Title: Structure of S-layer protein Sap reveals a mechanism for therapeutic intervention in anthrax

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Anthrax is an ancient and deadly disease caused by the spore-forming bacterial pathogen *B. anthracis*. Today, anthrax mostly affects wildlife and livestock, but remains a concern for human public health primarily in persons handling contaminated animal products and as a bioterror threat due to the high resilience of spores, high case-fatality rates and the lack of a civilian vaccine program[1,2]. The bacterium's cell surface is covered by a protective paracrystalline monolayer composed of the S-layer proteins Sap or EA1. Here, we generated nanobodies to inhibit Sap self-assembly, determine the structure of the Sap S-layer assembly domain (Sap^{AD}) and show that S-layer disintegration attenuates *B. anthracis* growth and anthrax pathology *in vivo.* Sap^{AD} is found to consist of 6 beta-sandwich domains that fold and support S-layer formation independently of calcium. Sap inhibitory nanobodies prevented Sap assembly and depolymerized existing Sap S-layers *in vitro. In vivo,* nanobody-mediated disruption of the Sap S-layer resulted in severe morphological defects and attenuated bacterial growth. Subcutaneous delivery of Sap inhibitory nanobodies cleared *B. anthracis* infection and prevented lethality in a mouse model of anthrax disease. These findings expose disruption of S-layer integrity as a mechanism with therapeutic potential in S-layer carrying pathogens.

As part of its immune evasion strategy, *B. anthracis* presents a dynamic and complex cell surface. Atop a ~40 nm thick peptidoglycan cell wall, the vegetative bacilli are covered by one of two distinct proteinaceous paracrystalline arrays known as Surface layer or S-layer (Fig. 1a)[3]. In response to host-derived signals, *B. anthracis* becomes fully virulent by secretion of the anthrax exotoxins, and the expression of a poorly immunogenic and antiphagocytic poly-γ-D-glutamic acid (PGA) capsule[4-6], ultimately leading to local or life-threatening systemic disease[7]. PGA chains are covalently attached to the peptidoglycan layer and cross the porous S-layer to extend as 100-200 nm long threads from the cell surface (Fig. 1a)[8]. S-layers compose the cell surface of a range of different Bacteria and near all Archea[9]. They have proposed roles as exoskeleton, protection against harmful environments, scaffolding structure for surface-localized enzymes and adhesins, molecular sieve for nutrient uptake and as contact zone with the extracellular environment, including host cells in case of pathogenic bacteria[10]. In *B. anthracis*, mutually exclusive S-layers composed of *surface array protein* (Sap) or *extractable antigen 1* (EA1) sequentially appear at the cell surface during exponential and stationary growth phases, respectively[11]. Although together with the PGA capsule the two S-layer proteins (SLPs) are the primary contact area between the bacterium and its environment, little is known about their structure and function. Strains with either or both SLPs deleted are viable when grown *in vitro* in rich culture media despite reported cell division defects in a *sap* deletion mutant[12,13]. However, the virulence of S-layer deletion mutants in the context of infection has not been examined. To interrogate the physiological role of Sap, the major *B. anthracis* SLP associated with vegetative growth, we built on previous work showing that camelid single domain antibodies or nanobodies, can be used as a tool to inhibit SLP polymerization for structural and functional studies[14].

Sap is an ~800 residue protein highly conserved in *B. anthracis, B. cereus* and *B. thuringiensis,* with 80% average pairwise sequence identity among different isolates. An N-terminal signal peptide directs Sap to the cell surface, where it binds a ketal-pyruvylated ManNac unit in the peptidoglycan via an α-helical cell wall anchoring domain that consists of three S-layer homology (SLH) regions (Fig. 1b)[15,16]. The region corresponding to the predicted S-layer assembly domain (Sap^{AD}; residues 216-814 ref[17]) was cloned for recombinant expression in *E. coli*. Sap^{AD} was isolated as soluble protein, purified and used for immunization of a llama and the selection of 20 unique Sap-binding nanobodies. A combinatorial screen of Sap^{AD} - nanobody complexes resulted in a set of two, Nb^{AF684} and Nb^{AF694}, that together allowed crystallization and structure determination of Sap^{AD} to 2.7 Å resolution. Our analysis showed that Sap^{AD} consists of six beta-sandwich domains (D1-D6) connected via short linkers (Fig. 1c; Supplementary Fig. 1). Domains D1 (residues 216 – 296) and D2 (residues 297 – 384) give rise to an L-shaped structure with an interdomain interface burying a 322 $Å^2$ surface area comprising 6 and 2 conserved H-bonds and hydrophobic contacts, respectively (Supplementary Fig. 2a). Nb^{AF684} and Nb^{AF694} bind the D1-D2 hinge and the D1 domain respectively (Fig. 1d; Supplementary Fig. 2b and c). The D1-D2 'arm' is connected via a flexible linker to a central

