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A *Streptococcus pyogenes* derived collagen-like protein as a non-cytotoxic and non-immunogenic cross-linkable biomaterial

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Abstract

A range of bacteria have been shown to contain collagen-like sequences that form triple-helical structures. Some of these proteins have been shown to form triple-helical motifs that are stable around body temperature without the inclusion of hydroxyproline or other secondary modifications to the protein sequence. This makes these collagen-like proteins particularly suitable for recombinant production as only a single gene product and no additional enzyme needs to be expressed. In the present study, we have examined the cytotoxicity and immunogenicity of the collagen-like domain from *Streptococcus pyogenes* Scl2 protein. These data show that the purified, recombinant collagen-like protein is not cytotoxic to fibroblasts and does not illicit an immune response in SJL/J and Arc mice. The freeze dried protein can be stabilised by glutaraldehyde cross-linking giving a material that is stable at >37°C and which supports cell attachment while not causing loss of viability. These data suggest that bacterial collagen-like proteins, which can be modified to include specific functional domains, could be a useful material for medical applications and as a scaffold for tissue engineering.

1. Introduction

Collagens are the major structural proteins in the extracellular matrix of animals, including all vertebrates and invertebrates, and are defined by a characteristic triple-helix structure that requires a (Gly-Xaa-Yaa)_n repeating sequence [1]. The residues found in the Xaa and Yaa positions are frequently proline, where Pro in the Yaa position is post-translationally modified to hydroxyproline (Hyp) which enhances helical stability [2]. In humans, a family of 28 collagen types is found [3, 4] with type-specific biological and structural functions. The triple helical motif is also present in other proteins, such as macrophage scavenger receptors, collectins, adiponectin, and C1q [5, 6]. Recent analysis of bacterial genomes has indicated there are many putative proteins containing Gly as every third residue and a high proline content, suggesting that collagen-like structures may be present in a range of

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pathogenic and non-pathogenic bacteria [7, 8]. Collagen-like proteins have been shown to be expressed in several bacteria, forming cell surface proteins on *Streptococcus pyogenes* [9] and filaments on *Bacillus anthracis* spores [10], and to form stable triple-helical proteins which play a role in pathogenicity. Bacterial triple-helix proteins can be cloned into well developed, high yield *E. coli* expression systems to facilitate large scale production and allow manipulation of designed expressed collagen proteins for biomedical applications.

Although the repeating (Gly-Xaa-Yaa)_n tripeptide pattern and high Pro content are similar in bacterial and animal collagen proteins, bacterial collagen-like proteins differ from animal collagens in the absence of hydroxyproline, since they lack prolyl hydroxylase [11]. There are wide variations in amino acid composition and sequence among collagen-like proteins from different bacteria; some have extremely high concentrations of charged residues, while others are (usually) enriched in Thr or Gln [8, 12]. One of the best studied systems is *S. pyogenes*, where it has been demonstrated that two collagen-like proteins, Scl1 and Scl2 are simultaneously expressed on the cell surface in the logarithmic growth phase and help the bacteria adhere to human cells [13–15]. Both Scl1 and Scl2 proteins contain a signal sequence, an N-terminal variable globular domain (V), a highly charged collagen-like triple-helix domain (CL) consisting of (Gly-Xaa-Yaa)_n triplet repeats and a C-terminal Gram-positive cell wall attachment domain [16]. Biochemical studies have confirmed that in the absence of post-translational modifications, proteins Scl1 and Scl2 expressed in *E. coli* still form stable triple-helix structures similar to those seen in animal collagens. Their circular dichroism spectra show the characteristic features of a triple-helix and the (Gly-Xaa-Yaa)_n domains of the proteins show resistance to digestion by trypsin expected for typical triple-helices [16, 17]. Despite the absence of Hyp and having a shorter triple-helix domain length compared with the (Gly-Xaa-Yaa)₃₃₈ domain of human type I collagen, the thermal stabilities of Scl1 and Scl2 proteins are 36–38 °C [18, 19], close to the stability found in human collagens [20]. In the case of the *S. pyogenes* Scl2 collagen-like protein, which has a very high charge content in the (Gly-Xaa-Yaa) sequence (30%), electrostatic interactions were shown to make an important contribution to stability [19]. In addition, the collagen domain of one construct from *S. pyogenes* was shown to form fibrillar structures as the concentration increased, suggesting a propensity for formation of supramolecular structures [17]. Similar collagen-like features and high thermal stability were observed for the bacterially expressed Thr-rich BclA protein from *B. anthracis* [10].

