Transmembrane and Juxtamembrane Structure of αL Integrin in Bicelles

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Abstract

The accepted model for the interaction of α and β integrins in the transmembrane (TM) domain is based on the pair αIIbβ3. This involves the so-called outer and inner membrane association clasp (OMC and IMC, respectively). In the α chain, the OMC involves a GxxxG-like motif, whereas in the IMC a conserved juxtamembrane GFFKR motif experiences a backbone reversal that partially fills the void generated by TM separation towards the cytoplasmic half. However, the GFFKR motif of several α integrin cytoplasmic tails in non-bicelle environments has been shown to adopt an α-helical structure that is not membrane-embedded and which was shown to bind a variety of cytoplasmic proteins. Thus it is not known if a membrane-embedded backbone reversal is a conserved structural feature in α integrins. We have studied the system αLβ2 because of its importance in leukocytes, where integrin deactivation is particularly important. Herein we show that the backbone reversal feature is not only present in αIIb but also in αL-TM when reconstituted in bicelles. Additionally, titration with β2 TM showed eight residues clustering along one side of αL-TM, forming a plausible interacting face with β2. The latter orientation is consistent with a previously predicted reported polar interaction between αL Ser-1071 and β2 Thr-686.

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Introduction

Integrins are heterodimeric type I transmembrane (TM) proteins formed by non-covalent association of an α and β subunit. In humans, 18 α-chains can interact with 8 different β-chains to form 24 different αβ heterodimers [1]. Each subunit contains a large extracellular domain, a single membrane-spanning α-helix and a short cytoplasmic tail [2]. By spanning the membrane, integrins serve as a dynamic linkage between the cytoplasm and extracellular space, transducing signals across the membrane, although in some cases they were myristoylated and found previously for the homodimer glycoporphin A (GpA) [12]. For the second proposed mode of interaction [11,13], the β helix is rotated by approximately 90° from its orientation in the GpA-like model. This latter model has been validated for αIIbβ3 using activating mutations at the transmembrane domain [14], cysteine cross-linking of the full integrin [10,15], and NMR studies of the αIIbβ3 transmembrane heterodimer structure in bicelles [16]. Thus, this is currently accepted to represent the interaction of the two TM domains of αIIb and β3 in the resting state.

This accepted model has been further refined in the case of αIIbβ3 [15–17]. Specifically, the two TMcs are in close proximity at the extracellular half of the membrane, forming an outer membrane association clasp (OMC) that involves the GxxxG-like motif in αIIb, Gly-972xxGly-976, and Gly-708 in β3. The TMcs separate towards the cytoplasmic half, and the void is partially filled with an inner membrane association clasp (IMC), formed by a juxtamembrane backbone reversal in αIIb that participated by two phenylalanines, Phe-992 and Phe-993. The latter are part of a conserved α chain GFFKR motif. Lastly, a salt bridge between Arg-995 of αIIb and Asp-723 of β3 has also been identified [16]. At present, no other detailed structural model for other integrin TMcs is available.

In contrast to the above model for the juxtamembrane backbone reversal, the GFFKR motif in several α integrins e.g., in αIIb, α1, αM, αL, αX or αV [18–24], formed an α-helical structure that was not membrane-embedded. However, most of these reports included the cytoplasmic tail without the TM domain, although in some cases they were myristoylated and
attached to detergent micelles. Other reports included the TM domain, but the structure was determined in the presence of organic solvents or in presence detergent micelles. Thus, it is possible that the failure to observe such reverse turn in the above examples was due either to the absence of the TM domain, or to the fact that detergent micelles or organic solvents do not provide the appropriate environment for such structural feature.

Several reports show binding of several cytosolic proteins to a region in the β chain that encompasses this conserved motif, e.g., serine-threonine phosphatase 2A (PPP2A) [25], nsislan [26–28], calcium- and integrin-binding protein 1 (CIB1) [29–31], sharpin [32], or mammary-derived growth inhibitor (MDGI) [33]. Although the precise binding location of these proteins has not been elucidated, these data suggest that an α-helical solvent exposed structure is physiologically relevant.

Although the membrane-embedded reverse backbone has been observed in the presence of the TM domain and lipids, whether in bicelle systems or membranes [15,16], it has only been reported for αIIbβ3 integrin. Therefore, observing a membrane-embedded reverse turn in an α integrin–TM different from αIIb would support that this structural feature is conserved, and that it represents one of the possible conformations of the GFFKR motif in all integrins.

