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Counteractive functions are encrypted in the residues of CD154

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ABSTRACT

CD40, as a single receptor that binds CD154 (CD40-ligand or CD40L), regulates counteractive effector functions such as production of pro- and anti-inflammatory cytokines. Therefore, we examined whether such dual messages are encrypted in CD40L. As such message encryption was never investigated, we hypothesized that mutation of certain amino acid residues should in principle enhance pro-inflammatory cytokine production whereas mutation of some others would enhance anti-inflammatory cytokine secretion. We mutated six such residues, which were previously showed to participate in CD40L function. Here, we report that the mutant CD154 129E → V was superior to the wild-type CD154 in killing of *Leishmania donovani*, induction of inducible nitric oxide synthase (iNOS) and production of IL-12 and relative phosphorylation of p38MAPK and ERK-1/2 in PBMC-derived macrophages. By contrast, 128S → V promoted *L. donovani* survival, reducing iNOS, but increasing IL-10 expression and predominant ERK-1/2 phosphorylation. The mutant 144G → V did not have significant effects. Other mutants (142E → V, 143K → A, 145Y → F) mimicked the wild-type CD154. Molecular dynamics simulation suggested that these mutations induced differential conformational changes in the CD40–CD154 complex. Therefore, assortment of the contrasting messages encrypted in a given ligand performing counteractive functions presents a novel fundamental biological principle that can be used for devising various therapies.

1. Introduction

Physiological processes are regulated within a range of values defining the state of health. As these values go beyond the range, the process of pathogenesis ensues. In immune system, the processes such as macrophage or T cell activation can be maintained by two counteractive receptors. While optimal T cell proliferation requires potentiation of T cell receptor-derived signals by CD28 [1], absence of CD152 (CTLA-4) leads to lymphoproliferative disorders [2]. Similarly, leishmanicidal functions of macrophages are activated by IFN-γ but deactivated by IL-10 [3,4]. Thus, a given process is reciprocally regulated by two counteractive receptors expressed on the same cell. By contrast, the macrophage expressed costimulatory molecule CD40 that binds CD154 (CD40L) signaled reciprocally to counteractively regulate leishmanicidal functions and IL-12:IL-10 ratio [5–7]. Our experiments using dodecameric phage peptides, which do not have any similarities with the primary structure of CD40L, suggested that the phage-derived peptides imparted functional duality to CD40 by differential binding [8]. This was the first ever implication that different ligands binding to the same receptor may invoke differential responses. It is therefore hypothesized that a bigger polypeptide with multiple contact points on its corresponding receptor might have messages encrypted in these amino acid residues. These messages are decoded by the interacting receptor-triggered signaling leading to counteractive effector functions. We hypothesized that being bigger than the dodecameric peptides that served as non-natural CD40 binders [8], the natural CD40 ligand (CD154) might have multiple CD40 binding residues and each of those might have different functions. According to this hypothesis, mutation of some amino acid residues would specifically enhance leishmanicidal functions in macrophages whereas the mutation of some other residues would result in opposite function.

In order to verify the above hypothesis, we generated a panel of human CD154 mutants to assess their binding to human CD40 and ability to induce CD40 signaling and effector functions. Residues were selected based on their involvement in CD40L function and

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previous structural studies. The CD40-binding site consists of a shallow groove sandwiched between two monomers of a trimeric CD154 [9–11]. Mutagenesis study indicated substantial contribution of K143 and Y145 in CD154 to the binding [11]. Our mutagenesis study includes CD154's four key positions (128S, 129E, 143K and 144G) where naturally occurring mutations are observed in x-chromosome associated hyper-IgM (xIgM) syndrome patients [12,13] and the other two positions (E142 and Y145) are found crucial for binding to CD40 [11]. We observed that 129E → V, a ligand with a stronger anti-leishmanial activity than that showed by wild-type CD154, elicited higher CD40-induced p38MAPK activation and IL-12 production. By contrast, the 128S → V mutant CD154 promotes parasite survival, accompanied by reduced p38MAPK, but increased ERK-1/2 phosphorylation and reduced IL-12:IL-10 ratio. 144G → V failed to show any effect. Other mutants functionally mimicked the wild type. Thus, we discover that the receptors signaling and effector functions can be attributed to its ligand-specific residues and holds promise for context-dependent therapeutic usage.

2. Materials and methods

2.1. Reagents

Antibodies to p-p38MAPK, p38MAPK, p-ERK1/2, ERK1/2, β-actin, hexahistidine, CD40 and CD154 were from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid miniprep kit, gel elution kit, PCR purification kit, TRI-reagent, Ni–NTA beads and columns were from Sigma–Aldrich (St. Louis, MO). Cytokine ELISA Kits, MCSF, IFN-γ were from Becton Dickinson (San Diego, CA).

2.2. Parasites and generation of macrophages from PBMC

PBMCs were stimulated with MCSF (100 ng/ml) for 7 days and IFN-γ (10 ng/ml) [8]. *Leishmania donovani* (AG83) was maintained in RPMI 1640 with 10% FCS (Gibco, Grand Island, NY). All studies were performed in strict accordance with the recommendations of the institutional ethics committee.