tile-shaped Sap^{AD} 'body' formed by domains D3 to D6 (Fig. 1c). A hinge region between D4 and D5 gives rise to pairwise alignment of D3 and D6, and D4 and D5, respectively, an interface that buries 962 $Å^2$ surface area and contains, respectively, 14 and 6 conserved H-bonds and charge interactions, as well as 7 hydrophobic contacts (Fig. 1c, d; Supplementary Fig. 2). Together, the Sap^{AD} arm and body form a flat supertertiary structure of 70 Å by 130 Å (Fig. 1c). SAXS analysis and the close superimposition of the Sap^{AD} in two unrelated crystal forms using Nb^{AF694}-Nb^{AF684} or Nb^{AF694}-Nb^{AF683} as crystallization aids suggest that this domain organization reflects the one of a Sap^{AD} monomer in solution (Supplementary Fig. 3).

Phylogenetic analysis of known S-layer proteins in *Bacillaceae* and *Paenibacillaceae* points to the presence of 12 SLP families (Supplementary Fig. 4a). The modular architecture of Sap^{AD} comprising six β-sandwich domains and the tile-like assembly of the D3-D6 'body' are strikingly similar to that seen in the assembly domains of *Geobacillus stereathermophilus* SbsB[14] and SbsC [18] (Supplementary Fig. 4b), suggesting that despite a low pairwise sequence identity (23 % on average; Supplementary Fig. 4c) SLPs in these *Bacillales* have a common origin and architecture. Different to SbsB or SbsC, folding of the individual Sap^{AD} domains into the tile-like supertertiary structure does not show any requirement for calcium binding. Many S-layer proteins require divalent metals, particularly calcium for folding and/or self-assembly[14, 18, 19, 20]. However, we find that purified Sap^{AD} readily folds and aggregates into high molecular weight species, also in the absence of free calcium (Fig. 2a; Supplementary Fig. 5c). Examination of Sap^{AD} aggregates via transmission electron microscopy (TEM) showed they consist of tubules, and two-dimensional sheets with a uniform lattice with P2 symmetry and unit cell dimensions of a=211.4 ±1.9 Å, b=89.1 ± 0.7 Å and γ=84.0° ± 1.0 (±SD, *n*=36; Fig. 2b). 2D projections of Sap^{AD} sheets and tubules show quasi isomorphous subunit packing, consistent with two Sap^{AD} copies per unit cell (Fig. 2c). Subunit packing gives rise to a cross-like contact across the central twofold axis, and higher density ridges formed by contacts along the short cell edge (Fig. 2c). The monomer packing and unit cell parameters are similar to that reported for Sap S-layers measured on deflated *B. anthracis* cells[3], suggesting that the tubules and 2D sheets seen in Sap^{AD} solutions at least partially reflect the subunit packing found in native Sap S-layers. The observed Sap^{AD} tubules have a diameter ranging from 50 to 300 nm, approaching the average ~1.3 μm width of *B. anthracis* cells. The intrinsic curvature in the lattice of the Sap^{AD} tubules may thus be representative of that found on the bacterial cell surface.