We have chosen to use the system αLβ2 because of its importance in leukocytes, where it is involved in adhesion, migration, cytotoxicity, proliferation, and antigen presentation [34]. The importance of the GFFKR motif in αL has been demonstrated by looking at the effect of deletion of this motif in a transgenic mouse, which led to a constitutive deactivation is particularly important, and we proposed previously that the latter could be facilitated by hydrogen bond interaction between TMs [36–38]. Thus, to elucidate if the reverse turn at the GFFKR motif is also found in αL when the peptide is reconstituted in bicelles, and to delineate the αL interfacial residues involved in αL/β2 interaction, we have expressed, purified and determined the structure of αL TM in bicelles by solution NMR. Further, titration experiments with non-labeled β2 TM show which residues in αL are most affected by this interaction.

**Experimental Procedures**

**Integrin αL TM and β2 TM Expression and Purification**

The DNA sequence encoding the TM region of human αL integrin, residues 1057–1103 (Fig. 1A), was cloned into a pET-28b expression vector, whereas the sequence for human TM β2 integrin, residues 675–710, was cloned into pNIC28-Bsa4. G685F and G689F mutations at in the GxxxG-like region of integrin, residues 675–710, was cloned into pNIC28-Bsa4. G685F and G689F mutations at in the GxxxG-like region of integrin, residues 1057–1103 (Fig. 1A), was cloned into a pET-28b (80/20 (v/v) with 0.1% TFA) linear gradient on a Phenomenex Jupiter C18 semipreparative column (250×10 mm, 300 Å pore size, 5 μm particle size). The peptides were lyophilized and stored in −20°C.

**NMR Sample Preparation**

The phospholipid bicelle system was composed of 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPC, Avanti Polar Lipids) and 1,2-dihexanoyl-sn-glycero-3-phosphate (DHPC, Avanti Polar Lipids). Peptides-DMPC mixture in methanol was dried under N₂ stream followed by high vacuum. DHPC solution in appropriate buffer was added into the dried peptide-DMPC mixture, vortexed and sonicated. In this way, lyophilized αL TM peptides were reconstituted into DHPC-DMPC bicelle (3% w/v, q = 0.3) at 0.6 mM (corresponding to approximately 1:100 peptide-to-lipid ratio) buffered with 50 mM potassium phosphate at pH 6.5.

Partial alignment of the αL-TM/bicelle complexes relative to magnetic field was obtained by using compressed and stretched polyacrylamide hydrogels [41,42]. Axially symmetric alignment tensor coefficients (axiality and rhombicity) were calculated using MODULE [43]. Briefly, 250 μL gels were co-polymerized with 0.6 mM αL TM-bicelle in 50 mM potassium phosphate at pH 6.5, from a 4.2% or 4.6% w/v solution of acrylamide, bis-acrylamide, and 2-acrylamido-2-methyl-1-propanesulfonate (AMPS) with acrylamide-bis ratio of 49:1 (w/w) and acrylamide-AMPS molar ratio of 9:46.

**NMR Spectroscopy** – NMR experiments were performed at 30°C using Bruker Avance-III 800 and Avance-II 700 NMR spectrometers with cryogenic probes. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal reference for ¹H nuclei. The chemical shifts of ¹C and ¹N nuclei were calculated from the ¹H chemical shifts. The NMR data were processed using TopSpin 3.1 (www.bruker-biospin.com) and analyzed using CARA (www.nmr.ch), Sequence-specific assignment of backbone ¹H, ¹N, ¹C and ¹C₀ was achieved by using 2D [¹H-¹³N]-TROSY-HSQC, 3D TROSY-optimized HNCO, HNCA, HN(CO)CA, HN(CA)CB, and HN(COCA)CB experiments on a fully-deuterated...
ated 15N/13C-labeled αL TM. Side-chain resonances were assigned using 3D 15N-resolved NOESY-HSQC (120 ms mixing time), (H)CCH-TOCSY and 13C-resolved NOESY-HSQC (120 ms mixing time) on non-deuterated and partially-deuterated 15N/13C-labeled αL TM. To identify membrane-embedded residues, the NMR sample was lyophilized overnight and reconstituted in 99% D2O. Immediately after reconstitution, 2D [1H-15N]-TROSY-HSQC was collected. The spectra measuring 1H-15N heteronuclear steady-state NOE were acquired with and without a 3s period of proton saturation. Titration experiments were performed by adding increasing amounts of non-labeled β2 TM to 50 μM 15N-labeled αL TM until there were no further changes in the [1H-15N]-HSQC spectra, from 1:0.5 to 1:6 α/β molar ratio.

