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Short communication

Evidence of direct interactions between the CC-chemokines CCL3, CCL4 and CCL5 and *Leishmania* promastigotes

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Parasites of the genus *Leishmania* are very successful parasites, possessing multiple host evasive machineries and unique molecules that aid in their survival. It is presumed that a freshly inoculated parasite needs to survive in the hostile extracellular environment for several hours before it is internalized by a macrophage [1,2]. How the parasite survives within this extracellular environment and find their way to a macrophage are matters of great interest and enigma to clinicians and biologists alike. Chemotactic molecules secreted by the parasite, that are to this day uncharacterized, can home certain host cells and probably play a major role in the process of inviting host cells to the site of infection [3]. Host derived chemotactic molecules, especially chemokines also play an important role in the homing of cells to the site of infection [4]. What is not clear is whether these host derived chemotactic factors can also be sensed and utilized by the parasites.

Chemokines are proteins that act by binding to certain transmembrane G-protein coupled receptors. Chemokines are specific for the receptors they bind to although a degree of degeneracy exists [5]. Thus, the chemokines CCL3, CCL4 and CCL5 (formerly designated MIP1α, MIP1β and RANTES) bind to the receptors CCR5 and CCR1, but not to any of the other 50 or so receptors reported [5]. CCL2, although produced from a similar source and perform a similar function, binds to its cognate receptor CCR2. Many human chemokine receptor homologues are found in pathogenic obligatory parasites like viruses [6]. These viruses use the host chemokine–chemokine receptor system to infect their target cells. Chemokine-binding proteins have been reported from other parasites like *Schistosoma mansoni* [7].

Earlier reports from our group indicate an early activation of the host chemokine system when cells are infected with *Leishmania* parasites in vitro [8]. It has been shown that the presence of certain CC-chemokines, specifically CCL3, CCL4 and CCL5 facilitates the uptake of both *Leishmania* and *Trypanosoma* parasites by macrophages during the early hours of in vitro infection [9,10]. What is less clear is whether any of these or other chemokines has any direct interaction with the *Leishmania* promastigotes. To address this issue, we tested binding of these three chemokines, CCL3, CCL4 and CCL5 to early passage *Leishmania donovani* (*Ld*) strain MHOM/IN/1983/AG83 (AG83) and *Leishmania major* (*Lm*) strain MHOM/JL/BO/Friedlin. Stationary phase early passages *Ld* promastigotes were used for the rest of the investigations. Due to limitations in obtaining cell derived chemokines in pure form, recombinant human chemokines expressed in *Escherichia coli* (Sigma, St. Louis, USA) were used throughout the study. These recombinant molecules are known to exhibit characteristics similar to their natural counterparts and have been extensively used in research involving chemokines [11].

To test the hypothesis that chemokines bind to *Ld*, we studied the binding of human recombinant ^{125}I-labeled CCL3, CCL4, CCL5 and CCL2 (Amersham, UK) (all produced in *E. coli*) to *Ld* and *Lm* by the displacement-binding assays and saturation-binding assays as described [12,13]. The data were analyzed using the SCAHOT program of the LIGAND software. When *Ld* were saturated with ^{125}I CCL3 and this radioligand was

Abbreviations: *Ld*, *Leishmania donovani*; CCL2, 3, 4 and 5, CC chemokine ligand 2, 3, 4 and 5, respectively (also known as the chemokines MCP-1, MIP1α, MIP1β and RANTES, respectively)

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Table 1
K_d's of chemokine–*Leishmania* binding

	K_d by displacement-binding assay (pM)		K_d by heterologous chemokines displacing ^{125}I CCL3 (pM)		K_d by saturation-binding assay (pM)
Parasite	(A) *L. donovani*	(B) *L. major*	(C) *L. donovani*	(D) *L. major*	(E) *L. donovani*
CCL3	31	5	–	–	32
CCL4	34	4	64	1	36
CCL5	4	87	15	1	26

