nature portfolio

Peer Review File

Generating a mirror-image monobody targeting MCP-1 via TRAP display and chemical protein synthesis

Corresponding Author: Professor Hiroshi Murakami

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is another solid paper from the Murakami group on the application of their TRAP display technology, an improved version of mRNA display. In this paper, the authors developed D-monobodies targeting L-MCP-1 by using the mirror-image TRAP display which allows in vitro selection of L-monobodies against synthetic D-MCP-1. The authors confirmed that the resulted D-monobodies had high proteolytic resistance and undetectable immunogenicity in comparing with L-monobodies. Although these advantages also lie in the macrocyclic peptide binders made from unnatural amino acids that they have so far selected, the authors suggest that monobodies have the advantage of higher affinity than peptides.

(1) Since the authors have already reported a paper in which monobody selection was performed using TRAP display [Ref. 37], one of the novelty of this work is thought to be that it is combined with a mirror-image target. However, since the authors state they are co-submitting a paper that applies the same method to different targets (lines 106-108), I think the novelty of the method will not be highly evaluated. Therefore, in order to judge whether this paper is suitable for Nature Communications, I think it is important to know whether the performance of the D-monobodies against MCP-1 obtained in this study is significantly superior to conventional MCP-1 inhibitors. However, the issues with conventional MCP-1 inhibitors and their performance comparisons are not described in this paper, so they should be described in Introduction and Discussion.

(2) At the beginning of Discussion (lines 307-322), the authors state the following two possibilities as reasons why the dissociation constants of the L-monobodies against D-MCP-1 obtained in the initial TRAP display selection was lower than usual. The first reason is that the size of the target MCP-1 is smaller than usual, and the second reason is that "the interactions between D- and L-configured polypeptides are less favorable than those found in L-configured polypeptides". I think the first reason feels commonplace, while the second reason is novel and unexpected. Is there any prior literature that suggests this second possibility? In order to eliminate the first possibility, I recommend performing TRAP display selection of L-monobodies for "L-MCP-1" and determining the dissociation constants of the obtained L-monobodies.

(3) Although the authors evaluated the binding affinity and "specificity" of the D-monobody (lines 252-268), the only antigens tested for "specificity" were D-MCP-1 and L-MCP-1. The authors should confirm that the D-monobody, which is expected to be used as a biopharmaceutical, does not bind to other human proteins to evaluate the specificity.

Specific comments

Figure 2A: Why is there no difference between the 6th round and the 7th round of the cDNA recovery? It seems likely that the recovery rate would increase in round 7 after decreasing in round 6 when selection pressure was applied, just as it decreased in round 3 and then increased in rounds 4 and 5 in Figure 2A.

line 169: Generally, clones with a high appearance ratio, such as Mb1 and Mb2, should have high affinity, but Mb5 and Mb8 had higher affinity than Mb1 and Mb2 in this experiment. This reason should be explained in Discussion.

line 185: (Figure 3D) should be (Figure 2D).

lines 273-274: Although the authors described 'about 90% of full-length 9L was degraded within 30 min', Figure 4C (blue

line) shows that about 30% remains after 30 min.

lines 525-526: Insert '(solid lines)' and '(a dashed line)' after 'disulfide bonds' and 'a ligation site', respectively.

line 637: Ref. 14: The title should be written in lower case.

Reviewer #2

(Remarks to the Author)
Hayashi, et al. describe the selection of TRAP (mRNA) displayed L-monobodies with mirror D-MCP-1 antigen that can be converted into D-monobodies that retain affinity for natural L-MCP-1 in their native contexts on-cell. The bulk of the manuscript describes hit affinity maturation and execution of previously described D-protein synthesis via native chemical ligation. Overall, this work reiterates previously described methods and would benefit from additional biological validation to underscore the authors claims of pharmaceutical relevance. We would recommend accepting this article with revisions.

Major comment:
• This manuscript would benefit from additional downstream/phenotypic assays to demonstrate the utility of the binders. The claimed “resistance to proteolytic degradation, minimal immune response, and a potent inhibitory effect on MCP-1 binding to its cell membrane receptor” are supported, but this story comes along a slew of additional ones from this group describing application of this same workflow to other antigens (GFP, vWF-A1, VEGF-A). Its impact would benefit from leveraging the specific biology pursued, namely perturbation of the MCP-1/CCR2 signaling axis. Previous studies elucidating the role of MCP-1 in cancer could be mimicked here with monobody treatment, including downstream signaling effects, especially at phenotype level (migration or immune cell activation) in cellular assays or in vivo.