Structure Calculation

NOE distance restraints to calculate the structures of αL TM domain were obtained from 15N-NOE-NOESY-TOCSY and 13C-NOE-NOESY-HSQC spectra, respectively. Backbone dihedral angle restraints (φ and ψ) were derived from 15N, 13Cα, 13Cβ, 1Hα and 1Hβ chemical shift values using TALOS [44]. The short-range and medium range NOE connectivities were used to establish the sequence-specific 1H NMR assignment and to identify elements of the regular secondary structure. Structure calculations were performed using CYANA 3.0 [45,46] and visualized using MOLMOL [47] and Cn3MOL (Delano Scientific). CNS 1.3 [48,49] was used to refine the structure using the standard simulated annealing protocol. A total of 20 structures have been calculated and the structure statistics are summarized in Table S1 in File S1. The structure of integrin αL TM domain has been deposited on the Protein Data Bank (PDB) with ID 2m3e, and the assigned chemical shifts have been deposited on the Biological Magnetic Resonance Bank (BMRB) with ID 18958.

Results

Structure of the αL TM Domain in Phospholipid Bicelles

The structure of bicelle-reconstituted integrin αL TM domain (αL) was determined by using a set of 2D and 3D NMR experiments on the 15N/13C-labeled peptide with varying deuteration levels. Upon reconstitution into DHPC-DMPC bicelles, αL forms a linear α-helix which extends approximately from Leu-1065 to Val-1089 (Fig. 1A-B and Fig. S2 in File SS). This α-helix comprises ~50% (25 residues) of the whole peptide.
This is significantly lower than \(~80–90\%\) \(\alpha\)-helix estimated by FTIR when this peptide was reconstituted in lipid bilayers (Fig. S3A in File S1), but the value is comparable to \(~60\%\) (30 residues) estimated from CD when the peptide was reconstituted in biciples (Fig. S3B in File S1).

The conserved GFFKR motif follows immediately the \(\alpha\)-helix, with Phe-1091 and Phe-1092 reinserting into the biciples (Fig. 1B–C) and forming a backbone reversal, only observed previously in GFFKb in biciples [16]. The structure is consistent with Phe-1091 making hydrophobic contacts to Leu-1086 and Val-1089, whereas the side-chain of Phe-1092 packs against Phe-1083 and Leu-1086, as indicated by NOE connectivities between these residues (Fig. 1C and Fig. 2). Thus Phe-1083, Leu-1086, Phe-1091 and Phe-1092 constitute a hydrophobic surface which potentially forms an interaction site with \(\beta\)2-TM. The superposition of our \(\alpha\)L-TM model and that reported for GFFKb-TM shows high structural similarity between the two proteins (Fig. 1D). This strongly suggests that the backbone reversal of the GFF motif is a characteristic structural feature of \(\alpha\) integrin subunits when reconstituted in biciples. Alignment of the rest of the TM domains produced an \(\alpha\)C RMSD of 0.83 Å. In comparison, the \(\alpha\)C RMSD with a recently published \(\alpha\)1 TM structure in micelles for which no reverse turn was observed [24] was 1.3 Å.

The membrane-embedded region of \(\alpha\)L was identified through an H\(^2\)D\(_2\)O exchange experiment. From a comparison between \([^{1}\text{H},^{15}\text{N}]\)-TROSY-HSQC spectra in water and in 99\% D\(_2\)O, amide protons of a stretch of 21 residues from Val-1069 to Val-1089, plus Phe-1092 in the GFFKR motif, are protected from exchange (Fig. 3A). This number is considerably lower than the 35 exchange-protected residues estimated from FTIR using the peptide reconstituted in lipid bilayers (see IR spectra before and after exchange in Fig. S3C in File S1).