Collagen has proven safe and effective in a wide variety of medical products in a range of distinct clinical applications [21]. For medical applications, collagen is usually extracted from animal sources, especially bovines, but there has been a growing concern of transmissible diseases, especially bovine spongiform encephalopathy ('mad cow disease'), ovine and caprine scrapie, and other zoonoses. Other concerns include the lack of standardisation for extracted bovine collagen preparations and an inability to make any modifications to the sequence to improve biological properties. To circumvent these difficulties with extracted bovine collagen, considerable effort has gone into developing systems for production of recombinant human collagens [22–27]. Human cell lines only result in moderate yields which are not suited to cost-effective, larger scale production, while yeast systems are complicated by the need to introduce genes for proline hydroxylase to form the Hyp residues needed for stability of animal collagens.

The set of bacterial collagen-like structures that are stable near body temperature and are capable of forming fibrillar structures provides new opportunities for the development of biomaterials. Recombinant constructs of these proteins have been shown to be amenable to production in *E. coli* without the need to introduce prolyl-4-hydroxylase [12, 17, 19]. If a bacterial collagen source is to be used in medical applications, then its biological properties must be determined to validate their safety. In the present study, the cell compatibility of the

purified, soluble recombinant Scl2 collagen domain from *S. pyogenes* was examined by two independent methods, and no cytotoxicity was observed. An immunological study was then carried out on the Scl2 collagen domain, presenting the purified protein to two different mouse strains, both with and without adjuvant, and no immunological reaction was present. To investigate its biomaterial potential, the freeze dried Scl2 collagen domain was stabilised by glutaraldehyde cross-linking and this material was found to have the stability, cell attachment and proliferation suitable for biomedical applications.

2. Materials and Methods

2.1. Preparation of bacterial collagen-like CL protein

Bacterial collagen like protein was obtained using the Cold-shock vector (Takara Bio, Shiga, Japan) [28] for expression of pColdIII-V-CL in *E. coli*, following methods previously described [17]. Briefly, this construct was derived using pColdIII-163 encoding p163 polypeptide based on Scl 2.28 [16, 19]. A His₆ tag sequence was introduced at the N-terminal of the p163 polypeptide sequence and a thrombin/trypsin cleavage LVPRGSP sequence was inserted between the N-terminal globular domain (V) and the following (Gly-Xaa-Yaa)₇₉ collagen-like domain (CL) sequences [17]. All constructed plasmids were confirmed by DNA sequencing. The pColdIII-V-CL was expressed in *E. coli* BL21 strain. Cells were grown in M9-Casamino acid with ampicillin (50 µg/ml) medium at 37 °C until A600 reached 1.2. Cells were harvested by centrifugation and resuspended in 2 × concentrated L broth with ampicillin (50µg/ml). The culture was then incubated at 21 °C and 1 mM isopropyl beta-D-thiogalactopyranoside was added to induce protein expression. After overnight incubation, cells were harvested by centrifugation and disrupted by a French press and cell debris was removed by centrifugation at 4°C. V-CL was in the supernatant fraction and was purified using a Ni-NTA-Sepharose column. The column was washed by 20 mM imidazole in 500 mM NaCl, 20 mM sodium phosphate, pH 7.4 followed by elution by stepwise addition of 58, 96 and 115 mM imidazole in the same buffer [17]. Purity was verified by SDS-PAGE [29]. The collagen-like domain was obtained from purified V-CL by incubating with trypsin for 4 h in 50 mM glycine buffer, pH 8.6, at room temperature, which removes the V-domain. Cleaved CL protein was purified by chromatography on DEAE Sephadex and fractions containing CL domain, as determined by SDS-PAGE, were further purified by gel filtration using a Superdex 200 column [17]. Protein purity was checked by SDS-PAGE and MALDI-TOF mass spectrometry.

2.2 Cytotoxicity evaluation of soluble CL protein

Cytotoxicity of CL protein was examined by 2 methods. In one, cytotoxicity was assessed using mouse lung fibroblast L929 cells in a Live/Dead® Viability/Cytotoxicity Kit assay (Molecular Probes). Solutions of 400 µg/ml CL in PBS were filter sterilised using a 0.2µm filter. Aliquots of 50 µl were placed into wells of 96 well microtitre plates (tissue culture treated and non-tissue culture) for 24 h absorption at 4 °C. After 24 h incubation, the supernatant was transferred to fresh wells and was tested along with the absorbed layer by incubation for 16 h at 37 °C with 2 × 10⁴ L929 cells per well in MEM culture medium containing 1% NEAA and 120 µg/ml penicillin and 200 µg/ml streptomycin and supplemented with and without 10% (v/v) FCS. Cell morphology and spreading was evaluated after 16 h, and samples then tested with a Live/Dead® Viability/Cytotoxicity Kit assay, according to the manufacturer's instructions, using a Eclipse TE2000-U fluorescent microscope (Nikon). Live cells were stained green by calcein AM while dead cells stained red with ethidium homodimer-1.

