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Efficient production of fluorophore-labeled CC chemokines for biophysical studies using recombinant enterokinase and recombinant sortase

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Abstract

Chemokines are important immune system proteins, many of which mediate inflammation due to their function to activate and cause chemotaxis of leukocytes. An important anti-inflammatory strategy is therefore to bind and inhibit chemokines, which leads to the need for biophysical studies of chemokines as they bind various possible partners. Because a successful anti-chemokine drug should bind at low concentrations, techniques such as fluorescence anisotropy that can provide nanomolar signal detection are required. To allow fluorescence experiments to be carried out on chemokines, a method is described for the production of fluorescently labeled chemokines. First, a fusion-tagged chemokine is produced in *Escherichia coli*, then efficient cleavage of the N-terminal fusion partner is carried out with lab-produced enterokinase, followed by covalent modification with a fluorophore, mediated by the lab-produced sortase enzyme. This overall process reduces the need for expensive commercial enzymatic reagents. Finally, we utilize the product, vMIP-fluor, in binding studies with the chemokine binding protein vCCI, which has great potential as an anti-inflammatory therapeutic, showing a binding constant for vCCI:vMIP-fluor of 0.37 ± 0.006 nM. We also show how a single modified chemokine homolog (vMIP-fluor) can be used in competition assays with other chemokines and we report a K_d for vCCI:CCL17 of 14 μM. This work demonstrates an efficient method of production and fluorescent labeling of chemokines for study across a broad range of concentrations.

KEYWORDS
chemokines, enterokinase, fluorescence anisotropy, sortase, vCCI

1 | INTRODUCTION

Chemokines are small immune system proteins that bind 7-transmembrane receptors and mediate chemotaxis of leukocytes. Given their size (around 70 amino acids), these proteins are amenable to structural characterization by NMR, and the groups of Marius Clore

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EK, enterokinase; His_6-Tag, hexahistidine tag; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; NaCl, sodium chloride; NaOAc, sodium acetate; NaPi, sodium phosphate; Ni-NTA, nickel-nitrilotriacetic acid; O.D., optical density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Trx-KaiC, residues 1–247 of KaiC from *Thermosynechococcus elongatus*, with mutations Arg41Ala and Lys173Ala, and an N-terminal thioredoxin fusion tag; Trx-vMIP, thioredoxin-vMIP-II.

and Angela Gronenborn were instrumental in obtaining early high resolution structures of chemokines, such as CCL4 (MIP-1β) and CXCL8 (Interleukin-8).[1,2] There are four subfamilies of chemokines, based on the placement of conserved cysteines near the N-terminus of the proteins. So-called CC chemokines have contiguous cysteines and form one of the largest subfamilies, with about two dozen members, having roles from T cell maturation to inflammation upon injury and infection.[3,4] Notably, due to their tight binding to the chemokine receptor CCR5, several CC chemokines (CCL3, CCL4, and CCL5) have also been identified as inhibiting infection by HIV-1, which uses CCR5 as a co-receptor for infection.[4,5] Subsequently, variants of these chemokines have proven to be highly potent HIV inhibitors.[6–9]

Due to their role in inflammation, maturation, and cell homing, chemokines play a role in both health and disease, and have been implicated in several pathologies including arthritis, multiple sclerosis, and traumatic brain injury.[4,10–12] The study of these proteins remains robust, along with investigation of strategies to control their action. Therefore, there remains a need to optimize the production of chemokines, and in particular, to modify chemokines for biophysical study.[7,13–15] While isotopic labeling of chemokines for NMR is already well-documented, many binding studies rely on fluorescence spectroscopy, especially when working at the low concentrations that are necessary to investigate the binding constants of these proteins.

The poxvirus protein vCCI (also called p35), is a 243 amino acid beta sheet protein that tightly binds CC chemokines, with equilibrium dissociation binding constants often at low nanomolar or even sub nanomolar concentrations.[16–20] Due to its ability to inhibit chemokine function, vCCI is a potent anti-inflammatory agent and a possible therapeutic, especially if it can eventually be targeted to particular chemokines.[21–24] While nanomolar concentrations are not suitable for most protein NMR experiments, binding experiments observed via fluorescence spectroscopy can be carried out at these concentrations if the chemokine is labeled with a fluorophore. We have pursued methods to label CC chemokines in order to allow a variety of fluorescence experiments, particularly to study the vCCI:chemokine interaction.[19]

Many chemokines can be expressed in high amounts from *Escherichia coli* using the T7 expression system, although often an N-terminal fusion tag is required. Generally, for CC chemokines, it is most efficient to unfold the protein in denaturant such as guanidinium chloride and then refold it using any of a variety of conditions.[13,18,25,26] But there are still several impediments to the efficient production of fluorophore-labeled chemokine. One impediment is the cost of specific proteases to cleave the fusion tag, which can be hundreds of dollars per aliquot from commercial sources, but which is necessary because the activity of a CC chemokine is sensitive to the exact sequence at its N-terminus.[26,27] Several well-known proteases such as TEV and thrombin leave extra amino acids at their cut site, making them unsuitable for use in chemokine production. Another roadblock is the often-low yield (and sometimes low specificity) of chemical conjugation of a fluorescent tag, although this can be mitigated if the protein is small enough to allow solid phase synthesis, followed by efficient chemical addition of the fluorophore.[28]

We present here a comprehensive set of methods for producing and purifying fluorescently labeled CC chemokines (Figure 1), including reducing costs for cleaving a fusion tag (by inexpensively producing the highly specific protease enterokinase in-lab) and similarly by implementing enzymatic fluorescent labeling of the chemokine with in-house produced sortase. We then show the use of a fluorescently labeled model chemokine (the chemokine homolog vMIP-II) in both direct fluorescence anisotropy titration experiments and as a reagent

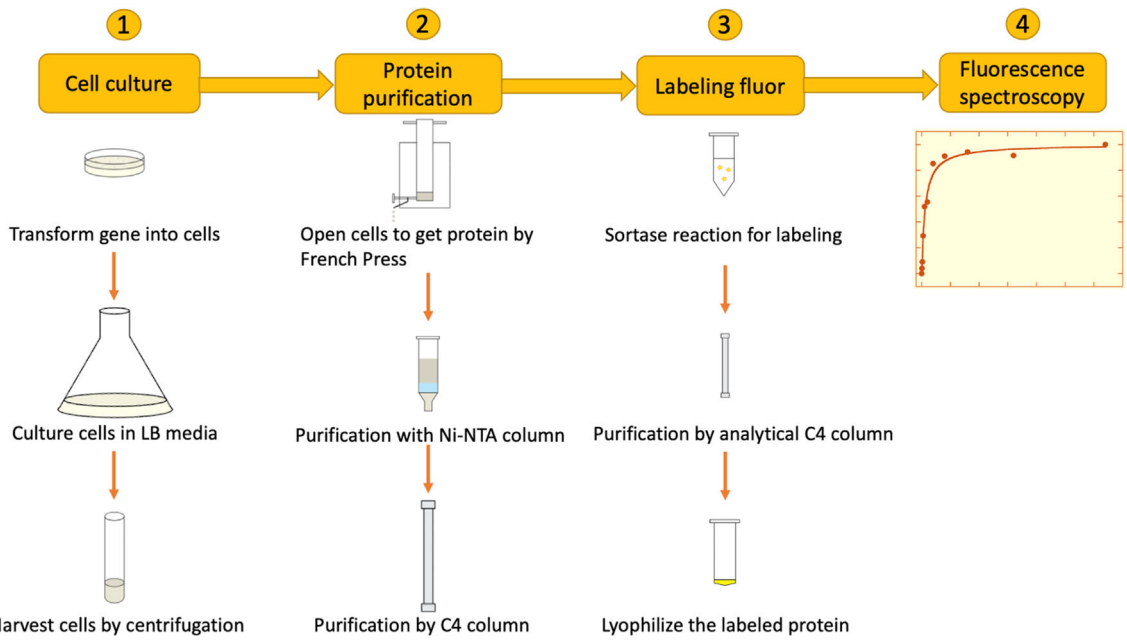

FIGURE 1 Flow diagram showing the steps of purification of fluorescently labeled chemokines.

to test binding by an unlabeled chemokine (CCL17) in competition anisotropy experiments.