Minor comments:
• The initial, repeated emphasis on increased library size correlating to higher affinity (reference 32) seems somewhat unwarranted despite the logic underlying this claim. The reference cites the limitations of this claim (that different libraries were used in the comparison between high diversity mRNA and phage display libraries) and that antigen immunogenicity is also important (“the degree of binding site complementarity is correlated to the size and complexity of the ligand.” This study may be worth citing for the claim made in line 313-315). This caveat is later underscored by the ultimate KDs of Mb5 and Mb8 and the explanation provided as to why.
• P/N value is undefined in the text (line 166)
• Figure 2D would benefit from a final x-axis label “Round of selection” as in Figure 1A. If this makes the axis too crowded, an inset color legend could also be used.
• The logic used to create the rational affinity matured clones is somewhat unclear without also viewing Supplemental figure 14. From the main text, it sounds as if all 12 are combinations of library hits. It is unclear that Mb5-1 through -8 consisted of hits from one to two libraries and only -9 through -12 are combinations of hits of all 3 libraries. Further, it isn’t clear that final clones consist of all combinations of only two BC and two FG loops (with only one residue mutation different between each loop’s pair, perhaps bold this residue). Additional elaboration would highlight the successful rational design approach.
o Further, do you find the combinatorial KDs to suggest an additive or synergistic effect? It would be nice to have KDs of Mb5-1 through -8, explanation of which 6-residue motifs were chosen, or some added speculation as to the effects of other library hit combinations.
• The naming convention in Figure 3A seems inconsistent, where 1D and 2D NCL yields 3D and 5D and 6D NCL yields 9D, but 6D and 7D NCL yields 6D-7D, unless there is a nuance oversight on my part. Similarly in Supplementary figure 20, the equivalent L-enantiomer is called 6L+7L (rather than 6L-7L).
• Cultured cell assays:
o Please cite the PathHunter® β-Arrestin eXpress GPCR Assay by name in the main text. This will make the description more easily understood.
o Further, it is worth clarifying towards the beginning of this section that this assay is a functional proxy and the goal is to decrease signal due to competition of CCR2 binding by monobody binding. These assay results represent the biological relevance of this work and highlighting them as such would amplify their impact.
o Perhaps adding a dotted horizontal line in Figure 4E to denote the basal RLU seen with 7 nM L-MCP-1 treatment alone (Supplementary figure 24) would make the claim in line 302 clearer.
• Discussion:
o Perhaps start this section reiterating what was done (well) by the study and the utility of the pipeline presented before launching into what comes across as disappointment with the initial KDs. What gap in the field does this address? What future opportunities does this open?
o It may make sense to organize the discussion around the three hypotheses that may have improved the selection: library diversity, antigen immunogenicity/real estate, and D-/L- interactions
Hypothesis 1 (library diversity): There is a grammatical error in lines 325-326, the sentence that supports exploring library diversity.
Hypothesis 3 (D-/L- interactions): This seems most pressing to the field of mirror-proteins and worth fleshing out. Could you further encourage exploration of this? In addition to structural studies, what would enable further understanding of this potential phenomenon?

Reviewer #3

(Remarks to the Author)

This paper seeks to develop mirror-image monobodies against MCP-1 using a more efficient version of mRNA display. After this initial screening, an additional cycle of affinity maturation led to a low-nM binder. The mirror-image synthesis of the 76-residue MCP-1 in mirror-image was performed following a previously described 2-segment strategy using NCL. One of the highest affinity candidates underwent affinity maturation (via regional hard mutagenesis) to produce the highest affinity binder (Mb5-11). This winning monobody was then sythesized in 3 segments (with 2 Cys substitutions, as developed for a previous monobody synthesis). MB5-11 is evaluated via BLI, CD, proteolytic/plasma stability, and immunogenicity. All of these validate the D-protein, except that the fairly rapid proteolytic degradation of a D-protein (Fig. 4C) is very surprising - other studies on D-protein stability show minimal degradation, so this result requires explanation. Fig. 1D shows significant differences in CD spectrum intensity between L and D-MCP-1 without explanation. Also, CD appears to be smoothed (not mentioned in minimal CD experimental methods).
Finally, a cellular assay was used to measure MCP-1 inhibition, showing low nM inhibitory activity (with no inhibition observed with the corresponding L-monobody). While this result is a good control for stereospecificity, there is no data provided to demonstrate MCP-1 specificity. The statement that D-proteins may have “less favorable binding patterns” with L-targets does not seem well supported by this one example.
Overall, this is an interesting study that provides solid supporting evidence for the discovery of a high-affinity D-monobody. Together with the co-submitted manuscript, these studies support the potential of D-monobodies as an emerging therapeutic class.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revised version, the authors have fully and appropriately addressed all of the points I raised in the initial review session, with the exception of the correction of the title of Ref. 14.
I support publication of the manuscript with this correction.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed our questions and concerns

Reviewer #3

(Remarks to the Author)
The authors were highly responsive to previous critiques and have addressed all of my significant concerns. This manuscript is now suitable for publication. One minor suggestion - the difference in CD spectra between L and D-proteins is small, but significant, and suggests that the D-protein likely has some impurities (e.g., deletion products) that are affecting its secondary structure (a common issue with synthetic D-proteins). This minor limitation should be acknowledged.