In the second method, CL protein was assessed for cytotoxicity using human lung fibroblast WI-38 and human fibrosarcoma HT1080 cells in a Neutral Red assay [30]. Bovine skin

collagen (BSC), prepared by differential salt precipitation of a pepsin extract [21] was used as a control. Samples containing CL protein or BSC (1, 10 and 50 µg/ml) were prepared using serum-free DMEM (for WI-38) or MEM (for HT1080) and sterilised using a 0.2 µm filter. WI-38 cells and HT1080 cells were seeded onto a 96-well plate at 1.5×10^4 cells/well in MEM or DMEM, respectively, with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. After 24 h, medium was replaced with 150 µl of the sample or medium control and incubated for an additional 24 h. Viability of the cells was then determined using a Neutral red assay at 37°C in PBS [30].

2.3. Animal studies

Mice were used for assessment of antibody responses. All animal experimentation was approved by the CSIRO Animal Care and Ethics Committee. The site of injection and volumes used for immunisations are those recommended by the NHMRC guidelines on monoclonal antibody production.

2.3.1. Mouse strains—Two strains of mice of either sex were used; SJL/J, an inbred strain [H-2^s], which has been shown previously to be most responsive to collagens [31, 32], and Arc [Arc:Arc (s) (Swiss)], an outbred albino mouse (Animal Resources Centre, Canning Vale, WA). All were approximately 8-9 weeks of age before initial immunisation. Eight mice (4 male and 4 female) half without adjuvant, half with adjuvant of each mouse strain were used.

2.3.2 Mouse immunisation—Both strains of mice were immunised without adjuvant as well as with Incomplete Freund's Adjuvant (IFA) to assess the maximum immunogenic potential of the injected immunogens. Mice treated without adjuvant were injected subcutaneously on Days 1, 14 and 35 with 100µl of protein (50µl of 0.4mg/ml bacterial collagen in phosphate buffered saline (PBS) mixed with 50µl of PBS). Mice treated with adjuvant were injected intraperitoneally on Days 1, 14 and 35 with 100µl of protein (50µl of 0.4mg/ml bacterial collagen in PBS mixed with 50µl of IFA). A pre-bleed from the saphenous vein was taken prior to the first injection and then test saphenous vein bleeds were taken 7 days after each immunisation. Approximately 20 – 40µl of blood was drawn at each bleed. At 42 days animals were killed and a terminal bleed taken. All blood samples were allowed to clot, ringed and centrifuged to separate and collect sera. Samples were stored at –20 °C prior to analysis.

2.3.3 Antibody analysis—Mouse sera were analysed by a standard enzyme linked immuno-sorbent assay (ELISA) for antibodies against the collagen preparation. For all sera, a 1/50 dilution was examined against the CL protein. The secondary antibody used was a 1/500 dilution of an affinity purified goat anti-mouse Ig coupled to horseradish peroxidase (Silenus, Melbourne), using 2,2'-azino bis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) as substrate.

2.4. Preparation of stabilised CL protein samples

For sponge preparation, purified CL protein was prepared in 20 mM acetic acid and freeze dried. Dry collagen was held at 20 °C over vapour from 20% w/v glutaraldehyde (GA) for 18 h in a closed vessel [33]. Stabilised samples were then held covered in air and stored at room temperature until analysis. Prior to cell evaluations samples were washed with 3 changes of sterile PBS.

2.4.1. Thermal stability of stabilised CL protein samples—The thermal stability of the GA stabilised samples was examined by differential scanning calorimetry (DSC) on a Mettler Toledo DSC 821° instrument using samples in PBS.

2.4.2. Cell evaluation of stabilised CL protein samples—For assessment of cell attachment, stabilised, PBS washed CL samples were treated for 2 h with 120 µg/ml penicillin and 200 µg/ml streptomycin in MEM and then seeded with 1×10^4 L929 cells per sample in MEM supplemented with 1% NEAA and 10% FCS in 96 well plates. Attachment was assessed at 3 h and 16 h after samples were rinsed twice in PBS. Cell viability was tested after 16 h at 37 °C with a Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes) assay, according to the manufacturers instructions, as described above (Section 2.2.).