Dissociation constants of the binding of the chemokines CCL3, CCL4 and CCL5 to *Ld* and *Lm*. 10^5 *Leishmania* (*Ld* or *Lm*) promastigotes in 100 μl were added into wells of 96-well plates and to each well, 0.5 nM labeled chemokine was added along with increasing concentration of cold chemokine of the same type (columns A and B). In a separate set of experiments, cold chemokines of heterologous types were added to study cross-ligand competition (columns C and D). The plates were then incubated at 4 °C for 1 h with gentle shaking. Subsequently, the content of each well was aspirated into a microcentrifuge tube and the cells were washed twice in PBS. The cell pellet was lysed using 1 ml of 1% SDS at pH 10, 2 ml cocktail-W was mixed and counts were obtained in a Packard Liquid scintillation analyzer. The counts were fed into the SCAPRE program of the LIGAND software (Courtesy: Munson PJ, NIH, Bethesda, USA), *X–Y* scatters were obtained using the SCAFIT program of the same software. The obtained co-ordinates were graphed and from the slope of their Scatchard (bound/free vs. bound) regressions K_d values were determined. In an independent set of experiments, saturation binding was studied. 10^6 parasites were added into each well of a 96-well plate and increasing concentrations of ^{125}I-labeled ligands (^{125}I CCL3, ^{125}I CCL4 or ^{125}I CCL5) were added in the presence of 100-fold excess cold ligand of the same type. The remaining steps and analysis methodologies (this time using the SCAHOT program) were the same as described above for the displacement binding.

competed with increasing concentrations of unlabeled CCL3, a gradual displacement of the labeled chemokine was observed up to a certain point, beyond which no amount of cold ligand could reduce the counts. The displacement curve generated using the LIGAND software yielded typical sigmoidal pattern of receptor ligand bindings (data not shown). Scatchard plots obtained from linear regression of these data showed the dissociation constant (K_d) of this binding to be about 31 pM, indicating a high affinity binding of CCL3 to *Ld*. According to the manufacturer, K_d for the supplied labeled CCL3 for CHO cells transfected with CCR1 receptor appears be 100 pM. Similarly we tested the ability of other β-chemokines CCL4 and CCL5 to bind to *Ld*. Both CCL4 and CCL5 showed specific binding with K_d values of 34 and 4 pM, respectively. To see if this chemokine-binding property is unique for *Ld*, we tested the binding of the three chemokines to *Lm*. Similar to *Ld*, CCL3, CCL4 and CCL5 also bound to *Lm* in a similar displaceable manner. Since CCL2 did not show any specific binding, no further analysis was done with it (data not shown). The binding constants are presented in (A and B) in Table 1.

The next question we asked was whether the three β-chemokines CCL3, CCL4 and CCL5 bind to the same or distinct binding site on the parasite. To answer this question, we tried to displace ^{125}I CCL3 bound to *Ld* using CCL4 and CCL5. Using the same methodology as used for the previous studies we obtained similar sigmoidal displacement curves for both CCL4 and CCL5 against ^{125}I CCL3, indicating that these ligands were competing with CCL3 for the same binding site, albeit with different affinities. Identical studies performed with *Lm* yielded essentially similar results. K_d's for CCL3, CCL4 and CCL5 are presented in (C and D) in Table 1.

To verify our results and check for the specificity of binding, we also performed saturation-binding experiments using ^{125}I-labeled CCL3, CCL4 and CCL5 in the presence and absence of 100-fold excess respective unlabelled ligand. Scatchard plots generated from the binding data also yielded K_d values in the picomolar range (E) in (Table 1). These results are in agreement with those obtained by the displacement-binding method. Thus, we conclude that *Leishmania* express receptor-like molecules on their surface that can bind certain specific host derived chemokines with high affinity.