2 | MATERIALS AND METHODS

2.1 | Materials

The gene sequence encoding the human Enterokinase (EK) catalytic subunit with an N-terminal tag (MGPINQTNDDDDK, which contains the EK recognition site for self-activation of EK), a single mutation Cys112Ser, and a C-terminal His_6-tag, was codon optimized in the pUC57 vector and purchased from GenScript (Piscataway, NJ). The DNA sequence is as follows:

ATGGGCCCTATTAACCAGACCAACGATGACGACGATAAAATT
GTGGGTGGCAGCAACGCTAAAGAAGGCGCGTGGCCTTGGGT
CGTTGGCCTGTACTATGGCGGTCGCCTGCTGTGTGGCGCGAG
CCTGGTTAGCTCCGATTGGCTGGTTAGCGCGGCGCACTGTGT
CTACGGCCGTAACCTGGAACCGAGCAAATGGACCGCCATCCT
GGGCCTGCACATGAAAAGCAACCTGACGAGCCCGCAAACCG
TACCGCGTCTGATCGATGAAATTGTGATCAATCCGCACTATAA
CCGCCGTCGTAAAGATAACGATATTGCGATGATGCATCTGGA
GTTTAAAGTTAACTATACCGATTATATTCAGCCGATTAGCCTG
CCAGAGGAGAATCAGGTTTTCCCGCCGGGCCGTAATTGTAGC
ATTGCCGGTTGGGGTACGGTCGTCTACCAGGGCACCACTGCC
AACATTCTGCAGGAGGCAGATGTGCCGCTGCTGAGCAACGA
ACGTTGCCAACAACAAATGCCGGAATATAATATTACGGAGAA
CATGATCTGCGCAGGTTACGAAGAAGGCGGTATCGATTCTTG
TCAGGGTGACAGCGGTGGTCCTCTGATGTGTCAGGAAAATAA
CCGCTGGTTTCTGGCAGGCGTTACTTCCTTTGGTTACAAATGC
GCACTGCCGAATCGCCCGGGTGTGTACGCACGCGTGTCCCG
TTTCACGGAATGGATTCAATCCTTTCTGCATCATCATCATCAC
CATTAA.

E. coli BL21(DE3) cells, XL1-Blue cells, plasmids pET15b, pET28a, and pET32a were purchased from Novagen (Madison, WI). Ni-NTA agarose was purchased from Qiagen (Valencia, CA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). The Broad-Range Molecular Weight Markers and 4%–20% Mini-PROTEAN® TGX™ Gels were purchased from Bio-Rad (Hercules, CA). The peptide GGGK-FAM (fluorescein amidite) was obtained from Bon Opus Biosciences.

2.2 | Production of chemokines and vCCI

Purification of chemokines (both vMIP-II and CCL17) was carried out as reported previously.[19,20] The chemokine gene with a thioredoxin tag followed by an enterokinase cleavage site on the N-terminus and a LPMTGHHHHHH tag on the C-terminus was transformed into *E. coli* BL21(DE3) cells (Novagen, Madison, WI, United States) and cultured at 37°C. When the cells' optical density OD_{600} reached ~0.7, protein expression was induced by adding IPTG to a final concentration of 0.5 mM and cells were allowed to grow for 18 h at 22°C. Cells were harvested by centrifugation at 4400 × g, 4°C for 10 min. The cell pellet was then resuspended in Lysis Buffer (6 M Guanidine HCl, 200 mM NaCl, 50 mM Tris, 10 mM benzamidine, pH 8.0) and French pressed twice at 16,000 p.s.i. After centrifuging the lysate for 1 h at 27,000 × g, the supernatant was purified by a home-packed Ni-NTA affinity column (Qiagen, Hilden, Germany). The protein was eluted with a pH gradient ending at pH 4. The purified protein was reduced with 10 mM β-mercaptoethanol (βME) at room temperature for 2 h while stirring. Then the protein was added dropwise with a 10-fold dilution into Refolding Buffer (550 mM L-arginine hydrochloride, 400 mM sucrose, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM reduced glutathione (GSH), 0.1 mM oxidized glutathione (GSSG), 50 mM Tris, pH 8.0) and allowed to refold for 24 h at 4°C with stirring. The protein was then dialyzed in 4 L of High Salt Buffer (200 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7.4) four times at 4°C. Subsequently the protein was cleaved using ~650 nM enterokinase for 1–3 days. (Due to low concentration of chemokine upon refolding and cutting at 4°C, higher concentrations of EK are used here.) Finally, the cleaved protein was purified by a C4 reversed phase chromatography column (Vydac, Hesperia, CA, United States) using an acetonitrile gradient.

vCCI was produced from *E. coli* as previously described.[20]

2.3 | Construction of the enterokinase plasmids

The gene encoding the EK in the pUC57 vector is flanked by several restriction sites on the 5′-end (Xba I, Bgl II, and Nco I) and 3′-end (EcoR I, Sac I, Sal I, Hind III, Not I, Xho I, and BamH I) of the DNA. The EK gene was cloned into various pET expression plasmids by using the restriction sites of Nco I and BamH I to generate pET15b-EK, Nco I and Xho I to generate pET28a-EK, and Bgl II and Xho I to generate pET32a-EK. All constructs were verified by DNA sequencing to ensure correct sequences.

2.4 | Expression of recombinant enterokinase

E. coli BL21(DE3) cells were transformed with individual EK plasmids, and grown overnight on LB agar plates containing 50 μg/mL kanamycin for pET28a-EK, or 100 μg/mL ampicillin for pET15b-EK and pET32a-EK. Colonies from each plate were inoculated into 5 mL LB starter cultures containing antibiotics (50 μg/mL kanamycin or 100 μg/mL ampicillin) and grown in a shaker at 37°C, 220 rpm for several hours until the cultures reached log phase of growth. The starter cultures were then added into 1 L of pre-warmed LB (37°C) containing antibiotics and allowed to grow at 37°C, 220 rpm until reaching an $O.D._{600}$ of 0.6–0.8, at which protein production was initiated by the addition of IPTG (1 mM final concentration). After 4 h, the cells were harvested by centrifugation at 4200 × g, 4°C for 10 min and the supernatant was discarded. The harvested cell pellets were stored at −20°C.

2.5 | Extraction and refolding of enterokinase

The harvested cell pellets were resuspended with 20 mL of Lysis Buffer (500 mM NaCl, 50 mM Tris, pH 8), and lysed by three passages through a French press at 16,000 psi (Thermo Fisher, PA). The resulting cell lysate was then centrifuged at 27,000 × g, 4°C for 1 h. The supernatant was discarded, and the pellet was dissolved in 20 mL of Resuspension Buffer (6 M Guanidinium chloride, 200 mM NaCl, 50 mM Tris, pH 8) at room temperature for 2 h with agitation. This mixture was then centrifuged at 27,000 × g, 4°C for 1 h and the supernatant was collected. The supernatant was passed through a 3 mL home-packed Ni-NTA affinity column equilibrated with Resuspension Buffer. The column containing the bound EK was washed with 10 column volumes of Resuspension Buffer, and then with 10 column volumes of Wash Buffer (6 M Guanidinium chloride, 200 mM NaCl, 20 mM NaPi, pH 7.2). The EK was eluted from the Ni-NTA affinity column with Elution Buffer (6 M Guanidinium chloride, 200 mM NaCl, 60 mM NaOAc, pH 4). Fractions containing the eluted EK were identified by absorbance at 280 nm, or by SDS-PAGE after removal of guanidinium chloride by trichloroacetic acid (TCA) precipitation as described in the next paragraph.[29] The fractions were combined (5–10 mL total volume) and reduced with 5 mM DTT at room temperature for 2 h with agitation. The solution was then added dropwise into a 15-fold volume of Refolding Buffer (700 mM L-arginine hydrochloride, 1 mM EDTA, 200 mM NaCl, 50 mM Tris, pH 8, modified from FoldIt Screen, Hampton Research, Aliso Viejo, CA) and incubated overnight at 4°C with gentle stirring.

For TCA (trichloroacetic acid) precipitation, all the samples and reagents are kept on ice throughout. Ten microliters of TCA is added into each 40 µL of protein sample, vortexed 5 s, then placed on ice for 10 min. The samples are then centrifuged at 15 k rpm for 5 min and placed back on ice immediately. The supernatant is then removed without disturbing the pellet (which may not be visible). The pellet is washed with 180 µL ice-cold acetone and then centrifuged at 15 k rpm for 5 min, and the supernatant is discarded; this wash step is repeated. The sample pellet is placed in a 60°C heat block for 10 s to evaporate the remaining acetone and 20 µL of desired SDS-PAGE sample buffer is added. The sample is heated for 1 min in a 95°C heat block before running SDS-PAGE.