3. Results

3.1. Collagen-like protein production

The previously described procedure for production of *S. pyogenes* collagen-like domain [17] gave good yields of highly purified protein. The V-CL Scl2 protein construct containing an N-terminal globular domain (V) connected by an artificial linker with a thrombin cleavage site to a (Gly-Xaa-Yaa)₇₉ collagen domain was expressed in *E. coli* using the Cold-shock vector system and gave yields of ~0.4 – 0.6 g/l of purified V-CL protein [17]. After absorption onto the Ni-NTA-Sepharose column, unbound protein was removed by washing with 20 mM imidazole in 500 mM NaCl, 20 mM sodium phosphate, pH 7.4 (Fig. 1a). Increasing the imidazole to 58 mM did not elute the V-CL protein. There was some elution after increasing the imidazole content to 96 mM, and efficient elution was achieved at 115 mM imidazole (Fig. 1a). The linker region was not readily cleaved by thrombin, possibly due to steric hindrance (data not shown). Trypsin did cleave at the Arg site in the linker between the V and CL domains and also digested the V domain, but not the CL triple-helical domain. Ion-exchange and gel permeation chromatography were used to obtain purified CL domain following trypsin digestion, with a yield of typically around 0.1– 0.2 g/l. SDS-PAGE as well as mass spectrometry indicated that V-CL and CL preparations were pure and did not contain significant quantities of other proteins (Fig. 1b). The CL domain was observed to have a thermal stability of $T_m = 36^\circ\text{C}$ even though it lacks Hyp [17].

3.1. Cytotoxicity evaluation of soluble CL protein

Qualitative examination of CL Protein by a Live/Dead® Cytotoxicity/Viability assay using murine L929 cells showed a lack of cytotoxicity. The L929 cells attached to CL coated plates after 16 h when serum was present and were starting to spread (Fig. 2a) when compared to control plates treated with PBS without CL protein (Fig 2b). Examination of cells by a Live/Dead® Cytotoxicity/Viability assay after 16 h incubation on absorbed CL protein or in the presence of supernatant aliquots after CL protein absorption, showed that almost all cells were viable for both the absorbed and supernatant samples, with < 1% dead cells present (Fig. 2c, 2d). The results were comparable to bovine collagen type 1 standard (data not shown).

The second assay was the quantitative Neutral Red assay [30]. No significant changes were observed in the cell viability nor cell morphology after 24 h for WI-38 cells and HT1080 cells when compared to cells incubated in serum-free DMEM and MEM, which were taken as the 100 % reference level (Fig. 3). The bovine skin collagen (BSC) controls showed 20–30% enhanced viability at 24 h compared to the medium only controls which is probably due to the better cell attachment by BSC in the serum free media.

Thus, these results indicate that the collagen like CL domain is not cytotoxic when assessed using HT1080, WI-38 or L929 cells.

3.2. Immunological analysis of CL protein

Antibody responses were assessed in two different mouse strains. One was an outbred, albino mouse strain, Arc [Arc:Arc (s) (Swiss)], while the other was an inbred strain, SJL/J [H-2^s]. While BALB/c mice are normally used for generating antibodies to globular proteins, and can be responsive to collagens [34], a comparative study has shown that SJL/J mice are the most responsive to mammalian collagens [31,32]. In the present study, the total Ig response against the CL protein after 3 immunisations without adjuvant, with sera collection over 42 days, was minimal by ELISA, even without any significant dilution of the sera (Fig. 4a). In the absence of adjuvant, the CL protein was non-immunogenic, even in the most responsive SJL/J mice. Even in the presence of adjuvant, a negligible response was observed for both mouse strains (Fig. 4b). The test sera were comparable to the PBS control. A positive control of the V-CL protein detected by an anti-His₅ MAb (Qiagen) was used. As the total Ig response from the test sera was negligible, differentiation to specific antibody isotypes was not examined. In addition to the lack of immune response to the CL protein, the 3 repeat injections of CL protein into the mice over the 42 day time period did not lead to adverse reactions indicating that acute toxicity and sub-chronic toxicity were also absent. These data were comparable to the commercial bovine collagen (Zyderm®), which was also non-responsive in these mice strains [35]

3.3. Evaluation of stabilised CL protein samples

The GA stabilisation of the freeze dried CL protein produced samples that were stable and did not disperse nor appear to denature (shrink) when incubated at 37 °C. DSC indicated that the stability of the stabilised material was 28-30 °C higher than that of the uncrosslinked CL protein. Examination of samples 3 h after seeding with murine L929 fibroblasts showed that these cells had attached well to the CL protein network in the presence of serum (Fig. 5a). Microscopy showed that the sample was a mixture of thin fibrils together with larger aggregates of protein fibres, with the cells attaching to both (Fig. 5a). After 16 h in culture, the cells were still attached and were examined by a Live/Dead® Cytotoxicity/Viability assay. All cells were observed to be viable (green) and dead (red staining) cells were rare (<0.1%), while some cell spreading was starting (Fig. 5b). The GA stabilised CL protein is visible due to low level autofluorescence, arising from the GA stabilisation [36].