The dynamic properties of \(\alpha\)L were studied using a steady-state \([^{1}\text{H},^{15}\text{N}]\) heteronuclear NOE experiment. The majority of the residues correspond to a well-folded structure, with the central part, Val-1059 to Asn-1095, being the most stable (Fig. 3B). The backbone RMSD of the central TM region from a set of 20 conformers is 0.23 Å, indicating a well-defined structure. On the other hand, the N and C termini, especially the latter, are highly dynamic regions (see also Fig. 1B).

Interaction between the TMs of \(\alpha\)L and \(\beta\)2

To determine what residues in \(\alpha\)L mediate interaction with \(\beta\)2, we titrated unlabeled \(\beta\)2 TM into 15N-labeled \(\alpha\)LTM, and monitored the changes in the \([^{1}\text{H},^{15}\text{N}]\)-HSQC spectra. Qualitative examination of the spectra at a 1:2 \(\alpha\)L/\(\beta\)2 molar ratio revealed eight residues with pronounced intensity reduction upon addition of \(\beta\)2 peptide, namely Leu-1067, Tyr-1068, Ser-1071, Gly-1072, Gly-1074, Gly-1075, Tyr-1087 and Phe-1091 (Fig. 4A). Met-1064 also showed intensity reduction, albeit less obvious, whereas Asn-1095 experienced a chemical shift change. Intensity of these resonances was reduced with increasing \(\beta\)2 peptide, until

Figure 2. NOE connectivity in the IMC region of \(\alpha\)L TM. Four selected strips from \(^{13}\text{N}\)-NOESY-HSQC and \(^{13}\text{C}\)-NOESY-HSQC show some NOE contacts among Leu-1086, Val-1089, Phe-1091, and Phe-1092. doi:10.1371/journal.pone.0074281.g002

Figure 3. Integrin \(\alpha\)L TM \(^{1}\text{H},^{2}\text{H}_{2}\text{O}\) chemical exchange and backbone dynamics. (A) \([^{1}\text{H},^{15}\text{N}]\)-TROSY-HSQC spectra in 99\% D\(_2\)O. The cross peaks are labeled by one-letter code and the residue number; (B) \([^{1}\text{H},^{15}\text{N}]\) steady-state heteronuclear NOE experiment. The three extra N-terminal residues, 5NA, are grayed out. The residues protected from H/D exchange are indicated by a line, and labeled as TM. doi:10.1371/journal.pone.0074281.g003
the signal disappeared (Fig. 4B). A summary of these changes for the α/β molar ratio 1:2 is shown in Fig. 4C. The same results were observed when the titration was performed with a β2 TM mutant where the ‘G’ residues in the GxxxG-like motif were changed to Phe, i.e., G685F and G689F (data not shown). The latter confirms that β2 does not interact with αL via its GxxxG-like motif, consistent with the reported αIIb/β3 model of interaction for resting integrins [10,16,50]. The affected residues cluster on one side of the αL-TM, with residues Ser-1071 and Gly-1075, located at positions 1 and 5 in the OMC region GxxxG-like motif, having the same orientation as the residues involved in the IMC (Fig. 5). At the same time, these locations are separated by a stretch of non-interacting residues, consistent with the TM separation previously observed for the αIIb/β3 system (see above). Thus, the putative IMC and OMC regions of αL TM are oriented to form a plausible interacting face with β2 TM.

The presence of αβ heterodimerization in our titration is supported by comparison of the TROSY-HSQC spectrum of 0.1 mM αL with that of 0.6 mM concentration obtained in identical conditions shows that increasing αL concentration does not lead to intensity reduction of the peaks (Fig. S4 in File S1). Therefore, the peak intensity reduction observed in our titration experiment is not caused by αL homodimerization. Also, membrane-mimicking screening studies [51] show a predominant presence of αβ heterodimers in a 1:1 integrin αβ-TM mixture at 0.1 mM, identical concentration to that used here, or even at 0.01 mM. In the latter report, in contrast, it was shown that homomeric species increase in DPC and SDS micelles, and also in organic solvents [51].