Responses to the referees' comments

Reviewer #1 (Remarks to the Author):

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Answer: We would like to emphasize that the selection strategy used in our study (i.e. mirror-image TRAP display) is different from that used in the co-submitted paper, which utilized conventional mirror-image phage display (MIPD). TRAP display can provide even higher library diversity (~10^{13}) than conventional phage display (~10^{10}). To make this clear, we added a phrase in the introduction part of the main text (line 114).

As for the performance comparison with a previous MCP-1 inhibitor, we conducted additional experiments as shown in Figure 4F, in which the inhibitory effect of D-monobody on cultured monocyte migration was compared with previously developed anti-MCP-1 IgG antibody, Carlumab. As a result, D-monobody showed inhibitory effect equal to Carlumab.

We added a paragraph describing these experiments at the end of "Results" section and also added a phrase in "Abstract" section (line 39) and a sentence in "Introduction" section (lines 109). Further, we added a new paragraph into "Discussion" section.

(2) At the beginning of Discussion (lines 307-322), the authors state the following two possibilities as reasons why the dissociation constants of the L-monobodies against D-MCP-1 obtained in the initial TRAP display selection was lower than usual. The first reason is that the size of the target MCP-1 is smaller than usual, and the second reason is that "the interactions between D- and L-configured polypeptides are less favorable than those found in L-configured polypeptides". I think the first reason feels commonplace, while the second reason is novel and unexpected. Is there any prior literature that suggests this second possibility? In order to eliminate the first possibility, I recommend performing TRAP display selection of L-monobodies for "L-MCP-1" and determining the dissociation constants of the obtained L-monobodies.

Answer: According to the reviewer's suggestion, we added results of TRAP display against L-MCP-1 using the same monobody library. Single-digit nM clones were obtained against L-MCP-1 without additional affinity maturation process (Figure S14). While the significant difference in the affinity of obtained clones between D- and L-MCP-1 might be due to chance, this result at least seems to support the hypothesis. However, we could not find any literature supporting this possibility. We think that this hypothesis is novel and interesting for future study. We added the sentences describing these results and discussion in the main text (line 188 and 443).

(3) Although the authors evaluated the binding affinity and "specificity" of the D-monobody (lines 252-268), the only antigens tested for "specificity" were D-MCP-1 and L-MCP-1. The authors should confirm that the D-monobody, which is expected to be used as a biopharmaceutical, does not bind to other human proteins to evaluate the specificity.

Answer: We conducted additional binding experiments by BLI with 6 pharmaceutically important target proteins (IL6, LIF, IL-6R, CD266, CTLA4 and PD1), and demonstrated that D-monobody obtained in this study selectively bound to MCP-1 (Figure S23). Explanation of the experiments were added to the result section of the main text (line 346).

Specific comments

Figure 2A: Why is there no difference between the 6th round and the 7th round of the cDNA recovery? It seems likely that the recovery rate would increase in round 7 after decreasing in round 6 when selection pressure was applied, just as it decreased in round 3 and then increased in rounds 4 and 5 in Figure 2A.

Answer: We assume that the library components after the 6th and 7th round could be same probably because of the completed enrichment process after the 6th round selection.

line 169: Generally, clones with a high appearance ratio, such as Mb1 and Mb2, should have high affinity, but Mb5 and Mb8 had higher affinity than Mb1 and Mb2 in this experiment. This reason should be explained in Discussion.

Answer: From our experiences, the high appearance ratio values do not necessarily promise the high affinity. Monobody molecules in TRAP display selection are placed in a unique environment different from those after E. coli expression. For example, a monobody clone in TRAP display is always conjugated with mRNA via puromycin linker and the concentration of the conjugate is even lower than that after the E. coli expression. These environmental differences can affect the properties of individual clones such as conformational stability and aggregation tendency. We added this explanation in “Results” section of the main text (line 179).

line 185: (Figure 3D) should be (Figure 2D).

Answer: The typo was corrected.

lines 273-274: Although the authors described 'about 90% of full-length 9L was degraded within 30 min', Figure 4C (blue line) shows that about 30% remains after 30 min.