4. Discussion

Collagen is the best known natural material that has been widely used in biomedical applications, largely due to its natural structural integrity and information content that supports biological compatibility [21]. The present study, which examined a *S. pyogenes* bacterial collagen-like protein indicates that this alternate source of collagen is an immunologically safe and non-cytotoxic material for biomedical and tissue engineering applications.

The bacterial collagen-like protein construct V-CL based on the *S. pyogenes* Scl2 protein was readily expressed in *E. coli*. The protein triple-helix is stable, $T_m \sim 35-37^\circ\text{C}$, without the need for incorporation of hydroxyproline [17]. This secondary modification is needed for stability in mammalian collagens [2], and adds extra complexity to recombinant expression of mammalian collagens [23–25]. The use of the Cold-shock vector system incorporating a single gene gives a high yield of purified recombinant bacterial CL protein either through trypsin cleavage of the V-CL protein [16, 17, 19] or through expression of His₆-CL directly [16; Yu, X. pers. comm.]. The large amounts of protein obtained from this system together with the ease of purification and the potential for scaling up provide advantages compared with currently used recombinant collagen producing systems [27].

The biological properties of purified CL protein are compatible with its potential use as a biomaterial. The protein when absorbed onto tissue culture plastic was not cytotoxic to three different mammalian cell lines. Although the CL protein adhered to plates at low temperature, any tests run at 37 °C or the immunogenicity studies in mice raise the possibility that some of the bacterial collagenous protein may be in a partially denatured form.[37, 38] Previously, antibodies to the streptococcal collagen-like proteins Scl2 were detected in paediatric patients and mice infected with M28 GAS [39], although the response to purified protein had not been reported. However, in the present study the response to purified protein has been shown to be negligible, suggesting that the intact organism enhances the response. Thus, when examined in 2 different mouse strains, the protein showed negligible immunogenicity, even when adjuvant was used. Compared with most globular proteins that are clearly immunogenic, the bacterial collagen-like protein is like mammalian and avian collagens which are particularly poor immunogens [35], consistent with their potential use as biomaterials where lack of immunogenicity is a critical issue. Nevertheless, mammalian (bovine) collagen can on occasion be immunogenic; for commercial bovine collagen materials there is a small percentage of patients (< 3.0 %) who show an adverse skin (delayed type IV immune) response [40, 41].

The CL protein can also be fabricated into sponge-like materials. Preliminary studies showed that freeze dried protein tended to disperse when water or 50% v/v EtOH was added, for example for EDC cross-linking, so that sponge structure could be lost (data not shown). For this reason a GA vapour treatment was selected, although it is possible that other non-solvent methods such as dehydrothermal crosslinking [42] may also be effective. The increase in stability suggests that less stable bacterial collagens could also be used, so long as the stabilised material has a T_m of around 40 °C or greater. The stabilised collagen was also able to support cell attachment. Fibroblasts attached readily by 3 h and were observed to be spreading after 16 h, with full cell viability.

A major advantage of bacterial collagen-like protein as a potential biomaterial is the ability to easily modify the expressed construct, to introduce desirable biological activities within or adjacent to the triple-helix protein. The sequence of the recombinant collagen-like domain from *S. pyogenes* protein Scl2 studied here lacks any identifiable cell binding sites and was not found to support cell adhesion in the absence of serum at 90 min [43]. In contrast, the *S. pyogenes* Scl1 protein interacts with the $\alpha 2\beta 1$ integrin, supporting cell attachment and inducing intracellular signaling under the same conditions [43, 44]. In the present study, serum was included and culture was for up to 16 h, which is more akin to the environment that would be encountered by a collagen implant. Under these conditions cell attachment was observed. These results indicate that functional sequences in human collagen will act to promote appropriate biological activity when placed in a bacterial collagen context. The inclusion of specific cell binding sites, other interaction sites, or degradation sites within the bacterial construct greatly expands the potential biomaterial applications, allowing them to be tailored to specific needs. In addition, it has been shown that designed collagens based on repeating individual bacterial collagen sequences can be made [17], and the incorporation of functional domains using mammalian sequences [45] within repeating units [46] should enhance activity.