Nb^{AF684} and Nb^{AF694} facilitate crystallization of Sap^{AD} by providing additional lattice contacts and slowing down polymerization of the protein. When systematically screening the twenty isolated Sap binding Nbs, we found six that maintained the protein in a monomeric form for extended times (at least 7 days; Supplementary Fig. 5a). Strikingly, the Nbs not only inhibited *de novo* Sap assembly, but also destabilized existing Sap lattices. The addition of a 15 μM cocktail of five of the **S**ap **a**ssembly **i**nhibitory Nbs (Nbs^{SAI}: Nb^{AF683}, Nb^{AF692}, Nb^{AF702}, Nb^{AF704} and Nb^{AF707}, 3 μM each) led to a rapid fragmentation of the tubules (Fig. 2e, f) and resulted full dissolution of Sap^{AD} polymers in a matter of minutes (Fig. 2d-f). Equivalent concentrations of a control Nb that binds an unrelated target (Nb11) neither blocked *de novo* Sap

S-layer assembly, nor had an effect on tubule length or number (Fig. 2e, g). 15 μM of a single representative inhibitory nanobody (Nb^{AF692}), however, led to Sap^{AD} tubule dissolution in a timeframe corresponding to that of the Nbs^{SAI} cocktail (Supplementary Fig. 5d). The depolymerization activity of Nbs^{SAI} appeared to be specific to the single domain antibodies. Sera obtained from mice immunized with monomeric Sap^{AD}, suppressed *de novo* polymerization of monomeric Sap^{AD}, but did not result in tubule dissolution (Fig. 2e, g). Thus, although antibodies in mouse serum can bind polymerization sensitive epitopes, this did not lead to a rapid destabilization of pre-existing S-layers as seen for Nb^{SAI}.

Next, we evaluated the effect of Nbs^{SAI} on *B. anthracis* growth. When grown at 37º C degrees on brain hearth infusion (BHI) medium under static conditions, *B. anthracis* forms long multicellular filaments that clump together at higher cell density. Addition of a 20 μM Nbs^{SAI} cocktail to the inoculum resulted in reduced growth rates compared to the buffer control or a 20 μM cocktail of Sap binding nanobodies lacking an assembly inhibitory activity (Nbs^{S2}) Nb^{AF679}, Nb^{AF687}, Nb^{AF694}, Nb^{AF695} and Nb^{AF703}; 4 μM each) (Fig. 2h). Whilst cells treated with buffer reached confluency within 5 hours post inoculation, a Nbs^{SAI} treated inoculum showed dispersed chains of *B. anthracis* cells only (Fig. 2h, i, Supplementary Fig. 6a, Supplementary Movie 1), suggesting that the disruption of Sap S-layer assembly attenuates bacterial growth. Individual SAI nanobodies varied in their attenuation of *B. anthracis* growth, with Nb^{AF692} approaching that of the Nbs^{SAI} cocktail (Supplementary Fig. 6b). Although Nbs^{SAI} attenuated *B. anthracis* filament growth, treatment did not lead to full growth inhibition. Following optical density at 600nm (OD_{600}) showed Nbs^{SAI}-treated cultures approached equivalent stationary phase densities to cultures treated with buffer or a non-SAI Sap-binding Nb, albeit with an approximate delay of 2 hours (Supplementary Fig. 6c). This delay is primarily due to an extended lag phase in the Nbs^{SAI} or Nb^{AF692} treated samples, and treated and non-treated cultures have comparable maximal growth rates, suggesting cultures become insensitive to Nb treatment (Supplementary Fig. 6c). We therefore tested whether Nbs^{SAI} become titrated below their minimal effective concentration at higher cell densities. When added at the moment of inoculation the inhibitory activity for both Nbs^{SAI} and Nb^{AF692} reached saturation near 100 nM concentration (Supplementary Fig. 6d). Free Nbs^{SAI} stayed well above this minimal effective concentration throughout culturing (Supplementary Fig. 7a). In wildtype cells, EA1 starts replacing Sap from mid exponential phase (Supplementary Fig. 7 b, c)[11,12], and previous studies have shown that cells lacking *sap* are viable and derepress *eag*[11,12]. Possibly, a change from a Sap to an EA1 expressing physiology leads to an insensitivity to Nbs^{SAI} activity. When Nbs^{SAI} or Nb^{AF692} was added at 2 hours post inoculation, i.e. upon entering exponential growth, treated cultures showed a 20 to 25% reduction in maximal growth rate compared to buffer treated cultures (Supplementary Fig. 6c).