2.3. Cloning of extracellular domain (ECD) of CD40 and CD154

CD40ECD (residues 21–193, using FP 5′-ATTCTTCATATGCCA GAACCACCCACT-3′ and RP 5′-ATACTCGAGTTCTCAGCCGATCCTG GG-3′) and CD154ECD (residues 47–261; using FP1: 5′-GCTCTGTA GTCATATGGCACGCCGCTTGG-3′, and RP2: 5′-GCATCCGCTCTCGAGT CAGAGTTTGAGTAAGCCAAAGGACGTGAAGCCAG-3′) were cloned in pET28a+. For site directed mutagenesis, two different PCR reactions were performed each time (using forward CD154 cloning primer, namely FP1 and mutagenic reverse primer, namely RP1; and reverse CD154 cloning primer, namely RP2 and mutagenic forward primer, namely FP2) to generate the overlapping fragment of double stranded DNA containing the desired mutation. Overlapping PCR fragments were joined in 5 cycles of PCR and after 5 cycle of PCR, FP1 and RP2 were used to amplify the mutagenic insert, which was cloned in pET28a+ [14,15]. The primers (5′ → 3′) are as follows: 128S → V: FP2: *TGCTGGCCTCCACTATGACATGTGCCGCAA* and RP1: *TTGCGGCACATGTCATAGTCGAGGCCAGCA*, 129E → V: FP2: *TTACTGCTGGCGACACTTATGACATGTGC* and RP1: *GCACATGTCATAAG TGTCGCCAGCAGTAA*, 144G → V: FP2: *TCATGGTGTAGTAGACTTTTT CAGCCCACT* and RP1: *AGTGGGCTGAAAAAGTCTACTACACCATGA*, 143 K → A: FP2: *ATGGTGTAGTATCCTGATTCAGCCCACTGT* and RP1: *A CAGTGGGCTGAAGCAGGATACTACACCAT*, 145Y → F: FP2: *TGCTCATGG TGTAGAATCCTTTTTCAGCCC* and RP1: *GGGCTGAAAAAGGATTCTACA CCATGAGCA*.

2.4. Expression and purification of extracellular domain of CD40, wild type and mutants of CD154

Escherichia coli BL21 (DE3) codon plus were induced with 0.75 mM IPTG at for CD40 and 0.50 mM for CD154, for 6 h at 25 °C at λ_{600} 0.5 180 rpm. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C; pellet was resuspended in ice-cold 50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, 10% glycerol (pH 8.0 for CD40ECD and pH 7.3 for CD154ECD). Lysozyme (1 mg/ml) was added to disrupt the bacterial cell wall. Pellet was subjected to sonication (10 s On, 10 s Off cycle for 5 min) in chilled condition with taking care that no or minimum frothing in the tube. The recombinant proteins were purified by Ni–NTA chromatography. Briefly, the lysate was incubated with 1 ml of Ni–NTA slurry (Qiagen, Hilden, Germany) for 4 h at 4 °C and the passed through a chromatography column where the beads were settled down at the bottom but excess resuspension buffer flowed off. Column was washed three times with wash buffer (50 mM NaH_2PO4, 300 mM NaCl, 50 mM imidazole, 10% glycerol, pH 8.0 for CD40ECD and pH 7.3 for CD154ECD) and finally eluted in 1 ml of elution buffer (50 mM NaH_2PO4, 500 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0 for CD40ECD and pH 7.3 for CD154ECD). Eluted protein was dialyzed extensively against 1× PBS in a step down manner to remove excess imidazole. Presence of protein was confirmed by SDS–PAGE. Proteins were re-purified by either size exclusion chromatography (in case of rhCD154ECD and its mutants) or ion exchange chromatography (in case of CD40ECD) and eluted in 1× PBS. LPS was <0.1 EU/μg, by LAL assay (MP Biomedical, Mumbai, India).

2.5. Surface plasmon resonance (SPR) studies for binding of wild type and mutants of CD154 to CD40

SPR measurements were performed on a ProteOn XPR36 protein interaction array system (Bio-Rad). The GLC chip was used in the assay [8]. The ligand rhCD40ECD was immobilized using the common amine coupling chemistry, which activated the GLC chip surface with newly made EDC (2 mM) and NHS (0.5 mM, as per the manufacturer's instruction); rhCD40 was injected in sodium acetate buffer, pH 5.0) and excess reactive esters were blocked with ethanolamine. rhCD154ECD was dissolved in phosphate buffered saline containing 0.05% TWEEN 20, pH 7.0 at 25 °C. Following this, rhCD154ECD was injected as analyte with a concentration of 5 μg/ml and 2.5 μg/ml at a flow rate of 50 μl/min for a total of 600 s. The SPR binding constants were calculated using Langmuir 1:1 binding model [8]. CD154 mutants were flown over the CD40 immobilized chip as described above. Binding constants were obtained from the Proteon-manager software.

2.6. Parasite killing and nitrite generation and cytokine ELISA

Macrophages were infected with *L. donovani* at 1:10 ratio and washed after 8 h; stimulated with wild type or mutants of CD154. 72 h later, macrophages were stained with Giemsa for 15 min. For NO generation or cytokine ELISA, macrophages were stimulated with wild type or mutants of CD154ECD, supernatants were collected 72 h after treatment, and levels of nitrite (μM) were estimated using Griess reagent [5], or the culture supernatants were assayed for the generation of IL-10 and IL-12 using Opt-EIA kits [8].

2.7. Western blotting

Macrophages were stimulated with wild type or mutants of CD154ECD for 15 min. Cells were lysed in lysis buffer [12]. SDS–PAGE was run followed by transfer. The membrane was blocked