5. Conclusions

A bacterial collagen protein from *S. pyogenes* has been shown to form a stable triple-helix structure without the need for post-translational modification to form hydroxyproline. The present results indicate the safety and suitability of this bacterial collagen protein for biomaterial applications. Thus, purified recombinant Scl2 collagen protein from *S. pyogenes* was not cytotoxic, nor immunogenic when presented to two different mouse strains. Freeze

dried Scl2 collagen protein could be further stabilised by glutaraldehyde cross-linking and this material supported cell attachment and proliferation. Recombinant bacterial collagen protein from *S. pyogenes*, may fill the need for a well defined, standardized collagen that addresses concerns on contamination by infectious agents and product standardisation in collagen extracted from animal tissues. This protein is readily expressed and purified from an *E. coli* system, and is amenable to easy sequence modifications and large scale production. The authors wish to thank Dr Wendy Tian for help with DSC determinations and Teresita Silva for assistance with protein preparations. This work was supported in part by NIH grant R21 EB007198 (B.B.)

References

1. Brodsky B, Ramshaw JAM. The collagen triple-helix structure. *Matrix Biol.* 1997; 15:545–554. [PubMed: 9138287]
2. Rosenbloom J, Harsch M, Jimenez S. Hydroxyproline content determines the denaturation temperature of chick tendon collagen. *Arch Biochem Biophys.* 1973; 158:478–484. [PubMed: 4592982]
3. Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathologie Biologie.* 2005; 53:430–442. [PubMed: 16085121]
4. Veit G, Kobbe B, Keene DR, Paulsson M, Koch M, Wagener R. Collagen XXVIII, a novel von Willebrand factor A domain-containing protein with many imperfections in the collagenous domain. *J Biol Chem.* 2006; 281:3494–3504. [PubMed: 16330543]
5. Brodsky B, Shah NK. Protein motifs. 8. The triple-helix motif in proteins. *FASEB J.* 1995; 9:1537–1546. [PubMed: 8529832]
6. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem.* 1996; 120:803–812. [PubMed: 8947845]
7. Sylvestre P, Couture-Tosi E, Mock M. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol Microbiol.* 2002; 45:169–178. [PubMed: 12100557]
8. Rasmussen M, Jacobsson M, Björck L. Genome-based identification and analysis of collagen-related structural motifs in bacterial and viral proteins. *J Biol Chem.* 2003; 278:32313–32316. [PubMed: 12788919]
9. Lukomski S, Nakashima K, Abdi I, Cipriano VJ, Ireland RM, Reid SD, Adams GG, Musser JM. Identification and characterization of the scl gene encoding a group A *Streptococcus* extracellular protein virulence factor with similarity to human collagen. *Infect Immun.* 2000; 68:6542–6553. [PubMed: 11083763]
10. Boydston JA, Chen P, Steichen CT, Turnbough CL. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *J Bacteriol.* 2005; 187:5310–5317. [PubMed: 16030225]
11. Myllyharju J. Recombinant collagen trimers from insect cells and yeast. *Methods Mol Biol.* 2009; 522:51–62. [PubMed: 19247604]
12. Xu C, Yu Z, Inouye M, Brodsky B, Mirochnitchenko O. Expanding the family of collagen proteins: recombinant bacterial collagens of varying composition form triple-helices of similar stability. *Biomacromolecules.* in press.
13. Rasmussen M, Edén A, Björck L. SclA, a novel collagen-like surface protein of *Streptococcus pyogenes*. *Infect Immun.* 2000; 68:6370–6377. [PubMed: 11035747]
14. Whatmore AM. *Streptococcus pyogenes* sclB encodes a putative hypervariable surface protein with a collagen-like repetitive structure. *Microbiology.* 2001; 147:419–429. [PubMed: 11158359]
15. Caswell CC, Lukomska E, Seo NS, Höök M, Lukomski S. Scl1-dependent internalization of group A *Streptococcus* via direct interactions with the $\alpha 2\beta 1$ integrin enhances pathogen survival and re-emergence. *Mol Microbiol.* 2007; 64:1319–1331. [PubMed: 17542923]
16. Xu Y, Keene DR, Bujnicki JM, Höök M, Lukomski S. Streptococcal Scl1 and Scl2 proteins form collagen-like triple helices. *J Biol Chem.* 2002; 277:27312–27318. [PubMed: 11976327]