To gain insight into the physiological effect of Sap assembly inhibition and Sap S-layer disassembly, treated *B. anthracis* cultures were examined using scanning electron microscopy

(SEM) and light and fluorescence microscopy. Nbs^{SAI} or Nb^{AF692} treated cultures contained cells with striking morphological defects as well as unaffected, normal looking cells resembling control cultures (Fig. 3a). The affected population presented as cells with irregular, scoured cells surfaces as well as collapsed cell masses that had lost the bacilliform cell shape (Fig. 3a,b, Supplementary Fig. 7d). Scoured cells represented up to 30% of Nbs^{SAI} or Nb^{AF692} treated cultures, whilst no affected cells were seen in buffer or Nb^{AF703} treated cultures (Fig. 3c, d). Like Nbs^{SAI} or Nb^{AF692} treated WT, cultures of the *sap* deletion strain RBA91 showed a mixed population of cells with normal and scoured morphology (Fig. 3a, b). This is unlike the *eag* deletion mutant SM91 [11,12], which showed defects in cell morphology only when treated with Nbs^{SAI} or Nb^{AF692} (Supplementary Fig. 7e). Notably, the *sap* deletion mutant lacks the collapsed cell masses seen in Nbs^{SAI} treated WT or SM91 cells, suggesting that the disruption of an existing Sap S-layer by Nbs intervention may be more detrimental than a genetic lack of *sap*, which may be (partially) compensated by changes in cell physiology. Although scoured cells had intact cell membranes as judged from a lack of propidium iodide staining, it is unclear whether they represent a viable part of the population (Supplementary Fig. 8a). Scoured cells in Nbs^{SAI} or Nb^{AF692} affected cultures showed a small (~0.6 μm) but significant increase in cell length compared to unaffected cells (Fig. 3d), though nothing like the multifold elongation into filamentous cells as reported for the *sap* deletion mutant RBA91 [11,12]. When examined by TEM, RBA91 and Nbs^{SAI} affected cells show a loss of the ordered surface monolayer seen in WT cells, indicating that Sap lost its S-layer structure and/or detached from the cell surface (Fig. 3c). However, scoured and collapsed cells showed a clear and even increased staining with fluorescently labeled Nbs^{SAI} or Nb^{AF692} compared to unaffected cells, showing that Sap remained surface-bound in Nbs^{SAI} affected cells (Fig. 3a). The lack of morphological defects and the poor staining of Sap in unaffected cells suggests these may have had EA1 rather than Sap containing S-layers. However, staining with anti-Sap or anti-EA1 polyclonal antibody pointed to Sap as dominant S-layer constituent in both affected and unaffected cells, with only sparse punctuate staining of EA1 (Supplementary Fig. 8b). Instead, we propose that the increased staining of Nbs^{SAI} or Nb^{AF692} as well as anti-Sap or anti-EA1 is more likely indicative of an increased antigen accessibility in scoured and collapsed cells. It is presently unclear whether unaffected cells represent a resilient or an as yet unaffected population of the culture.

We next evaluated virulence and viability of Nbs^{SAI} and Nb^{AF692}-treated cultures in a rodent model of lethal *B. anthracis* infection. For mouse infection, the *B. anthracis* 34F2 inoculum was grown on RM+ medium to induce expression of the anthrax exotoxins (Supplementary Fig. 9a). Cells grown on RM+ predominantly express the Sap S-layer (Supplementary Fig. 9c, d), and when treated with Nbs^{SAI} or Nb^{AF692} these cells show the morphological defects and collapsed cell masses also observed for BHI medium (Supplementary Fig. 9e). Whereas sham-operated and Nb11-treated mice succumbed to lethal anthrax disease within 3-5 days post-inoculation, all animals (n=8) that were subcutaneously treated with 10 doses of 20 nmole Nbs^{SAI} or Nb^{AF692} administered over a 6 days period survived (Fig. 4a).