Answer: The sentence was corrected from “within 30 min” to “within 2 h”.

lines 525-526: Insert '(solid lines)' and '(a dashed line)' after 'disulfide bonds' and 'a ligation site', respectively.

Answer: The phrases were added as the reviewer pointed.

line 637: Ref. 14: The title should be written in lower case.

Answer: The title of Ref.14 was corrected.

Reviewer #2 (Remarks to the Author):

Hayashi, et al. describe the selection of TRAP (mRNA) displayed L-monobodies with mirror D-MCP-1 antigen that can be converted into D-monobodies that retain affinity for natural L-MCP-1 in their native contexts on-cell. The bulk of the manuscript describes hit affinity

maturation and execution of previously described D-protein synthesis via native chemical ligation. Overall, this work reiterates previously described methods and would benefit from additional biological validation to underscore the authors claims of pharmaceutical relevance. We would recommend accepting this article with revisions.

Major comment:

• This manuscript would benefit from additional downstream/phenotypic assays to demonstrate the utility of the binders. The claimed “resistance to proteolytic degradation, minimal immune response, and a potent inhibitory effect on MCP-1 binding to its cell membrane receptor” are supported, but this story comes along a slew of additional ones from this group describing application of this same workflow to other antigens (GFP, vWF-A1, VEGF-A). Its impact would benefit from leveraging the specific biology pursued, namely perturbation of the MCP-1/CCR2 signaling axis. Previous studies elucidating the role of MCP-1 in cancer could be mimicked here with monobody treatment, including downstream signaling effects, especially at phenotype level (migration or immune cell activation) in cellular assays or in vivo.

Answer: According to the reviewer’s suggestion, we conducted a phenotype-level experiment by using cultured THP-1 cells (monocyte cell line). As a result, D-monobody inhibited migration of THP-1 cells in a concentration dependent manner (Figure 4F). We added a new paragraph describing this experiment at the end of “Results” section.

Minor comments:

• The initial, repeated emphasis on increased library size correlating to higher affinity (reference 32) seems somewhat unwarranted despite the logic underlying this claim. The reference cites the limitations of this claim (that different libraries were used in the comparison between high diversity mRNA and phage display libraries) and that antigen immunogenicity is also important (“the degree of binding site complementarity is correlated to the size and complexity of the ligand.” This study may be worth citing for the claim made in line 313-315). This caveat is later underscored by the ultimate KDs of Mb5 and Mb8 and the explanation provided as to why.

We agree with the fact that antigen immunogenicity (target property) and library design can affect the affinity of the binder. On the other hand, we believe that if the same antigen and library design are used, the KD value would correlate well with library diversity. Therefore, we reasons that both target property and library size are essential factors for whether we

could obtain high affinity clones or not. We retained the first citation of reference 32, but as the reviewer pointed out, we removed the second citation to avoid redundancy.

• P/N value is undefined in the text (line 166)
Answer: The explanation of P/N value was added in the text.

• Figure 2D would benefit from a final x-axis label “Round of selection” as in Figure 1A. If this makes the axis too crowded, an inset color legend could also be used.
Answer: The label “Round of selection” was inserted into Figure 2D.

• The logic used to create the rational affinity matured clones is somewhat unclear without also viewing Supplemental figure 14. From the main text, it sounds as if all 12 are combinations of library hits. It is unclear that Mb5-1 through -8 consisted of hits from one to two libraries and only -9 through -12 are combinations of hits of all 3 libraries. Further, it isn’t clear that final clones consist of all combinations of only two BC and two FG loops (with only one residue mutation different between each loop’s pair, perhaps bold this residue). Additional elaboration would highlight the successful rational design approach.
o Further, do you find the combinatorial KDs to suggest an additive or synergistic effect? It would be nice to have KDs of Mb5-1 through -8, explanation of which 6-residue motifs were chosen, or some added speculation as to the effects of other library hit combinations.
According to our experience, the effects of enriched residues are additive in terms of binding. Our point here is that, even if some mutations do not show a significant effect individually, they could improve affinity when combined. From Figure 2E, we first aimed to create a clone with the highest affinity that includes all of the most abundant residues, that is Mb5-9. However, we are also interested in several other mutations. We decided to mutate Asn to Arg at the 4th position of the FG loop (Mb5-11) since it appeared as the second most abundant clone from Lib-C. Additionally, we mutated Gly to Ser at the 7th position of the BC loop, along with a Phe to Trp mutation at the 8th position (Mb5-10 and Mb5-12), as only the Gly and Phe or Ser and Trp combinations appeared among the top 10 sequences.
Clones with fewer mutations (Mb5-1 to -8) were supposed as sorts of negative control. We updated the explanation of these clone designs in the main text (line 216).