17. Yoshizumi A, Yu Z, Silva T, Thiagarajan G, Ramshaw JAM, Inouye M, Brodsky B. Self-association of *Streptococcus pyogenes* collagen-like constructs into higher order structures. *Protein Sci.* 2009; 18:1241–1251. [PubMed: 19472339]
18. Han R, Zwiefka A, Caswell CC, Xu Y, Keene DR, Lukomska E, Zhao Z, Höök M, Lukomski S. Assessment of prokaryotic collagen-like sequences derived from streptococcal Scl1 and Scl2 proteins as a source of recombinant GXY polymers. *Appl Microbiol Biotechnol.* 2006; 72:109–115. [PubMed: 16552563]
19. Mohs A, Silva T, Yoshida T, Amin R, Lukomski S, Inouye M, Brodsky B. Mechanism of stabilization of a bacterial collagen triple helix in the absence of hydroxyproline. *J Biol Chem.* 2007; 282:29757–29765. [PubMed: 17693404]
20. Leikina E, Merts MV, Kuznetsova N, Leikin S. Type I collagen is thermally unstable at body temperature. *Proc Natl Acad Sci USA.* 2002; 99:1314–1318. [PubMed: 11805290]
21. Ramshaw JAM, Werkmeister JA, Glattauer V. Collagen-based biomaterials. *Biotechnol Genetic Eng Rev.* 1995; 13:335–382.
22. Fertala A, Sieron AL, Ganguly A, Li SW, Ala-Kokko L, Anumula KR, Prockop DJ. Synthesis of recombinant human procollagen II in a stably transfected tumour cell line (HT1080). *Biochem J.* 1994; 298:31–37. [PubMed: 8129728]
23. Vaughan PR, Galanis M, Richards KM, Tebb TA, Ramshaw JAM, Werkmeister JA. Production of recombinant hydroxylated human type III collagen fragment in *Saccharomyces cerevisiae*. *DNA Cell Biol.* 1998; 17:511–518. [PubMed: 9655244]
24. Toman PD, Pieper F, Sakai N, Karatzas C, Platenburg E, de Wit I, Samuel C, Dekker A, Daniels GA, Berg RA, Platenburg GJ. Production of recombinant human type I procollagen homotrimer in the mammary gland of transgenic mice. *Transgenic Res.* 1999; 8:415–427. [PubMed: 10767986]
25. Nokelainen M, Tu H, Vuorela A, Notbohm H, Kivirikko KI, Myllyharju J. High-level production of human type I collagen in the yeast *Pichia pastoris*. *Yeast.* 2001; 18:797–806. [PubMed: 11427962]
26. Merle C, Perret S, Lacour T, Jonval V, Hudaverdian S, Garrone R, Ruggiero F, Theisen M. Hydroxylated human homotrimeric collagen I in *Agrobacterium tumefaciens*-mediated transient expression and in transgenic tobacco plant. *FEBS Lett.* 2002; 515:114–118. [PubMed: 11943205]
27. Ruggiero F, Koch M. Making recombinant extracellular matrix proteins. *Methods.* 2008; 45:75–85. [PubMed: 18442707]
28. Qing G, Ma LC, Khorchid A, Swapna GV, Mal TK, Takayama MM, Xia B, Phadtare S, Ke H, Acton T, Montelione GT, Ikura M, Inouye M. Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat Biotechnol.* 2004; 22:877–882. [PubMed: 15195104]
29. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
30. Borenfreund E, Puerner JA. A simple quantitative procedure using monolayer cultures for cytotoxicity assays. *J Tiss Culture Meth.* 1984; 9:7–9.
31. Werkmeister JA, Ramshaw JAM, Ellender G. Characterisation of a monoclonal antibody against native human type I collagen. *Eur J Biochem.* 1990; 187:439–443. [PubMed: 2105219]
32. Werkmeister JA, Ramshaw JAM. Multiple antigenic determinants on type III collagen. *Biochem J.* 1991; 274:895–898. [PubMed: 1707272]
33. Lickorish D, Ramshaw JAM, Werkmeister JA, Glattauer V, Howlett CR. Collagen-hydroxyapatite composite prepared by biomimetic process. *J Biomed Mater Res A.* 2004; 68:19–27. [PubMed: 14661245]
34. SundarRaj N, Martin J, Hrinya N. Development and characterization of monoclonal antibodies to human type III procollagen. *Biochem Biophys Res Comm.* 1982; 106:48–57. [PubMed: 7049168]
35. Peng YY, Glattauer V, Ramshaw JAM, Werkmeister JA. Evaluation of the immunogenicity and cell compatibility of avian collagen for biomedical applications. *J Biomed Mater Res A.* 2009 [Epub ahead of print].
36. Werkmeister JA, Tebb TA, Peters DE, Ramshaw JAM. The use of quenching agents to enable immunofluorescent examination of collagen-based biomaterials showing glutaraldehyde-derived autofluorescence. *Clinical Mat.* 1990; 6:13–20.