2.6 | Dialysis and concentration of enterokinase

After refolding overnight, trace amounts of precipitation in the refolded EK solution were removed by centrifuging at 3600 × g, 4°C for 10 min. The refolded EK solution was dialyzed against 4 L of EK Cleavage Buffer (200 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7.5) at 4°C. The buffer was changed every 8–10 h. Active EK was obtained after 20 h of dialysis, as evidenced by an SDS-PAGE gel showing the self-cleavage of its N-terminal fusion tag. Any precipitates were removed by centrifugation at 3600 × g, 4°C for 10 min. A stock solution of 5 M NaCl was added to the EK solution to adjust the final concentration of NaCl to 500 mM. The EK solution was then loaded onto a 3 mL home-packed Ni-NTA affinity column equilibrated with Native Equilibration Buffer (500 mM NaCl, 20 mM Tris, pH 7.5). The column was then washed with 10 column volumes of Native Wash Buffer (500 mM NaCl, 20 mM Imidazole, 20 mM Tris, pH 7.5), and finally, the protein was eluted with a small volume (10–15 mL) of Native Elution Buffer (500 mM NaCl, 250 mM Imidazole, 20 mM Tris, pH 7.5). Fractions containing EK were identified by SDS-PAGE and combined. The final EK solution was added with glycerol to 50% (vol/vol), aliquoted into individual tubes, and stored at −20°C. For concentration determination, prior to addition of glycerol, the EK solution may be dialyzed against 500 mM NaCl, 20 mM Tris, pH 7.5, and then its absorbance measured at 280 nm. The concentration of EK can be calculated using an extinction coefficient of 55,390 M^{-1} cm^{-1}.[30]

2.7 | Digestion of substrate proteins by EK

Substrate proteins thioredoxin-KaiC 1–247 R41A K173A (Trx-KaiC)[31] and thioredoxin-vMIP-II (Trx-vMIP)[14] were used to test the activity of EK. These proteins contain a thioredoxin fusion tag followed by a His_6-tag and the EK recognition site (DDDDK↓). These substrate proteins were produced following procedures as previously described.[14,31] To test the EK activity, 500 µg of the substrate proteins were digested with various amounts of EK in 1 mL Cleavage Buffer (200 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7.5) at 4°C and 25°C. The substrate proteins were quantified by measuring the absorbance at 280 nm, using the extinction coefficients of 30,035 M^{-1} cm^{-1} for Trx-KaiC and 33,835 M^{-1} cm^{-1} for Trx-vMIP.[30] Time point samples were taken and analyzed on 17% Tris-Glycine SDS-PAGE for Trx-KaiC, and 4%–20% Mini-PROTEAN® TGX™ Gels for Trx-vMIP.

2.8 | Production of the sortase enzyme from staphylococcus aureus

The gene for the sortase A enzyme was a gift of Dr. Archana Chavan, and was encoded with a 6-histidine tag on the N-terminus. This was transformed into *E. coli* BL21(DE3) cells, and cells were grown in M9 minimal media in a shaker at 37°C. Protein production was induced with IPTG (0.5 mM final concentration) at OD_{600}~0.7. The temperature was reduced to 18°C and shaking continued overnight (16 h). Cell pellets were collected by centrifugation for 10 min at 4400 × g at 4°C. The pellets were resuspended with ~20 mL Buffer A (150 mM NaCl, 50 mM Tris, 20 mM Imidazole, pH 7.5) and the cells lysed 3× using a French press at 16,000 psi. Lysate was clarified by centrifuging at 27,000 × g, 4°C for 1 h. Then the clarified lysate was introduced to a home-packed Ni-NTA affinity column equilibrated with Lysis Buffer and passed through the column three times. The column was washed with 10 column volumes of Buffer A, and then eluted with Buffer B (150 mM NaCl, 50 mM Tris, 500 mM Imidazole, pH 7.5). The fractions containing

His-Sortase A were identified by SDS-PAGE and combined followed by dialyzing in 4 L Sortase Buffer (150 mM NaCl, 50 mM Tris, pH 7.5) and then aliquoted and stored at −20°C. A 1 L prep can generally produce around 20 mL of 30–70 μM, which is about 20 mg protein per prep.

2.9 | Labeling chemokine with fluorescein

In order to obtain a high yield of chemokine-fluor, several related fusion tags on the C-terminus of the chemokines were tested using the sortase reaction, including LPMTGG, LPMTG-CHis, LPETG-CHis, and LPETG-2His. "CHis" is a 6-histidine tag, and "2His" is a 12 histidine tag, which was tested so that the cleavage of the tag during the reaction would be more easily visible on an SDS-PAGE gel due to the change in size of the starting protein. In our hands, the LPMTGHHHHHH tag could realize relatively high yield and had easy purification, while the others had lower yield and were more difficult to purify after the sortase reaction.

For the ligation of the fluorophore-peptide with the chemokine analog vMIP-II, 50 μM vMIP-II-LPMTG-CHis (powder was dissolved in Sortase Buffer [150 mM NaCl, 50 mM Tris, pH 7.5]), 10 mM $CaCl_2$, 5 μM His-Sortase A, and 150 μM GGGK5FAM (Bon Opus Biosciences) were incubated in the dark at 4°C for 2 days followed by incubating in ambient temperature for 17 h. The time required for a sortase reaction is heavily dependent on the substrate, with some proteins requiring less (as little as a few hours) or more time (as much as 3 days). There is also a dependence on the particular amino acids used in the sortase tag on the same target protein.[32] The target protein vMIP-II-LPMTGGGK5FAM, was then purified through an analytical C4 reversed-phase chromatography column (Vydac, Hesperia, CA, United States), and verified by SDS-PAGE and mass spectrometry. The target chemokine-fluor was then aliquoted, then lyophilized and stored in the dark at −20°C. The protein was rehydrated in 20 mM potassium phosphate and 100 mM NaCl, pH 7.0 for fluorescence assays.

2.10 | Fluorescence anisotropy

The fluorescence anisotropy titration assays were performed in 20 mM potassium phosphate and 100 mM NaCl, pH 7.0, on a fluorimeter (PC1, ISS, Champaign, IL) (excitation: 498.4 nm, emission: 523.9 nm) at 25°C controlled by a water bath (VWR International, Visalia, CA). The reading of anisotropy was carried out with the excitation filter: 497/16, emission filter: 524/24 (BrightLine Fluorescence band pass filter). The concentration of the vMIP-II-LPMTGGGK5FAM stock solution (buffer: 20 mM NaPi, pH 2.5) and vCCI stock (buffer: 100 mM NaCl, 20 mM NaPi, pH 7.0) were measured by the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific Pierce). The vMIP-II-LPMTGGGK5FAM was diluted to a desired concentration in a beaker and mixed 1 min by shaking, and then 1990 μL was aliquoted into each cuvette and kept in the dark. Then, 10 μL of desired stock concentration of vCCI (in random order to remove possible bias from using a regular order) was added into one cuvette and mixed for 1 min by shaking the cuvette in a 50 mL beaker. After that, the cuvette was incubated at 25°C for 30 min to allow equilibration before measurement. Anisotropy data were recorded, and upon hitting a plateau, the average data over 10 min was recorded, and all the anisotropy values were normalized to the proportion of 100% bound anisotropy value. The data were fit to a system of mass conservation equations that included the following equation as previously described[19]:

$$\theta = \frac{[L]_{\text{free}} \times K_a}{1 + [L]_{\text{free}} \times K_a} \quad (1)$$

where θ is the fraction of vMIP-II bound to vCCI, $[L]_{\text{free}}$ is the free vCCI concentration approximated by the total vCCI concentration, and K_a is the equilibrium association constant ($1/K_d$).

Binding constants using competition for the interaction between CCL17 and vCCI were obtained by titrating this ligand into a preformed complex of vCCI and vMIP-fluor at 8 nM[20] and observing the competition displacement of vCCI as a function of concentration at equilibrium via the observed change in anisotropy. The data were then normalized fit to a system of equations describing the conservation of species concentrations and both equilibria to yield the K_d for the CCL17:vMIP interaction using Scientist software (Micromath, St. Louis, MO).[19] The data were fit utilizing a K_d for vCCI:vMIP of 0.37 nM (found in this work) which yielded a K_d for vCCI:CCL17 of 16 nM; then fit using a K_d for vCCI:vMIP of 0.06 nM (reported previously[20]) which yielded a very similar K_d for vCCI:CCL17 of 14 nM. The rationale for using competition experiments as opposed to direct binding to measure the K_d for CCL17/TARC was that while this protein can be labeled with fluorophore as described for vMIP-II, the resulting peptide-fluor on the C-terminus of the protein was too mobile to provide useful anisotropy values upon directly binding vCCI. Therefore, it was used in its unlabeled form to compete for binding with vMIP-fluor.

2.11 | Mass spectrometry

The purified protein from the sortase reaction was collected after running it through a C4-reversed phase column and then lyophilized. To determine the mass of the intact protein, an aliquot of the purified protein was directly injected into an electrospray ionization mass spectrometer (ESI-MS) (Q-Exactive Hybrid Quadrupole-Orbitrap, Thermo Scientific). Additionally, some unpurified protein samples from the sortase reaction were diluted with 95% acetonitrile, 5% water, and 0.1% formic acid and then injected into a mass spectrometer coupled with an ultra-high performance liquid chromatography (UHPLC) system (Vanquish Flex, Thermo Scientific) at a flow rate of 0.3 mL/min for scouting purposes. The spectra, shown as signal intensity versus mass/charge, were analyzed using BioPharma Finder 2.0 software (Thermo Scientific).