Discussion

Importance of Environment for Integrin TM Reconstitution

Phospholipid bicelles have been successfully used for structural determination of membrane proteins by solution NMR [52], especially with bicelles of low q-ratios. The inclusion of a small proportion of long-chain lipid makes this system more membrane-like than micelles, yet still reasonably small for solution NMR studies. The better suitability of bicelles, relative to micelles, has been demonstrated in the study of the integrin αIIbβ3 TM heterodimer in bicelles [16,51]; in micelles, these two TMs were not found to heterodimerize [51,53].
In addition, the unique backbone reversal for zIIb, was reported in bicelles [17] and full length integrin in biological membranes [15]. In presence of detergent micelles or organic solvents, the region encompassing the GFFKR motif adopted an α-helical conformation in zIIb and several other integrins [18-23,34], even when the TM domain was also present (e.g., [24,34]). Thus, not only the reconstitution environment may play a critical role for the observation of this reverse turn, but cytoplasmic peptide length and proper conformation of the TM region may be important. Indeed, the reverse turn formed by the GFFKR motif makes contacts with several side-chains in the α TM domain, as we (this paper) and others [16] have observed. Herein a hydrophobic packing is proposed in the αzL-TM for the reinserted Phe-1091 and Phe-1092 against Phe-1083 and Leu-1086. The titration data also suggests that Phe-1091 is strongly involved in the interaction with β2 TM, which indicates that the hydrophobic surface formed by Phe-1091 and Phe-1092, Phe-1083 and Leu-1086 is important in the interaction with β2 TM.

We also note that in lipid bilayers (FTIR experiments) 14 more residues were calculated to be protected from H/D exchange relative to the bicelle system (NMR). This higher number is unlikely to only originate from the inevitably larger error incurred when measuring band areas in IR. Rather, these differences must be of experimental origin; in the NMR experiment, bicelles that have a high curvature are re-solubilized in D2O. In contrast, in the FTIR experiment, preformed planar lipid bilayers are exposed to D2O-saturated air. Whether or not the reverse turn is present in the FTIR experiment is not known, but even considering it is present and completely protected from H/D exchange, this is not sufficient to account for these results. We propose that the extra protection to H/D exchange in membranes is due to the formation of a larger proportion of α-helix in extramembrane domains. Indeed, a higher helical content was observed in the FTIR studies performed in membranes (see above), compared to NMR and CD techniques, where bicelles were used. The increased α-helical content in extramembrane regions in supported lipid bilayers relative to the solution NMR conditions may arise from a far more dehydrated environment in the former. Tight binding of these α-helical domains to the membrane may explain the increased H/D exchange protection. These regions then become unbound, disordered and exposed to solvent when reconstituted in bicelles. The above results suggest that data obtained in organic solvents, micelles or bicelles probably underestimate the helical content and membrane binding behavior of juxtamembrane domains, when compared to lipid bilayers. Indeed, in the present work even the reverse turn formed by the GFFKR motif is not totally protected against H/D exchange in bicelles: Phe-1092 is protected, but Gly-1090 and Phe-1091 are not (Fig. 3A), although as discussed above this may arise from experimental protocol differences. In αIIb-TM, both of the equivalent Phe residues, showed little broadening after exposure to Mn2+EDDA2 and H218O chemical exchange [17], consistent with some degree of protection. Differences in methodology might also account for the shorter membrane-embedded region for αL, relative to αIIb, determined in the present paper.

Structure of αL in Bicelles

The results of αL structure calculation show that the bicelle-embedded αL peptide adopts a well-defined, highly α-helical conformation at the membrane-spanning region, as expected from a single-pass TM domain. The TM boundaries we found here are generally in agreement with previous studies [17,54,55]. The rest of the αL structure is remarkably similar to αIIb, especially with regards to the two proposed interaction points with the β2 chain at the two ends of the TM domain, i.e. the OMC and IMC regions.

In the OMC region, both alternative models proposed for α-β heterodimer interaction predict the small residues in the α TM GxxxG-like motif to face the β TM [11], as observed in the αIIbβ3 interface [16]. In αL, a polar residue, Ser-1071, was hypothesized to form a polar interaction with Thr-686 at the binding partner β2 [36]. Both the alignment of Ser-1071 with Gly-1075 and the hydrophobic IMC region, together with the sensitivity of these residues to titration with the β2 peptide, are consistent with this part of αL forming a polar interaction with β2. As polar interactions are stronger in non-polar environments [56,57], this has been proposed to have an impact on integrin deactivation, which is important during leukocyte migration [35]. However, further characterization of the αL/β2 interaction is necessary to confirm that point. The OMC region of αL may extend as far as Tyr-1068 and Met-1064, as suggested by the titration experiments.