37. Egles C, Shamis Y, Mauney JR, Volloch V, Kaplan DL, Garlick JA. Denatured collagen modulates the phenotype of normal and wounded human skin equivalents. *J Invest Dermatol.* 2008; 128:1830–1837. [PubMed: 18200055]
38. Abraham LC, Zuenä E, Perez-Ramirez B, Kaplan DL. Guide to collagen characterization for biomaterial studies. *J Biomed Mater Res B Appl Biomater.* 2008; 87:264–285. [PubMed: 18386843]
39. Hoe NP, Lukomska E, Musser JM, Lukomski S. Characterization of the immune response to collagen-like proteins Sc11 and Sc12 of serotype M1 and M28 group A *Streptococcus*. *FEMS Microbiol Lett.* 2007; 277:142–149. [PubMed: 18031333]
40. Adelman BC, Kirrane J, Glynn LE. The structural basis of cell-mediated immunological reactions of collagen. Characteristics of cutaneous delayed hypersensitivity reactions in specifically sensitised guinea-pigs. *Immunology.* 1972; 23:723–737. [PubMed: 4636287]
41. Werkmeister, JA.; Ramshaw, JAM. Immunology of collagen-based biomaterials. In: Wise, DL., editor. *Handbook of Biomaterials Engineering*. New York: Marcel Dekker Inc; 2000. p. 739-759.
42. Ramshaw, JAM.; Glattau, V.; Werkmeister, JA. Stabilisation of collagen in clinical applications. In: Wise, DL., editor. *Handbook of Biomaterials Engineering*. New York: Marcel Dekker Inc; 2000. p. 717-738.
43. Humtsoe JO, Kim JK, Xu Y, Keene DR, Höök M, Lukomski S, Wary KK. A streptococcal collagen-like protein interacts with the $\alpha 2\beta 1$ integrin and induces intracellular signalling. *J Biol Chem.* 2005; 280:13848–13857. [PubMed: 15647274]
44. Caswell CC, Barczyk M, Keene DR, Lukomska E, Gullberg DE, Lukomski S. Identification of the first prokaryotic collagen sequence motif that mediates binding to human collagen receptors, integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$. *J Biol Chem.* 2008; 283:36168–36175. [PubMed: 18990704]
45. Sweeney SM, Orgel JP, Fertala A, McAuliffe JD, Turner KR, Di Lullo GA, Chen S, Antipova O, Perumal S, Ala-Kokko L, Forlino A, Cabral WA, Barnes AM, Marini JC, San Antonio JD. Candidate cell and matrix interaction domains on the collagen fibril, the predominant protein of vertebrates. *J Biol Chem.* 2008; 283:21187–21197. [PubMed: 18487200]
46. Peng YY, Werkmeister JA, Vaughan PR, Ramshaw JAM. Constructs for the expression of repeating triple-helical protein domains. *Biomed Materials.* 2009; 4:015006.

Fig 1. Purification of the *S. pyogenes* V-CL and CL domains. (a) Isolation of V-CL using Ni-NTA-Sepharose. SDS-PAGE analysis of samples from various fractions. L: Lysate, F: Flow through, W: Wash, and fractions eluted batch-wise with 58, 96 and 115, mM imidazole in 500 mM NaCl, 20 mM sodium phosphate, pH 7.4. (b) Purified protein samples, where the lanes are: MW: molecular weight standards (kDa), V-CL and CL.

Fig. 2. Cell adherence and spreading (a, b) and cell viability (c, d) after 16 h incubations with L929 cells. Cells seeded onto non-tissue culture plastic, in the presence of serum, where (a) the plate had been treated with CL protein, and (b) the plate was treated with PBS. Live/Dead® Cytotoxicity assay on cells grown on non-tissue culture plastic with serum, (a) on cells on CL coated plates, and (d) on cells treated with the post-absorption CL sample supernatant. Dead (red) cells are indicated by arrows.

Fig. 3. Cytotoxicity evaluation using a Neutral Red assay, showing cell viability after 24 h incubations with HT1080 and W1-38 cells. Samples were bovine skin collagen (BSC) control ■ and purified CL protein □.

Fig. 4. Assessment of the immunological response before immunisation (Pre-Im) and at various time points after immunisation of CL in SJL/J □ and Arc ■ mice. ELISA absorbance for mice immunised (a) without IF adjuvant and (b) with IF adjuvant. PBS and VCL controls are indicated.

Fig. 5. Growth of cells on GA stabilised CL protein scaffold. (a) Bright field microscopy, showing cell attachment after 3 h to CL protein fibres and larger CL aggregates, and (b) a Live/Dead® Cytotoxicity/Viability assay after 16 h showing live cells. Virtually no dead cells were observed. The stabilised protein is visible by faint autofluorescence.