3 | RESULTS AND DISCUSSION

3.1 | Expression and purification of CC chemokines

CC chemokines have been easily expressed by a variety of methods for many years, so will only be briefly described here. Expression is usually effected using a T7 expression system (such as the pET system) in *E. coli* such as BL21(DE3), encoding the T7 RNA polymerase. Since solubility of the chemokine is sometimes limited in the supernatant of disrupted cells, it is often desirable to adjust disruption conditions to place the chemokine into the inclusion body, followed by dissolution of the inclusion body in 6 M guanidinium chloride, or to simply disrupt the cells in 6 M guanidinium chloride. This is generally followed by a His-tag affinity column such as a Ni-NTA column, followed by refolding the semi-purified protein and dialysis. Since it is common to produce chemokines with an N-terminal fusion partner, this can be selectively cleaved at this point by an appropriate protease, such as by enterokinase (below) or by ULP-1 protease if the fusion tag is the SUMO protein.[20] Each of these proteases leaves no extra amino acids on the chemokine N-terminus upon cutting. Finally, CC chemokines are amenable to final purification by a C4 reversed phase column, which has the benefit of de-salting the protein (so that it can be lyophilized without other components like salts or buffer) and of removing bacterial endotoxins (which is important if the chemokine will be used in cell or animal studies).

For the present work, the CC chemokine homolog vMIP-II containing a C-terminal LPMTG sequence to allow eventual sortase labeling, was expressed with a thioredoxin tag, partially purified using its

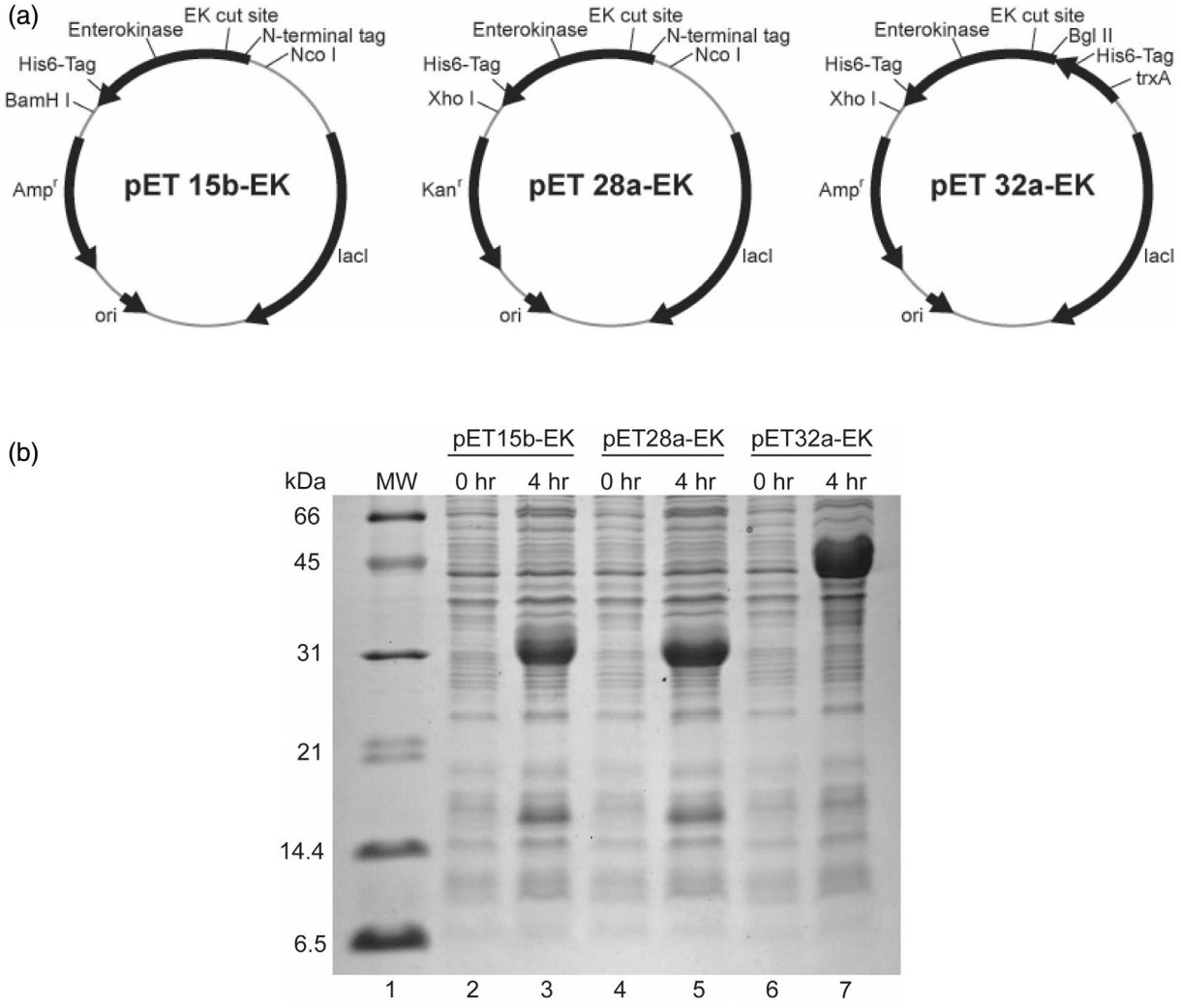

FIGURE 2 (a) Schematic representation of the enterokinase expression vectors pET15b-EK, pET28a-EK, and pET32a-EK. (b) Expression of Enterokinase from various vectors in BL21 (DE3) cells at 37°C. Lane 1: Broad range molecular weight marker. Lane 2: Pre-induction time point of pET15b-EK. Lane 3: 4 h post-induction of pET15b-EK with 1 mM IPTG. Lane 4: Pre-induction time point of pET28a-EK. Lane 5: 4 h post-induction of pET28a-EK with 1 mM IPTG. Lane 6: Pre-induction time point of pET32a-EK. Lane 7: 4 h post-induction of pET32a-EK with 1 mM IPTG. The pET32a-EK has a thioredoxin fusion tag at the N-terminus of EK, resulting in a higher apparent molecular mass compared with the other two constructs.

His-tag with a Ni-NTA column, refolded, and finally purified with a C4 reversed phase column (Figures 1 and S1). The human CC chemokine CCL17/TARC was similarly expressed and purified. In each case, several milligrams per liter of pure protein were produced; the yield can be improved if necessary by using more of the semi-pure protein from the Nickel column, although this leads to very high volumes upon the dilution necessary for refolding.

3.2 | Production of enterokinase

Assuming a chemokine construct with an EK cleavage site (DDDDK↓), the cleavage step of chemokine purification can be efficient but costly if commercial sources of protease are used. To more easily obtain EK in the lab, we have developed a method to purify the human EK light chain from *E. coli*. The human EK gene was codon optimized for expression in *E. coli*, and flanked with various restriction sites to allow convenient subcloning into the pET vectors 15b, 28a, and 32a (Figure 2a). Two of these vectors, pET15b-EK and pET28a-EK, confer different antibiotic resistance (ampicillin vs. kanamycin, respectively), but produce an identical fusion EK protein. The third construct, pET32a-EK, expresses a thioredoxin fused to EK (Trx-EK).

The EK expressed using our designed pET15b-EK and pET28a-EK vectors contains an N-terminal tag (MGPINQTNDDDDK) that includes the EK cleavage site. This tag was incorporated as a tell-tale marker, as its self-cleavage from the initial fusion protein makes it easy to monitor successful production of mature, functional EK. The N-terminal tag is immediately followed by the catalytic subunit of human enterokinase, with a single Cys112Ser mutation which has been shown to improve refolding yield.[33] At the C-terminus of the EK, a His_6-tag was added, enabling the purification of EK by a simple Ni-NTA affinity column, as well as its convenient removal after cleaving a target protein.

EK expressed in pET32a-EK includes original components from the pET32a vector: starting from the N-terminus, there is a thioredoxin fusion partner (105 amino acid residues), followed by a His_6-tag, an S-tag (the N-terminal 15 amino acids from RNase A that can be used in affinity purification) and an EK cleavage site. Following these components is the human EK catalytic subunit with the Cys112-Ser mutation, then a C-terminal His_6-tag. This thioredoxin fusion partner makes the self-cleavage of EK easier to monitor by SDS-PAGE. It is worth noting that by design, both the thioredoxin fusion partner and the final EK would each contain a His_6-tag, so they would both bind to Ni-NTA beads. Therefore, the Trx-EK can be purified and self-cleavage of the Trx tag can occur, but no additional purification is needed before adding this to a solution with target protein, since both Trx and EK can be removed together by binding to Ni-NTA beads. In cases where the co-existing thioredoxin tag may raise concerns, EK produced by the pET15b-EK and pET28a-EK vectors may be preferred.