• The naming convention in Figure 3A seems inconsistent, where 1D and 2D NCL yields 3D and 5D and 6D NCL yields 9D, but 6D and 7D NCL yields 6D-7D, unless there is a nuance oversight on my part. Similarly in Supplementary figure 20, the equivalent L-enantiomer is called 6L+7L (rather than 6L-7L).

Answer: We numbered all of the isolated peptides. However, the ligation product 6D-7D is an intermediate that is transiently generated in the reaction mixture and was not isolated. This is the reason why we did not number this peptide. However, we should unify the naming of this intermediate. “6L+7L” was corrected to “6L-7L” and parentheses that means “intermediate” was also added in Figure S21.

• Cultured cell assays:

o Please cite the PathHunter® β-Arrestin eXpress GPCR Assay by name in the main text. This will make the description more easily understood.

Answer: We added the name in the main text (line 391).

o Further, it is worth clarifying towards the beginning of this section that this assay is a functional proxy and the goal is to decrease signal due to competition of CCR2 binding by monobody binding. These assay results represent the biological relevance of this work and highlighting them as such would amplify their impact.

Answer: We added more explanation about the principle and significance of the experiment (line 390 and 396).

o Perhaps adding a dotted horizontal line in Figure 4E to denote the basal RLU seen with 7 nM L-MCP-1 treatment alone (Supplementary figure 24) would make the claim in line 302 clearer.

Answer: Dotted horizontal line was inserted to both D- and L-monobody graphs.

• Discussion:

o Perhaps start this section reiterating what was done (well) by the study and the utility of the pipeline presented before launching into what comes across as disappointment with the initial KDs. What gap in the field does this address? What future opportunities does this open?

Answer: We added a first sentence in “Discussion” section describing the summary of this study (line 426). Sentences describing contribution by this research and future opportunities provided by this research were also added in “Discussion” section (line 430-451, 532-539).

o It may make sense to organize the discussion around the three hypotheses that may have improved the selection: library diversity, antigen immunogenicity/real estate, and D-/L-interactions

♣ Hypothesis 1 (library diversity): There is a grammatical error in lines 325-326, the sentence that supports exploring library diversity.

♣ Hypothesis 3 (D-/L- interactions): This seems most pressing to the field of mirror-proteins and worth fleshing out. Could you further encourage exploration of this? In addition to structural studies, what would enable further understanding of this potential phenomenon?

Answer: We added an additional results describing results of TRAP display against L-MCP-1 using the same monobody library (Figure S14). And, we found that single-digit nM clones were obtained against L-MCP-1 without additional affinity maturation process. Therefore, we revised “Discussion” section by focusing on two possibilities about the reason why high affinity clones were selected not against D-MCP-1 but L-MCP-1 without affinity maturation.

Reviewer #3 (Remarks to the Author):

This paper seeks to develop mirror-image monobodies against MCP-1 using a more efficient version of mRNA display. After this initial screening, an additional cycle of affinity maturation led to a low-nM binder. The mirror-image synthesis of the 76-residue MCP-1 in mirror-image was performed following a previously described 2-segment strategy using NCL. One of the highest affinity candidates underwent affinity maturation (via regional hard mutagenesis) to produce the highest affinity binder (Mb5-11). This winning monobody was then sythesized in 3 segments (with 2 Cys substitutions, as developed for a previous monobody synthesis). MB5-11 is evaluated via BLI, CD, proteolytic/plasma stability, and immunogenicity. All of these validate the D-protein, except that the fairly rapid proteolytic degradation of a D-protein (Fig. 4C) is very surprising - other studies on D-protein stability show minimal degradation, so this result requires explanation.

Answer: We carefully re-conducted the tryptic digestion experiment and found that the degradation of D-monobody was ignorable (Figure 4C and S25). Now, we speculate that the decreased intensity of gel band could be not due to the degradation but due to the absorption to plastic tube.

Fig. 1D shows significant differences in CD spectrum intensity between L and D-MCP-1 without explanation. Also, CD appears to be smoothed (not mentioned in minimal CD experimental methods).

Answer: When we compare the absolute values of the spectrum intensity of L and D-MCP1, the difference is mostly within 1.2 times: for example, 3182.11 (L) and 3766.90 (D) at 200 nm, and 5482.59 (L) and 6209.20 (D) at 210 nm (peak top). We believe that this difference is not so significant given that previously published CD spectra showed similar or even larger

differences in the comparison of L and D-proteins (e.g. Fig 1F in 10.1002/anie.201506225, Fig 1C in 10.1021/acs.bioconjchem.7b00326, and Fig S6 in 10.1002/cbic.201900355). According to the reviewer's comment, we added precise conditions in CD spectrum measurements including a smoothing method into supporting information.

