BßR signals may favor IL-10 or IFN-γ production, most likely reflecting differences in the concentration levels of C5a and BK agonists, whether driven to lymphatics and/or targeting migratory DCs and/or affecting DC/T cell interaction in secondary lymphoid tissues.

In summary, our study supports the hypothesis that C5a and BK, two major proteolytic products generated during the cascade activation of complement, and the kinin systems may converge in parasite-laden tissues, from where they fuel inflammation and modulate innate/adaptive immunity through the cross-talk between C5aR/BßR. Future studies may clarify whether C5a and BK modulate APC function and shape Th development by activating C5aR/BßR expressed by migratory DCs and/or resident DCs localized in T cell–rich regions of secondary lymphoid tissues. Finally, our studies of TCT interaction with resident MΦ implicated C5aR/BßR cross-talk as an integrative signaling pathway that stimulates parasite internalization while conditioning host metabolism of the infected phagocytes in ways that limit intracellular parasite outgrowth through the upregulation of nitric oxide.

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Disclosures
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