A chemical shift change was also observed in Asn-1095. This residue was reported to be involved in heterodimerization of the αLβ2 cytoplasmic domain [21]. The equivalent residue in αIIb, Asn-996, experienced a large chemical shift change upon heterodimerization with β3 TM domain [16]. It is possible that the changes observed in αIIbβ3 Asn-996 are indirectly caused by a nearby salt bridge, Arg-995/αIIb-Asp723[β3] [16]. This salt bridge should also be present in αLβ2, between αL-Arg1094 and β2 Asp-731 [21]. Unfortunately, peak crowding of a large portion of the cytoplasmic tail prevented us from examining the behavior of other residues near this region, e.g. Arg-1094 and Lys-1097.

In summary, we have shown that the conserved GFFKR motif in α integrins forms a structural motif, a backbone reversal, that can be observed when TM domain, cytoplasmic tail and a lipid environment consisting or membranes or bicelles are present, although cytoplasmic tail length may also be an important variable for the stability of this turn [54]. This backbone reversal has been now observed in two different α integrins TMs, αIIb and αL. This is likely to be one of the structural forms of this motif, present in the inactive or intermediate activation state of integrins. We also

![Figure 5. Putative interacting face of αL TM. Stereo view ribbon representation of the αL TM domain (Val-1058 to Lys-1097) with residues most affected by heterodimerization with β2 shown in magenta. doi:10.1371/journal.pone.0074281.g005](image-url)
show that for αL/β2, interaction in the TM domain proper takes place through a remarkably similar structural organization to αIIb/β3, with features reminiscent of OMC and IMC claps. Further studies will include mutagenesis at the predicted α-β interface to confirm the observed interaction, and crucially, determination of interhelical NOE contacts that can unequivocally describe the precise nature of the polar interaction in αL/β2 pairs. This work is presently underway.

**Supporting Information**

File S1 Includes Table S1, Figures S1–S3. Table S1. Structure statistics for the selected 20 structures of integrin αL TM domain. Figure S1. Expression and purification of integrin αL TM and β2 TM peptides. (A) SDS-PAGE of methanol-extracted (meOH) and HPLC-purified (HPLC) αL-TM and β2-TM peptides. (B) MALDI-TOF MS spectra of the HPLC fractions corresponding to αL TM and β2 TM peptides. The letters inside square brackets identify the charged species forming a particular peak: M, H, Na, K corresponds to peptide, proton, sodium ion, and potassium ions, respectively. The charge of each species is indicated outside the square bracket. Figure S2. Secondary structure of the peptide αL used, based on NOE connectivity. Sequential and medium-range NOE connectivity between residues are displayed as bands under the respective residues. The dN(i,i+4) connectivities indicate the α-helical structure, extending from Leu-1065 to Val-1088. Figure S3. Secondary structure of integrin TM peptides. (A) FT-IR spectra of integrin TM α (upper) and β (lower) peptides reconstituted in DMPC lipid bilayers indicating that both TM peptides are almost completely α-helical. The amide I peak centered at 1657 cm⁻¹ (blue) and the Fourier self-deconvolved spectra (red) are shown; (B) CD spectra of integrin TM peptides in DHPC-DMPC bicelles, showing minima at 209 and 222 nm, indicating a high proportion of α-helical form for both peptides; (C) Amide I and amide II regions of integrin TM peptides when bulk water was removed (red) and after D2O-saturated air exposure (blue). The decrease in the amide II area at ~1550 cm⁻¹ indicates H-D exchange. Figure S4. Effect of increasing concentration on the [1H-15N]-TROSY-HSQC spectrum of αL-TM. αL TM peptide at 0.1 mM (A) or 0.6 mM (B). αL was reconstituted into DHPC-DMPC bicelles (3% w/v, q = 0.3) buffered with 50 mM potassium phosphate at pH 6.3 and spectra were recorded at 305 K.

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**Author Contributions**

Conceived and designed the experiments: WS YL OM TD JT. Performed the experiments: WS YL OM TD. Analyzed the data: WS YL. Contributed reagents/materials/analysis tools: OM TD JT. Wrote the paper: WS YL JT.

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