Finally, a cellular assay was used to measure MCP-1 inhibition, showing low nM inhibitory activity (with no inhibition observed with the corresponding L-monobody). While this result is a good control for stereospecificity, there is no data provided to demonstrate MCP-1 specificity.

Answer: To strengthen the binding specificity of D-monobody against MCP-1, we conducted additional binding experiments by BLI with 6 pharmaceutically important target proteins (IL6, LIF, IL-6R, CD266, CTLA4 and PD1), and demonstrated that D-monobody obtained in this study selectively bound to MCP-1 (Figure S23). Explanation of the experiments were added to the result section of the main text (line 346).

The statement that D-proteins may have “less favorable binding patterns” with L-targets does not seem well supported by this one example.

Answer: To evaluate the statement, we added results of TRAP display against L-MCP-1 using the same monobody library. Single-digit nM clones were obtained against L-MCP-1 without additional affinity maturation process (Figure S14). While the significant difference in the affinity of obtained clones between D- and L-MCP-1 might be due to chance, this result at least seems to support the statement. However, we could not find any literature describing this possibility. Therefore, we concluded that future study is necessary to claim the generality of the statement. We added the sentences describing these results and discussion in the main text (line 188 and 443).

Responses to the referees' comments

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Answer: We assume that the library components after the 6th and 7th round could be same probably because of the completed enrichment process after the 6th round selection.

line 169: Generally, clones with a high appearance ratio, such as Mb1 and Mb2, should have high affinity, but Mb5 and Mb8 had higher affinity than Mb1 and Mb2 in this experiment. This reason should be explained in Discussion.

Answer: From our experiences, the high appearance ratio values do not necessarily promise the high affinity. Monobody molecules in TRAP display selection are placed in a unique environment different from those after E. coli expression. For example, a monobody clone in TRAP display is always conjugated with mRNA via puromycin linker and the concentration of the conjugate is even lower than that after the E. coli expression. These environmental differences can affect the properties of individual clones such as conformational stability and aggregation tendency. We added this explanation in “Results” section of the main text (line 179).

line 185: (Figure 3D) should be (Figure 2D).

Answer: The typo was corrected.

lines 273-274: Although the authors described 'about 90% of full-length 9L was degraded within 30 min', Figure 4C (blue line) shows that about 30% remains after 30 min.

Answer: The sentence was corrected from “within 30 min” to “within 2 h”.

lines 525-526: Insert '(solid lines)' and '(a dashed line)' after 'disulfide bonds' and 'a ligation site', respectively.

Answer: The phrases were added as the reviewer pointed.

line 637: Ref. 14: The title should be written in lower case.

Answer: The title of Ref.14 was corrected.

Reviewer #2 (Remarks to the Author):

Hayashi, et al. describe the selection of TRAP (mRNA) displayed L-monobodies with mirror D-MCP-1 antigen that can be converted into D-monobodies that retain affinity for natural L-MCP-1 in their native contexts on-cell. The bulk of the manuscript describes hit affinity

maturation and execution of previously described D-protein synthesis via native chemical ligation. Overall, this work reiterates previously described methods and would benefit from additional biological validation to underscore the authors claims of pharmaceutical relevance. We would recommend accepting this article with revisions.

Major comment:

• This manuscript would benefit from additional downstream/phenotypic assays to demonstrate the utility of the binders. The claimed “resistance to proteolytic degradation, minimal immune response, and a potent inhibitory effect on MCP-1 binding to its cell membrane receptor” are supported, but this story comes along a slew of additional ones from this group describing application of this same workflow to other antigens (GFP, vWF-A1, VEGF-A). Its impact would benefit from leveraging the specific biology pursued, namely perturbation of the MCP-1/CCR2 signaling axis. Previous studies elucidating the role of MCP-1 in cancer could be mimicked here with monobody treatment, including downstream signaling effects, especially at phenotype level (migration or immune cell activation) in cellular assays or in vivo.

Answer: According to the reviewer’s suggestion, we conducted a phenotype-level experiment by using cultured THP-1 cells (monocyte cell line). As a result, D-monobody inhibited migration of THP-1 cells in a concentration dependent manner (Figure 4F). We added a new paragraph describing this experiment at the end of “Results” section.