Since we found that CCL3, CCL4 and CCL5 bind on the *Leishmania* parasites, we became interested to see if the binding is functional and can cause transduction of signal further downstream. One of the hallmarks of chemokine induced signal transduction is intracellular Ca^{2+} mobilization, which was monitored in parasites as described [14]. FURA2-AM loaded *Ld* gave a steady fluorescence over time. In the presence of 100 ng CCL3 there was an instantaneous and sharp rise in fluorescence ratio that lasted for a few seconds, indicating a rapid and very transient release of intracellular Ca^{2+} (Fig. 1A). The same amount of CCL4 also induced intracellular Ca^{2+} release, but with less intensity than that observed with CCL3 (Fig. 1B). Using CCL5, the release was still less, though quite detectable (Fig. 1C). MCP-1, another CC-chemokine that binds to a different receptor in the mammalian system failed to induce any Ca^{2+} release in *Ld*, which remained unaltered (Fig. 1D). Thus, we conclude that indeed the binding of CCL3, CCL4 and CCL5 to *Ld* causes transduction of signal as evident from the intracellular Ca^{2+} mobilization. Since it is fairly well established that the chemokine signal is mediated by G-protein coupled receptors, we studied the effect of pertussis toxin, a G-protein blocker, in chemokine induced signaling in parasites as described [15]. As CCL3 induced maximum Ca^{2+} mobilization we studied the CCL3 mediated Ca^{2+} signaling in the presence and absence of pertussis toxin. Interestingly, the mobilization of Ca^{2+} by CCL3 was almost completely abrogated by pretreatment of *Ld* with pertussis toxin (Fig. 1E), indicating that the signal transduction is likely to be G-protein mediated. Pertussis toxin at the concentration used had no toxic effects on the promastigotes (data not shown). It is apparent from the above results that not only the binding of chemokines to the promastigote surface is functional, but also the signal transduction is probably G-protein mediated, similar to that observed in the mammalian host. Although till date no G-protein coupled receptors have been crystallized from *Leishmania* parasites, several hypothetical G-proteins with unknown functions have been predicted from the recently completed *Lm* genome databases.

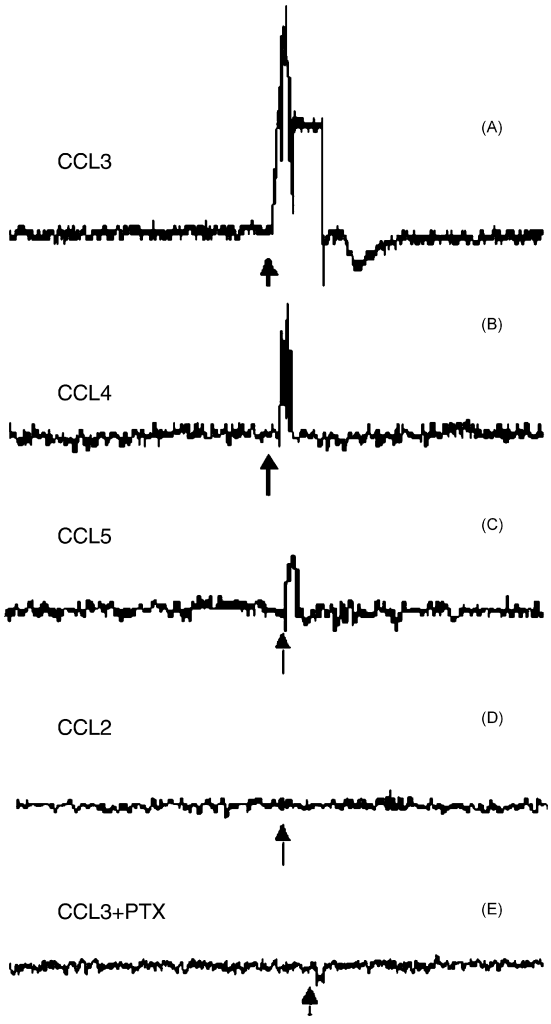

Fig. 1. Ca^{2+} mobilization in *Ld* promastigotes in response to CCL3 (A), CCL4 (B), CCL5 (C), MCP-1 (D) and calcium mobilization in pertussis toxin (PTX) treated parasites to CCL3 (E). 10^6 ml^{-1} FURA-2 AM loaded promastigotes were taken in a quartz cuvette and during fluorescent intensity emission scan (ratio of emission at 510 nm when excited at 340 and 380 nm), 100 ng chemokine was added using a Hamilton syringe to the continuously stirred cuvette. The plots were obtained in real time in a Hitachi Spectroflurometer. The arrowhead indicates the point where the chemokine was added.