When expressed in *E. coli* BL21(DE3) cells, all the three constructs were able to produce EK at high levels as evidenced by SDS-PAGE (Figure 2b). Despite the difference in antibiotic resistance, no apparent difference in expression level was observed for pET15b-EK and pET28a-EK (Figure 2b, lanes 3 and 5). Due to the similarity in experimental procedures for purification of EK from each of these constructs, only the purification of the EK from pET28a-EK will be discussed here; this protein has a 13 amino acid residue fusion tag at the N-terminus.

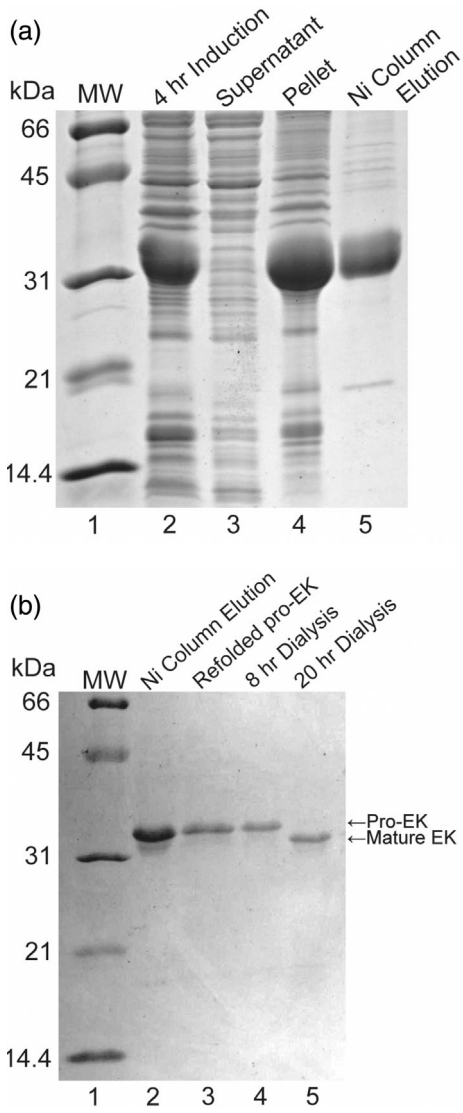

FIGURE 3 (a) Purification of enterokinase expressed by pET28a-EK using a Ni-NTA column. Lane 1: Broad range molecular weight marker. Lane 2: 4 h post-induction of pET28a-EK with 1 mM IPTG. Lane 3: Supernatant of cell lysate. Lane 4: Pellet of cell lysate. Lane 5: Elution from Ni-NTA column with pH 4 Elution Buffer containing a denaturing concentration of guanidinium chloride. TCA precipitation was performed to remove guanidinium chloride before preparation of gel samples. (b) Self-cleavage of the N-terminal tag during dialysis to produce mature EK. Lane 1: Broad range molecular weight marker. Lane 2: Elution from Ni-NTA column by pH 4 Elution Buffer. Lane 3: pro-EK after the refolding procedure and before the start of dialysis. Lane 4: Refolded pro-EK after 8 h of dialysis. Dialysis Buffer was changed once after the first 8 h. Lane 5: Self-cleaved, mature EK after 20 h dialysis. Samples containing guanidinium chloride were processed by trichloroacetic acid (TCA) precipitation.

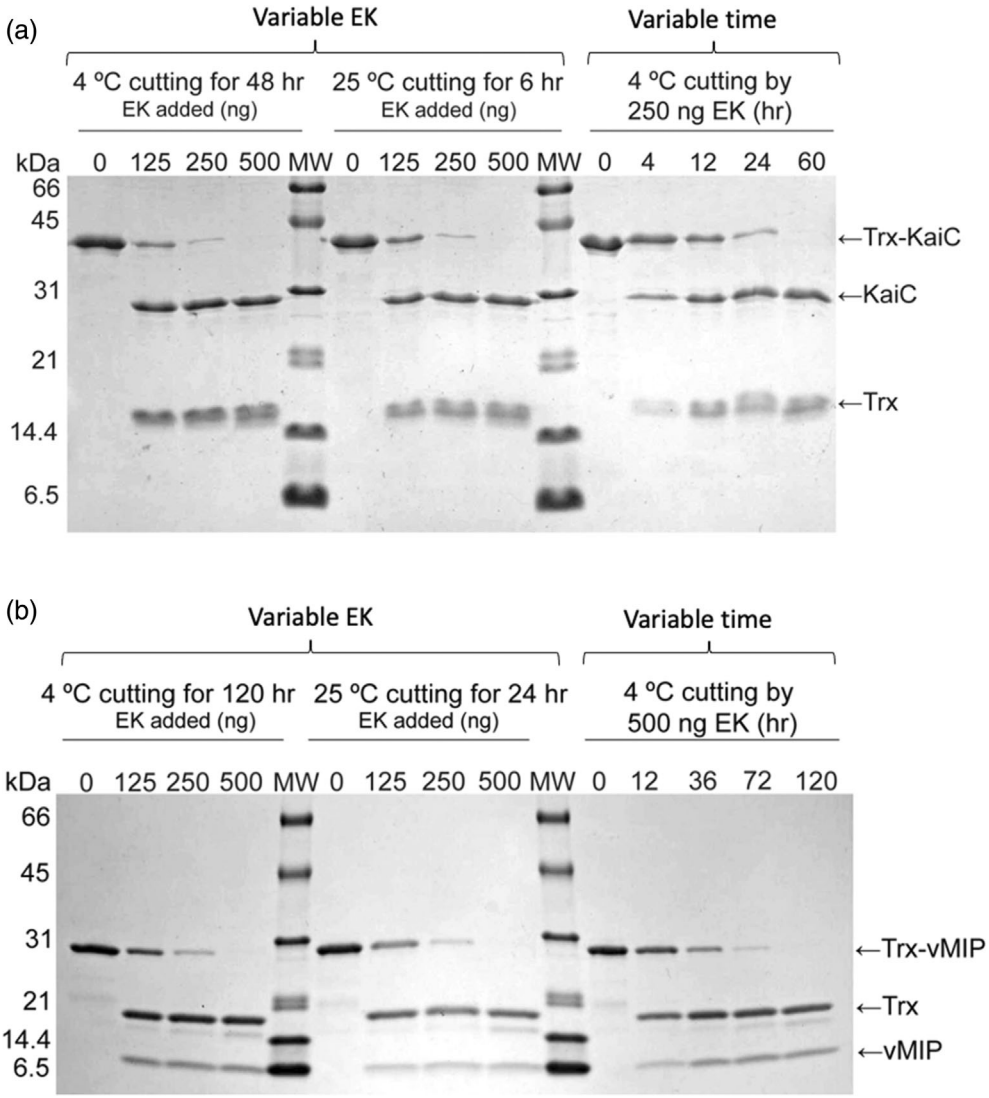

FIGURE 4 (a) Cleavage reaction of the Trx-KaiC fusion protein by EK. Samples containing 500 μg of Trx-KaiC in 1 mL Cleavage Buffer with various amounts of EK were incubated at 4 or 25°C. Left set: 4°C for 48 h with 0, 125, 250, and 500 ng of EK. Middle set: 25°C for 6 h with 0,125, 250, and 500 ng of EK. Right set: Time course of 0, 4, 12, 24, and 60 h at 4°C with a fixed amount (250 ng) of EK. (b) Cleavage reaction of the Trx-vMIP fusion protein by EK. Samples containing 500 μg of Trx-vMIP in 1 mL Cleavage Buffer with various amounts of EK were incubated at 4 or 25°C. Left set: 4°C for 120 h with 0, 125, 250, and 500 ng of EK. Middle set: 25°C for 24 h with 0, 125, 250, and 500 ng of EK. Right set: Time course of 0, 12, 36, 72, and 120 h at 4°C with a fixed amount (500 ng) of EK.