Minor comments:

• The initial, repeated emphasis on increased library size correlating to higher affinity (reference 32) seems somewhat unwarranted despite the logic underlying this claim. The reference cites the limitations of this claim (that different libraries were used in the comparison between high diversity mRNA and phage display libraries) and that antigen immunogenicity is also important (“the degree of binding site complementarity is correlated to the size and complexity of the ligand.” This study may be worth citing for the claim made in line 313-315). This caveat is later underscored by the ultimate KDs of Mb5 and Mb8 and the explanation provided as to why.

We agree with the fact that antigen immunogenicity (target property) and library design can affect the affinity of the binder. On the other hand, we believe that if the same antigen and library design are used, the KD value would correlate well with library diversity. Therefore, we reasons that both target property and library size are essential factors for whether we

could obtain high affinity clones or not. We retained the first citation of reference 32, but as the reviewer pointed out, we removed the second citation to avoid redundancy.

• P/N value is undefined in the text (line 166)
Answer: The explanation of P/N value was added in the text.

• Figure 2D would benefit from a final x-axis label “Round of selection” as in Figure 1A. If this makes the axis too crowded, an inset color legend could also be used.
Answer: The label “Round of selection” was inserted into Figure 2D.

• The logic used to create the rational affinity matured clones is somewhat unclear without also viewing Supplemental figure 14. From the main text, it sounds as if all 12 are combinations of library hits. It is unclear that Mb5-1 through -8 consisted of hits from one to two libraries and only -9 through -12 are combinations of hits of all 3 libraries. Further, it isn’t clear that final clones consist of all combinations of only two BC and two FG loops (with only one residue mutation different between each loop’s pair, perhaps bold this residue). Additional elaboration would highlight the successful rational design approach.
o Further, do you find the combinatorial KDs to suggest an additive or synergistic effect? It would be nice to have KDs of Mb5-1 through -8, explanation of which 6-residue motifs were chosen, or some added speculation as to the effects of other library hit combinations.
According to our experience, the effects of enriched residues are additive in terms of binding. Our point here is that, even if some mutations do not show a significant effect individually, they could improve affinity when combined. From Figure 2E, we first aimed to create a clone with the highest affinity that includes all of the most abundant residues, that is Mb5-9. However, we are also interested in several other mutations. We decided to mutate Asn to Arg at the 4th position of the FG loop (Mb5-11) since it appeared as the second most abundant clone from Lib-C. Additionally, we mutated Gly to Ser at the 7th position of the BC loop, along with a Phe to Trp mutation at the 8th position (Mb5-10 and Mb5-12), as only the Gly and Phe or Ser and Trp combinations appeared among the top 10 sequences.
Clones with fewer mutations (Mb5-1 to -8) were supposed as sorts of negative control. We updated the explanation of these clone designs in the main text (line 216).

• The naming convention in Figure 3A seems inconsistent, where 1D and 2D NCL yields 3D and 5D and 6D NCL yields 9D, but 6D and 7D NCL yields 6D-7D, unless there is a nuance oversight on my part. Similarly in Supplementary figure 20, the equivalent L-enantiomer is called 6L+7L (rather than 6L-7L).

Answer: We numbered all of the isolated peptides. However, the ligation product 6D-7D is an intermediate that is transiently generated in the reaction mixture and was not isolated. This is the reason why we did not number this peptide. However, we should unify the naming of this intermediate. “6L+7L” was corrected to “6L-7L” and parentheses that means “intermediate” was also added in Figure S21.

• Cultured cell assays:

o Please cite the PathHunter® β-Arrestin eXpress GPCR Assay by name in the main text. This will make the description more easily understood.

Answer: We added the name in the main text (line 391).

o Further, it is worth clarifying towards the beginning of this section that this assay is a functional proxy and the goal is to decrease signal due to competition of CCR2 binding by monobody binding. These assay results represent the biological relevance of this work and highlighting them as such would amplify their impact.

Answer: We added more explanation about the principle and significance of the experiment (line 390 and 396).

o Perhaps adding a dotted horizontal line in Figure 4E to denote the basal RLU seen with 7 nM L-MCP-1 treatment alone (Supplementary figure 24) would make the claim in line 302 clearer.

Answer: Dotted horizontal line was inserted to both D- and L-monobody graphs.

• Discussion:

o Perhaps start this section reiterating what was done (well) by the study and the utility of the pipeline presented before launching into what comes across as disappointment with the initial KDs. What gap in the field does this address? What future opportunities does this open?

Answer: We added a first sentence in “Discussion” section describing the summary of this study (line 426). Sentences describing contribution by this research and future opportunities provided by this research were also added in “Discussion” section (line 430-451, 532-539).

o It may make sense to organize the discussion around the three hypotheses that may have improved the selection: library diversity, antigen immunogenicity/real estate, and D-/L-interactions

♣ Hypothesis 1 (library diversity): There is a grammatical error in lines 325-326, the sentence that supports exploring library diversity.