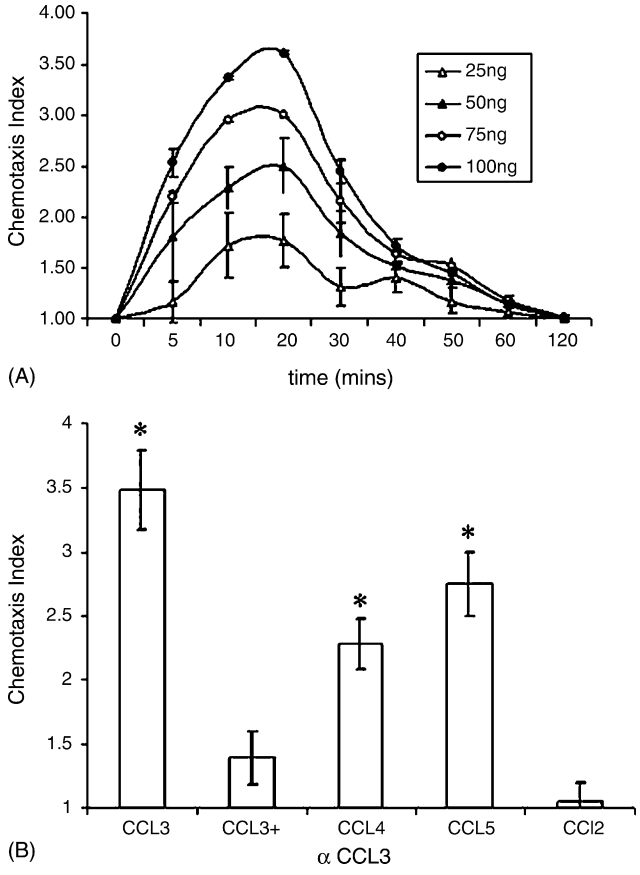

Fig. 2. Chemotaxis of *Ld* promastigotes towards CCL3: concentration and time kinetics. Promastigotes were taken in the upper chamber of a Transwell® plate and medium 199 containing indicated concentration of CCL3 in the lower chamber. Parasite migration to the lower chamber was monitored at the indicated time points microscopically by removing a small (20 μl) aliquot from the lower chamber and enumerating in a haemocytometer (A). The chemotactic indices of 100 ng/ml of different chemokines at 20 min are shown along with the effect of neutralizing antibody pretreatment of CCL3 prior to loading in the Transwell® plate (B). $^*p < 0.05$ represents significant difference as determined by paired Student's *t*-test (with respect to negative control MCP-1).

Chemokines usually chemoattract the cells they bind to. Finally, to see if CCL3 could actually chemoattract *Ld* parasites, we carried out chemoattraction assays in Transwell® plates as described [3] with minor modifications. Dose and time kinetics (Fig. 2A) showed that CCL3 can chemoattract *Ld* at a concentration of 50–100 ng/ml and the chemotaxis index (CI) peaks at 20 min post-incubation. To see if other chemokine ligands could also chemoattract *Ld*, we studied chemoattraction induced by CCL4, CCL5 and CCL2. Of the four chemokines, CCL3 appeared to have the highest CI, peaking 3.5. CI for CCL4 and CCL5 were 2.25 and 2.75, respectively. CCL2 had a CI of almost 1, meaning that it did not chemoattract *Ld* promastigotes (Fig. 2B). Neutralizing antibody pretreatment of the CCL3 almost completely inhibited the chemoattracting property of CCL3 (Fig. 2B). Equal concentration of CCL3 added to both chambers failed to influence the migration rate of *Ld* indicating that the migration was due to chemotaxis rather than chemokinesis (data not shown). Preincubation of the parasites with an equivalent amount of CCL4 and CCL5 prior to loading in the upper chamber significantly reduced chemotaxis (54 and 70% reduction in chemotactic indices, respectively; $p \leq 0.001$) while preincubation with CCL2 failed to show any significant reduction in chemotaxis (about 3% reduction; $p \geq 0.2$). Thus, we conclude that the host chemokine mediated signaling ultimately results in the chemoattraction of the parasites to the source of the chemokine secretion. It is known that macrophages produce the chemokines CCL3, CCL4 and CCL5 in response to *Leishmania* infection [8].