To summarize the purification of expressed EK, the cell pellet was resuspended in a Lysis Buffer containing a high concentration of NaCl (500 mM) to drive the EK fusion protein into the lysate pellet (Figure 3a, lanes 3 and 4). After cell disruption, this pellet was re-solubilized with buffer containing guanidinium chloride to extract tagged EK in an unfolded form. The crude supernatant was twice passed through a Ni-NTA affinity column and the EK was eluted by guanidinium chloride buffer at pH 4. Analysis by SDS-PAGE revealed that the EK was purified to around 90% purity after this step (Figure 3a, lane 5). To correctly fold EK into its native, functional tertiary structure, the purified EK was first fully reduced to break any disulfide bonds that may have formed, then the protein solution was refolded as described in Section 2 and dialyzed in Cleavage Buffer where self-cleavage to remove the N-terminal tag is observed (Figure 3b, lane 5).

The matured EK was purified once more by loading the solution onto a Ni-NTA affinity column, and eluting with a small volume of buffer that contained 250 mM Imidazole, resulting in concentrated EK solution that could be used as a stock. Approximately 2 mg of active EK was purified from a 1 L *E. coli* prep as determined by absorbance at 280 nm. Note that this amount of protease is sufficient for the needs of most labs for several months but could be optimized for higher yields if desired. The final EK product was stored in 50% glycerol (vol/vol) at either −20°C or −80°C. No special procedure was used to remove the imidazole, as upon usage, the EK solution will be diluted by at least several hundred fold when added into a target

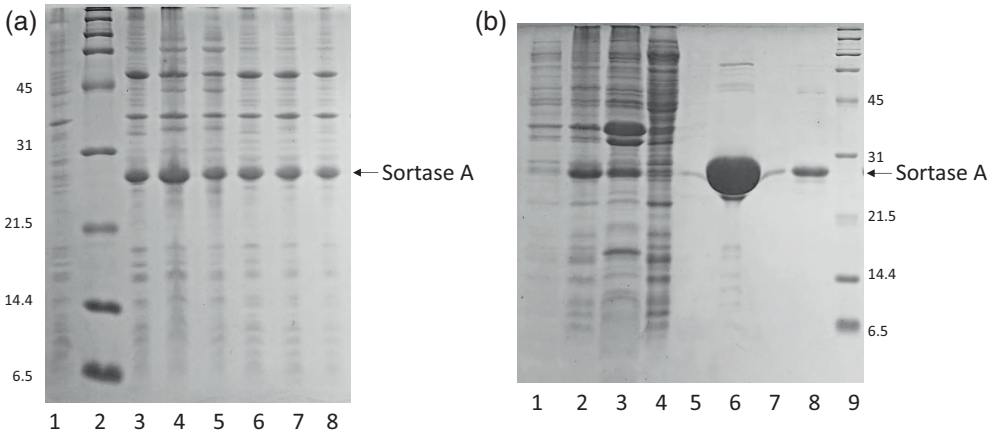

FIGURE 5 Expression and purification of sortase. (a) Expression of sortase in BL21 (DE3) cells at 37 and 22°C. Lane 1: Pre-induction. Lane 2: Molecular weight marker. Lane 3: 3 h post-induction at 37°C. Lane 4: 5 h post-induction at 37°C. Lane 5: 7 h post-induction at 37°C. Lane 6: 16 h post-induction at 22°C. Lane 7: 18 h post-induction at 22°C. Lane 8: 20 h post-induction at 22°C. (b) Purification of sortase A with a Ni-NTA column. Lane 1: Pre-induction. Lane 2: 5 h post-induction at 37°C. Lane 3: Pellets after lysing. Lane 4: Ni-NTA column flow through. Lane 5: Ni-NTA column wash. Lane 6: Elution 1 from Ni-NTA. Small amounts of impurities from *E. coli* proteins are observed if a large loading amount is used. Lane 7: Further elution from Ni-NTA. Lane 8: Result of further size exclusion column which is no longer used in lab due to sufficient purity after Ni-NTA column. Lane 9: Molecular weight marker.

protein, and it has been shown that moderate concentrations of imidazole do not appear to affect the proteolytic activity of EK.[34]

3.3 | Digestion of substrate fusion proteins with EK

To test the functionality of the purified EK, two recombinant proteins, each with an N-terminal thioredoxin fusion tag followed by an EK cleavage site, were chosen as substrates. vMIP-II is a small viral chemokine homolog of 8 kDa[13,14] that will be used in fluorescent labeling (below), and the 26 kDa domain of KaiC is from a critical circadian clock protein.[31] For the cutting reaction, 500 μg of Trx-vMIP-II or Trx-KaiC in 1 mL of EK Cleavage Buffer were digested with increasing amounts of enterokinase, with the mass ratio of substrate protein to EK kept at or above 1000:1 (125, 250, and 500 ng EK). In research labs, cutting reactions for recombinant proteins are frequently carried out either at room temperature, or at a colder temperature to limit bacterial or fungal growth when the cutting takes a longer period of time. To determine functionality under these conditions, the test cutting reactions were carried out at 25°C and 4°C. Time point samples were taken periodically and analyzed by SDS-PAGE (Figure 4a,b). The results showed that the time needed to achieve complete proteolytic cleavage for each substrate protein differed. Five hundred micrograms of Trx-KaiC was completely cleaved by 500 ng of EK in 48 h at 4°C, or in 6 h at 25°C (Figure 4a, left and middle section). For 500 ng of EK to completely cleave 500 μg of Trx-vMIP, the time needed was about 120 h at 4°C, or 24 h at 25°C (Figure 4b, left and middle section).

Although the purification of active recombinant EK has been reported previously by several groups,[35–37] we have made significant improvements on the production method, presenting a streamlined protocol that produces functional EK in as little as 4 days. The current protocol bypasses many drawbacks in previous reported methods, without the requirement of complicated multi-buffer exchange before initiation of refolding, or an extensive incubation period during the refolding process, or usage of multiple columns or specialized (and potentially expensive) affinity columns. In contrast to eukaryotic systems such as *Pichia pastoris* and *Schizosaccharomyces pombe*, our current method produces EK using the *E. coli* expression system, which greatly simplifies and expedites steps involved in plasmid construction and modification, transformation of expression host cells, and convenient induction of high levels of protein expression, which in turn, should make homemade EK readily accessible for most research labs.

3.4 | Production and use of the sortase enzyme from staphylococcus aureus

There are several strategies that can be used to fluorescently label proteins for subsequent biophysical experiments. Direct chemical reaction with a fluorophore can be carried out, generally using the free thiol group of Cys or the amino group of Lys.[19,38] This can be an effective strategy, but maintaining a free thiol in a protein that also has disulfides can be difficult, while labeling amino groups can lead to a variety of fluorescently labeled sites since a typical protein will have several Lys residues. For smaller proteins that can be entirely synthesized by solid phase synthesis, there is also the possibility of chemical steps added to the synthesis that allow conjugation of a fluorophore to the site of interest. This latter method has been shown to be successful with variants of the chemokine CCL5.[28] We chose to use enzymatic methods to fluorescently label our chemokines, due to the specificity of the enzyme used (sortase) and the simplicity of making all components with bacterial expression. The sortase enzyme has the ability to cleave a protein at a specific site (cleavage occurs after the T

in LPXTG, where X can be any amino acid), and then covalently add a peptide or protein (that has an N-terminal series of glycines) to the LPXT sequence.[32,39] When this procedure is applied to a chemokine bearing LPMTG at its C-terminus, adding to the reaction the peptide GGGK-fluor, the product is: Chemokine-LPMTGGGK-fluor. The utility of the sortase enzyme has been described for a variety of functions.[39–41] Briefly, the enzyme can be expressed in *E. coli* and purified with a single nickel column, then aliquoted for storage after brief dialysis (Figure 5).

When using sortase, the reaction should occur in the dark using foil to wrap the reaction tube, and it is advisable to set up several small scale (200 μL) test reactions to determine the optimal conditions for the ligation, including temperature and time scale. Once suitable conditions are obtained, larger scale reactions can be carried out, at sufficient scale to allow purification afterward. In our hands, reaction amounts of 2 mL containing 50 μM chemokine were used, with the reaction requiring several days for optimal efficiency (although others report a wide range of time scales from hours to days[32,42]). Following the sortase reaction, the labeled chemokine can be purified using reversed-phase chromatography (Figure 6a). Figure 6c shows an SDS-PAGE gel of the various fractions after reversed phase chromatography, and also shows the same gel under UV light to illuminate the successful fluorescent labeling of the chemokine. If further evidence of purification of the correct labeled product is desired, mass spec can also provide this (Figure 6b).