♣ Hypothesis 3 (D-/L- interactions): This seems most pressing to the field of mirror-proteins and worth fleshing out. Could you further encourage exploration of this? In addition to structural studies, what would enable further understanding of this potential phenomenon?

Answer: We added an additional results describing results of TRAP display against L-MCP-1 using the same monobody library (Figure S14). And, we found that single-digit nM clones were obtained against L-MCP-1 without additional affinity maturation process. Therefore, we revised “Discussion” section by focusing on two possibilities about the reason why high affinity clones were selected not against D-MCP-1 but L-MCP-1 without affinity maturation.

Reviewer #3 (Remarks to the Author):

This paper seeks to develop mirror-image monobodies against MCP-1 using a more efficient version of mRNA display. After this initial screening, an additional cycle of affinity maturation led to a low-nM binder. The mirror-image synthesis of the 76-residue MCP-1 in mirror-image was performed following a previously described 2-segment strategy using NCL. One of the highest affinity candidates underwent affinity maturation (via regional hard mutagenesis) to produce the highest affinity binder (Mb5-11). This winning monobody was then sythesized in 3 segments (with 2 Cys substitutions, as developed for a previous monobody synthesis). MB5-11 is evaluated via BLI, CD, proteolytic/plasma stability, and immunogenicity. All of these validate the D-protein, except that the fairly rapid proteolytic degradation of a D-protein (Fig. 4C) is very surprising - other studies on D-protein stability show minimal degradation, so this result requires explanation.

Answer: We carefully re-conducted the tryptic digestion experiment and found that the degradation of D-monobody was ignorable (Figure 4C and S25). Now, we speculate that the decreased intensity of gel band could be not due to the degradation but due to the absorption to plastic tube.

Fig. 1D shows significant differences in CD spectrum intensity between L and D-MCP-1 without explanation. Also, CD appears to be smoothed (not mentioned in minimal CD experimental methods).

Answer: When we compare the absolute values of the spectrum intensity of L and D-MCP1, the difference is mostly within 1.2 times: for example, 3182.11 (L) and 3766.90 (D) at 200 nm, and 5482.59 (L) and 6209.20 (D) at 210 nm (peak top). We believe that this difference is not so significant given that previously published CD spectra showed similar or even larger

differences in the comparison of L and D-proteins (e.g. Fig 1F in 10.1002/anie.201506225, Fig 1C in 10.1021/acs.bioconjchem.7b00326, and Fig S6 in 10.1002/cbic.201900355). According to the reviewer's comment, we added precise conditions in CD spectrum measurements including a smoothing method into supporting information.

Finally, a cellular assay was used to measure MCP-1 inhibition, showing low nM inhibitory activity (with no inhibition observed with the corresponding L-monobody). While this result is a good control for stereospecificity, there is no data provided to demonstrate MCP-1 specificity.

Answer: To strengthen the binding specificity of D-monobody against MCP-1, we conducted additional binding experiments by BLI with 6 pharmaceutically important target proteins (IL6, LIF, IL-6R, CD266, CTLA4 and PD1), and demonstrated that D-monobody obtained in this study selectively bound to MCP-1 (Figure S23). Explanation of the experiments were added to the result section of the main text (line 346).

The statement that D-proteins may have “less favorable binding patterns” with L-targets does not seem well supported by this one example.

Answer: To evaluate the statement, we added results of TRAP display against L-MCP-1 using the same monobody library. Single-digit nM clones were obtained against L-MCP-1 without additional affinity maturation process (Figure S14). While the significant difference in the affinity of obtained clones between D- and L-MCP-1 might be due to chance, this result at least seems to support the statement. However, we could not find any literature describing this possibility. Therefore, we concluded that future study is necessary to claim the generality of the statement. We added the sentences describing these results and discussion in the main text (line 188 and 443).

Reviewer #1 (Remarks to the Author):

In this revised version, the authors have fully and appropriately addressed all of the points I raised in the initial review session, with the exception of the correction of the title of Ref. 14.

I support publication of the manuscript with this correction.

The title of Ref.14 was corrected.

Reviewer #3 (Remarks to the Author):

The authors were highly responsive to previous critiques and have addressed all of my significant concerns. This manuscript is now suitable for publication. One minor suggestion - the difference in CD spectra between L and D-proteins is small, but significant, and suggests that the D-protein likely has some impurities (e.g., deletion products) that are affecting its secondary structure (a common issue with synthetic D-proteins). This minor limitation should be acknowledged.

Thank you for the valuable information. We also noticed that the lower purity of Fmoc-D-aa often caused the increased by-product formation.