To the best of our knowledge this is the first report of a chemokine-binding property amongst the kinetoplastids and indicates the presence of a chemokine-binding molecule on the parasite surface. It is imperative that such a molecule would provide the parasite with survival advantage. Several viruses are known to posses homologues of certain host molecules like chemokines and chemokine receptors, a phenomenon that has

been termed molecular mimicry [16]. These mimics are often believed to have been acquired horizontally from the host during the co-evolutionary history of the parasite and host in close proximity, a phenomenon termed molecular piracy [17]. However, whether the chemokine-binding site on the *Leishmania* parasite actually turns out to be a homologue of CCR5 or whether it is a totally unrelated molecule remains to be seen. Glycosaminoglycans, which are also known to bind chemokines in the mammalian system, are unlikely candidates since no glycosaminoglycans have been reported from *Leishmania*. Moreover, glycosaminoglycan binding of chemokines does not trigger signaling events or induce biological activity [18], which is contrary to our data on Ca^{2+} mobilization and chemotaxis. Genome wide BLAST searches conducted on *Lm*/*Leishmania infantum* databases at the Sanger Center failed to yield a significant match for human or murine CCR5 sequences (data not shown). The search is currently on for identification, cloning and characterization of the molecule responsible for chemokine binding on the *Leishmania* surface and the biological significance of the finding in terms of host–parasite interactions.

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References

[1] de Almeida MC, Vilhena V, Barral A, Barral-Netto M. Leishmanial infection: analysis of its first steps. A review. Mem Inst Oswaldo Cruz 2003;98:861–70.
[2] Dominguez M, Moreno I, Aizpurua C, Torano A. Early mechanisms of *Leishmania* infection in human blood. Microbes Infect 2003;5:507–13.
[3] van Zandbergen G, Hermann N, Laufs H, Solbach W, Laskay T. *Leishmania* promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. Infect Immun 2002;70: 4177–84.
[4] Roychoudhury K, Roy S. Role of chemokines in *Leishmania* infection. Curr Mol Med 2004;4:691–6.
[5] Baggiolini M, Dewald B, Moser B. Human chemokines: an update. Annu Rev Immunol 1997;15:675–705.
[6] Boomker JM, van Luyn MJ, The TH, de Leij LF, Harmsen MC. US28 actions in HCMV infection: lessons from a versatile hijacker. Rev Med Virol 2005;15:269–82.
[7] Smith P, Fallon RE, Mangan NE, et al. *Schistosoma mansoni* secretes a chemokine binding protein with antiinflammatory activity. J Exp Med 2005;202:1319–25.
[8] Dasgupta B, Roychoudhury K, Ganguly S, Akbar MA, Das P, Roy S. Infection of human mononuclear phagocytes and macrophage-like THP1 cells with *Leishmania donovani* results in modulation of expression of a subset of chemokines and a chemokine receptor. Scand J Immunol 2003;57:366–74.
[9] Bhattacharyya S, Ghosh S, Dasgupta B, Mazumder D, Roy S, Majumdar S. Chemokine-induced leishmanicidal activity in murine macrophages via the generation of nitric oxide. J Infect Dis 2002;185:1704–8.
[10] Lima MF, Zhang Y, Villalta F. Beta-chemokines that inhibit HIV-1 infection of human macrophages stimulate uptake and promote destruction of *Trypanosoma cruzi* by human macrophages. Cell Mol Biol 1997;43:1067–76.
[11] Broxmeyer HE, Sherry B, Lu L, et al. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. Blood 1990;76:1110–6.
[12] Munson PJ. A users guide to LIGAND. Bethesda, MD: Analytical Biostatistical Section, DCRT, National Institutes of Health; 1990. p. 9.
[13] Munson PJ. A users guide to LIGAND. Bethesda, MD: Analytical Biostatistical Section, DCRT, National Institutes of Health; 1990. p. 31.
[14] Sarkar D, Bhaduri A. Temperature-induced rapid increase in cytoplasmic free Ca^{2+} in pathogenic *Leishmania donovani* promastigotes. FEBS Lett 1995;375:83–6.
[15] Portilla D, Morrissey J, Morrison AR. Bradykinin-activated membrane-associated phospholipase C in Madin–Darby canine kidney cells. J Clin Invest 1988;81:1896–902.
[16] Murphy PM. Viral exploitation and subversion of the immune system through chemokine mimicry. Nat Immunol 2001;2:116–22.
[17] Murphy PM. Molecular piracy of chemokine receptors by herpesviruses. Infect Agents Dis 1994;3:137–54.
[18] Ali S, Plamer ACV, Banerjee B, Hitchley SJ, Kirby JA. Examination of the function of RANTES, MIP-1α and MIP-1β following interactions with heparin like glycosaminoglycans. J Biol Chem 2000;275:11721–7.