3.5 | Fluorescently labeled vMIP-II tightly binds vCCI and competes with CCL17/TARC

The fluorescently labeled chemokine can be used in many types of assays, as fluorescence spectroscopy is an effective technique to

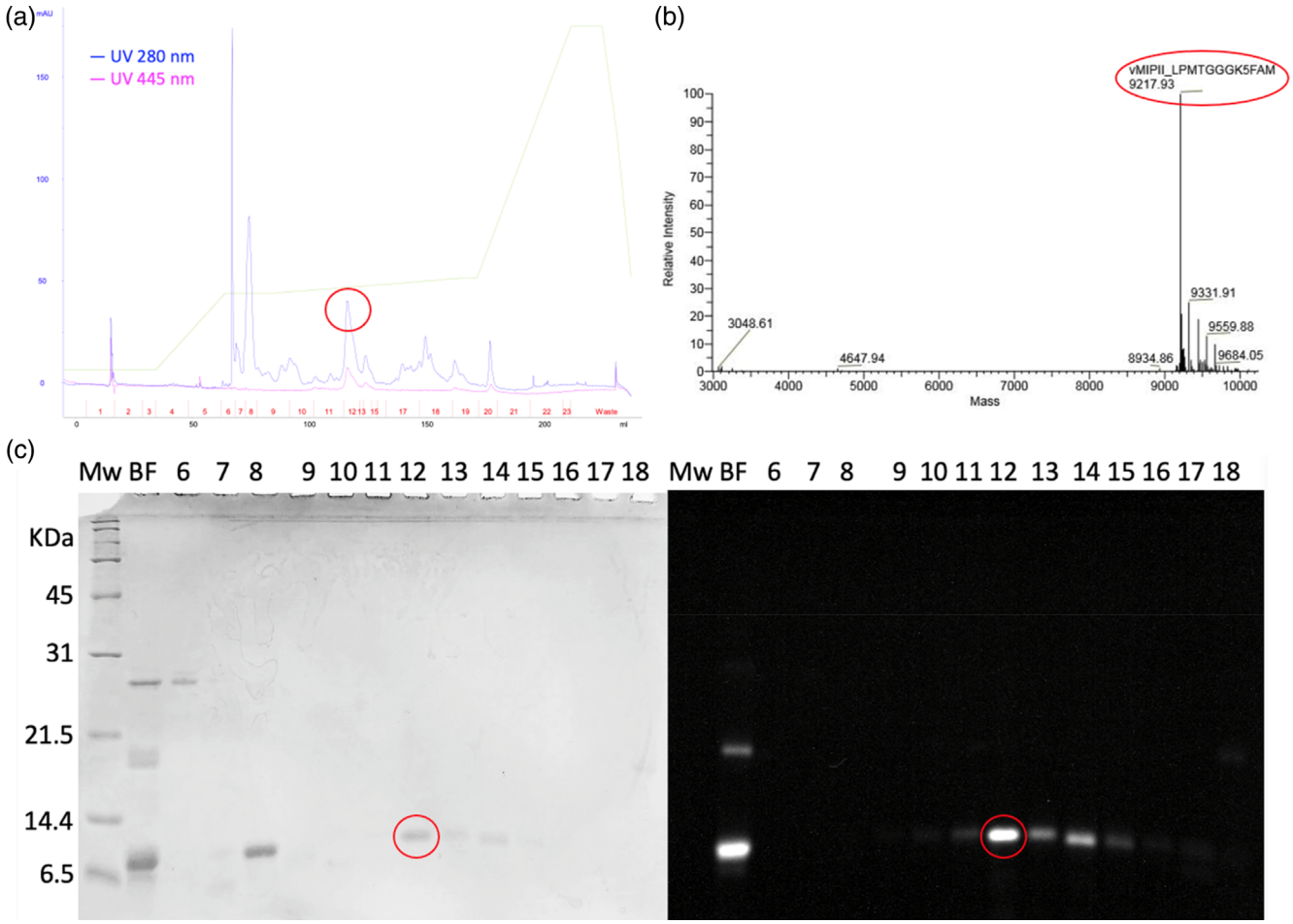

FIGURE 6 Purification of vMIP-II-fluor after the sortase reaction. (a) Analytical C4 column trace after the sortase reaction. The two large early peaks are the sortase and unreacted chemokine respectively. The peak for the correctly labeled chemokine is circled. (b) C4 fraction 12 verified as vMIP-II-fluor by mass spectrometry. The major peak on the mass spectrum is the expected size after reaction with the fluorophore. The smaller peaks at slightly higher molecular weight correspond to the protein-fluor adduct with trifluoroacetic acid that was used in the purification process. (c) (Left)The SDS-PAGE gel of the fractions from the analytical C4 column. Left lane is the molecular weight marker, second lane is the sortase reaction before the C4 column, followed by fractions of the C4 column. (Right) The same gel, in the presence of UV light. The overall yield from the sortase reaction is about 20%.

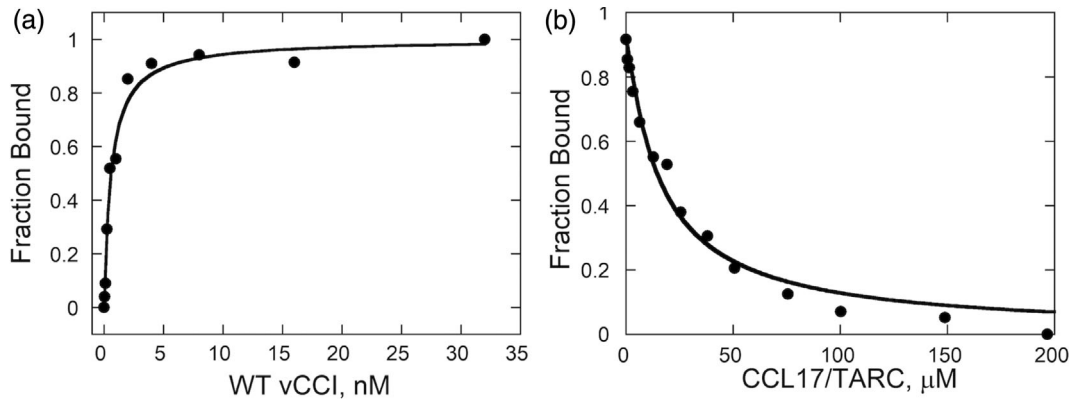

FIGURE 7 The use of fluorescent labeled chemokine to measure binding to chemokine binding protein vCCI. (a) Fluorescence anisotropy used to follow the titration of vCCI into a cuvette containing 0.5 nM vMIP-II-fluor solution. (b) Competition fluorescence anisotropy. Unlabeled chemokine CCL17 (also called TARC) is added to a pre-formed complex of vCCI:vMIP-fluor (8 nM). Increasing amounts of CCL17 compete off the vMIP-fluor, leading to a lower fraction bound of vMIP-fluor. Using a system of equations, this data can be fit to yield a binding constant of 14 μM for the interaction between CCL17 and vMIP-fluor. See Section 2 for additional details.

measure binding across orders of magnitude of affinity. Binding can be measured by observing fluorescence intensity, fluorescence anisotropy, and fluorescence correlation (and cross-correlation) spectroscopy, the latter of which has been used to study chemokine binding to the CCR5 receptor.[28] For the in vitro binding of a small chemokine with a larger binding partner (such as vCCI), fluorescence anisotropy is an effective tool that can be carried out in a cuvette or plate reader, and can also be used in a competition experiment where an unlabeled chemokine competes with a labeled chemokine for binding to vCCI. It should be noted that a plate reader can be quite convenient in its ability to measure all the data points of a titration at once, although it tends to have lower sensitivity than the larger path length of a cuvette. We proceeded with fluorescence anisotropy experiments. As shown in Figure 7a vCCI is titrated into a solution containing vMIP-fluor, revealing sub-nanomolar binding with a $K_d = 0.37 \pm 0.006$ nM. Further, when a vCCI:vMIP-fluor complex is preassembled, a competition experiment can be carried out to determine binding constants of unlabeled chemokines as they compete with vMIP-fluor for the vCCI binding site. As shown in Figure 7b, CCL17 (also known as TARC) requires high concentrations to effectively compete with vMIP-fluor, demonstrating a binding constant of 14 μM. This chemokine has been implicated in several inflammatory disorders, such as allergic asthma and atopic dermatitis.[43,44] CCL17 had been previously used in qualitative assays which did suggest low binding to vCCI,[16] but fluorescence spectroscopy allows a quantitative assessment. Higher throughput is also easily achieved with these modified chemokines as 96 well plates can be used for multiple simultaneous binding assays (data not shown).

4 | CONCLUSIONS

In conclusion, we describe techniques and constructs that allow the efficient and inexpensive production and purification of fluorescently labeled chemokines. The elements involved are broadly applicable to other proteins. Three options are presented for the production of recombinant enterokinase, which is used to specifically cleave fusion protein from a target; these varying constructs allow a variety of choices in antibiotic during expression of enterokinase, and maintain a simple purification thereafter. The straightforward production of the sortase enzyme allows fluorescent labeling of a target protein if the target protein contains a simple five amino acid tag, which again can be applied in a wide variety of experimental systems. As an example of the utility of these toolkit proteins, we show their application in the chemokine system. It is demonstrated that chemokines can be expressed, purified and fluorescently labeled efficiently. Overall, this set of techniques can be used in a wide variety of experiments where proteins are expressed with fusion partners and there is additionally the desire to fluorescently label them inexpensively.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors will provide the primary data presented here as well as protein expression vectors upon request.

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REFERENCES

[1] P. J. Lodi, D. S. Garrett, J. Kuszewski, M. L. S. Tsang, J. A. Weatherbee, W. J. Leonard, A. M. Gronenborn, G. M. Clore, *Science* **1994**, *1979*, 1762.
[2] G. M. Clore, A. M. Gronenborn, E. Appella, M. Yamada, K. Matsushima, *Biochemistry* **1990**, *29*, 1689.
[3] R. P. Bhusal, S. R. Foster, M. J. Stone, *Protein Sci.* **2020**, *29*, 420.
[4] I. F. Charo, R. M. Ransohoff, *N. Engl. J. Med.* **2006**, *354*, 610.
[5] T. Dragic, V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, W. A. Paxton, *Nature* **1996**, *381*, 667.
[6] L. Zhang, C. Herrera, J. Coburn, N. Olejniczak, P. Ziprin, D. L. Kaplan, P. J. Liwang, *ACS Biomater. Sci. Eng.* **2017**, *3*, 1654.
[7] B. Zhao, M. K. Mankowski, B. A. Snyder, R. G. Ptak, P. J. LiWang, *J. Biol. Chem.* **2011**, *286*, 28370.
[8] H. Gaertner, F. Cerini, J. M. Escola, G. Kuenzi, A. Melotti, R. Offord, I. Rossitto-Borlat, R. Nedellec, J. Salkowitz, G. Gorochov, D. Mosier, O. Hartley, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 17706.
[9] O. Hartley, K. Dorgham, D. Perez-Bercoff, F. Cerini, A. Heimann, H. Gaertner, R. E. Offord, G. Pancino, P. Debré, G. Gorochov, *J. Virol.* **2003**, *77*, 6637.
[10] A. E. I. Proudfoot, *Nat. Rev. Immunol.* **2002**, *2*, 106.
[11] C. L. Hsieh, E. C. Niemi, S. H. Wang, C. C. Lee, D. Bingham, J. Zhang, M. L. Cozen, I. Charo, E. J. Huang, J. Liu, M. C. Nakamura, *J. Neurotrauma* **2014**, *31*, 1677.
[12] M. T. Joy, E. Ben Assayag, D. Shabashov-Stone, S. Liraz-Zaltsman, J. Mazzitelli, M. Arenas, N. Abduljawad, E. Kliper, A. D. Korczyn, N. S. Thareja, E. L. Kesner, M. Zhou, S. Huang, T. K. Silva, N. Katz, N. M. Bornstein, A. J. Silva, E. Shohami, S. T. Carmichael, *Cell* **2019**, *176*, 1143.
[13] B. Zhao, P. J. Liwang, *Biochemistry* **2010**, *49*, 7012.
[14] A. C. LiWang, Z.-X. Wang, Y. Sun, S. C. Peiper, P. J. LiWang, *Protein Sci.* **1999**, *8*, 2270.
[15] T. M. Handel, P. J. Domaille, *Biochemistry* **1996**, *35*, 6569.
[16] J. M. Burns, D. J. Dairaghi, M. Deitz, M. Tsang, T. J. Schall, *J. Biol. Chem.* **2002**, *277*, 2785.
[17] A. Carfí, C. A. Smith, P. J. Smolak, J. McGrew, D. C. Wiley, *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 12379.
[18] L. Zhang, M. DeRider, M. A. McCornack, S.-C. Jao, N. Isern, T. Ness, R. Moyer, P. J. LiWang, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 13985.
[19] N. W. Kuo, Y. G. Gao, M. S. Schill, N. Isern, C. M. Dupureur, P. J. Li Wang, *J. Biol. Chem.* **2014**, *289*, 6592.
[20] A. F. Nguyen, N. W. Kuo, L. J. Showalter, R. Ramos, C. M. Dupureur, M. E. Colvin, P. J. LiWang, *Int. J. Mol. Sci.* **2017**, *18*, 1778.
[21] C. A. Bursill, R. P. Choudhury, Z. Ali, D. R. Greaves, K. M. Channon, *Circulation* **2004**, *110*, 2460.
[22] V. Buatois, S. Fagète, G. Magistrelli, L. Chatel, N. Fischer, M. H. Kosco-Vilbois, W. G. Ferlin, *J. Immunol.* **2010**, *185*, 2544.
[23] G. E. White, A. J. Iqbal, D. R. Greaves, *Pharmacol. Rev.* **2013**, *65*, 47.
[24] A. Ridiandries, C. Bursill, J. Tan, *Int. J. Mol. Sci.* **2017**, *18*, 155.
[25] A. F. Nguyen, M. S. Schill, M. Jian, P. J. LiWang, *Int. J. Mol. Sci.* **2017**, *18*, 1575.
[26] J. S. Laurence, C. Blanpain, J. W. Burgner, M. Parmentier, P. J. LiWang, *Biochemistry* **2000**, *39*, 3401.
[27] A. E. I. Proudfoot, C. A. Power, A. J. Hoogewerf, M. O. Montjovent, F. Borlat, R. E. Offord, T. N. C. Wells, *J. Biol. Chem.* **1996**, *271*, 2599.
[28] C. A. Rico, Y. A. Berchiche, M. Horioka, J. C. Peeler, E. Lorenzen, H. Tian, M. A. Kazmi, A. Fürstenberg, H. Gaertner, O. Hartley, T. P. Sakmar, T. Huber, *Biophys. J.* **2019**, *117*, 903.
[29] D. Rajalingam, C. Loftis, J. J. Xu, T. K. S. Kumar, *Protein Sci.* **2009**, *18*, 980.
[30] G. Y. Wiederschain, *Biochemistry* **2006**, *71*, 696.
[31] Y. G. Chang, R. Tseng, N. W. Kuo, A. LiWang, *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 16847.
[32] I. M. Piper, S. A. Struyvenberg, J. D. Valgardson, D. A. Johnson, M. Gao, K. Johnston, J. E. Svendsen, H. M. Kodama, K. L. Hvorecny, J. M. Antos, J. F. Amacher, *J. Biol. Chem.* **2021**, *297*, 100981.
[33] P. Simeonov, R. Berger-Hoffmann, R. Hoffmann, N. Sträter, T. Zuchner, *Protein Eng. Des. Sel.* **2011**, *24*, 261.
[34] M. E. Gasparian, V. G. Ostapchenko, D. A. Dolgikh, M. P. Kirpichnikov, *Biochemistry* **2006**, *71*, 113.
[35] W. Skala, P. Goettig, H. Brandstetter, *J. Biotechnol.* **2013**, *168*, 421.
[36] M. E. Gasparian, V. G. Ostapchenko, A. A. Schulga, D. A. Dolgikh, M. P. Kirpichnikov, *Protein Expression Purif.* **2003**, *31*, 133.
[37] S. Pepeliaev, J. Krahulec, Z. Černý, J. Jílková, M. Tlustá, J. Dostálová, *J. Biotechnol.* **2011**, *156*, 67.
[38] J. C. Kouokam, D. Huskens, D. Schols, A. Johannemann, S. K. Riedell, W. Walter, J. M. Walker, N. Matoba, B. R. O'Keefe, K. E. Palmer, *PLoS One* **2011**, *6*, e22635.
[39] M. W. L. Popp, J. M. Antos, H. L. Ploegh, *Curr. Protoc. Protein Sci.* **2009**, *56*, 15.3.1.
[40] G. Pasqual, A. Chudnovskiy, J. M. J. Tas, M. Agudelo, L. D. Schweitzer, A. Cui, N. Hacohen, G. D. Victora, *Nature* **2018**, *553*, 496.
[41] J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W. L. Popp, H. L. Ploegh, *J. Am. Chem. Soc.* **2009**, *131*, 10800.
[42] J. M. Antos, M. W. L. Popp, R. Ernst, G. L. Chew, E. Spooner, H. L. Ploegh, *J. Biol. Chem.* **2009**, *284*, 16028.
[43] J. Catherine, F. Roufosse, Seminars in Immunopathology. **2021**, *43*, 439.
[44] Y. Tsunemi, H. Saeki, K. Nakamura, D. Nagakubo, T. Nakayama, O. Yoshie, S. Kagami, K. Shimazu, T. Kadono, M. Sugaya, M. Komine, K. Matsushima, K. Tamaki, *Eur. J. Immunol.* **2006**, *36*, 2116.

SUPPORTING INFORMATION

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