Because in this experiment the first treatment dose was given together with the infectious inoculum, we tested effectiveness of a single dose treatment. 75% of the animals (*n*=8) succumbed to anthrax disease when a single dose of 20 nmole Nbs^{SAI} was given concomitant with inoculation (Fig. 4b), demonstrating the need for consecutive treatment doses during ongoing infection. On the other hand, the 10 dose treatment remained effective also when the first Nbs^{SAI} dose was administered 15 min after infection rather than concomitant with the infectious inoculum (Fig. 4b). Over the first days of Nb therapy, mice in the repeated dosing part of the cohorts fully recovered from anthrax symptoms and showed no signs of illness up to 10 days after coming off treatment when the experiment was halted, suggesting they had cleared the infection. Motivated by the therapeutic activity of the S-layer inhibitory nanobodies, we evaluated whether vaccination with Sap can protect mice from anthrax. However, mice immunized with monomeric Sap^{AD} or individual Sap domains (Sap^{D1}, Sap^{D4} or Sap^{D6}) were not protected from anthrax despite showing robust anti-Sap titers in their sera (Fig. 4c, d). Although disease development and point-of-death in vaccinated animals slowed two- to three-fold compared to control animals (Fig. 4c), 75 to 100% of the animals succumbed to lethal anthrax disease within a week of infection. Nevertheless, the sera isolated from animals immunized with monomeric Sap showed robust binding to cells expressing the Sap S-layer (Fig. 3e and Supplementary Fig. 7-9) and demonstrated a Sap assembly inhibitory activity even down to 1:1000 dilution (Fig. 2g). However, unlike Nbs^{SAI} or Nb^{AF692}, mice sera had no dissolving activity towards Sap^{AD} tubules. We speculate that the unexpected discrepancy in protective or therapeutic efficacy between antibodies in the Sap vaccinated mice sera and Nbs^{SAI} may relate to the S-layer disrupting activity of the Nbs. The ability to disassemble pre-existing S-layer and the associated cell surface defects may be critical for therapeutic efficacy.

In conclusion, we show that camelid single domain antibodies provide a unique platform to generate S-layer penetrating and disrupting affinity reagents that have growth inhibitory activity on *B. anthracis* and can provide a therapeutic potential during ongoing anthrax disease. We find that the disruption of the Sap S-layer leads to severe cell surface defects, pointing to a cell envelope supporting activity of the S-layer. These observations provide tantalizing evidence that *in vivo* S-layers disruption can be detrimental to bacterial growth and that S-layers may provide good therapeutic targets in additional human pathogens, including *Clostridium difficile*, *Serratia marcescens* and *Rickettsia*'s[21].

Methods

Cloning, production and purification of recombinant *B. anthracis* Sap^{AD} and $EA1^{AD}$. Synthetic codon-optimized genes encoding *B. anthracis* Sap^{AD} (residues 216-814 of UniProtKB - P49051, C-terminal 6-His tagged) and $EA1^{AD}$, (residues 214-862 of UniProtKB - P94217, N-terminal 6-His tagged) (Supplementary Table 1), were generated to ensure good overexpression in *E. coli*. The synthetic *sap^{AD} and eag^{AD} genes* were made by gene assembly using overlap PCR

and cloned into the Gateway T7 expression vector pDEST14 and pET300, creating pAFSLP1 and pAFSLP10, respectively. *E. coli* BL21 (DE3) cells were transformed with pAFSL1 or pAFSLP10. For Sap^{AD} production cells were grown in lysogeny broth (LB) medium in presence of 100 µg/mL ampicillin and 0.1 % glucose at 37 °C with shaking prior induction with 10 µM isopropyl 1-thio-D-galactopyranoside (IPTG) when an OD_{600} of 0.6-0.8 was reached. After overnight (O/N) induction at 37 °C, 50 mL of cells expressing Sap^{AD} were harvested by centrifugation and resuspended in 50 mL of buffer A (50 mM tris pH 8, 300 mM NaCl) supplemented with protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride and leupeptin, 0.1 mg/mL and 1 µg/mL final concentrations, respectively), 0.1 % Triton-100 and 20 mM imidazole pH 8. Cells were lysed by high-pressure homogenization using a Cell Disruptor (Constant Systems) and centrifugated at 20,000 *g*. The supernatant containing His_6-tagged Sap^{AD} was applied to 5 mL of 40 IDA^{high} agarose IMAC beads charged with Ni^{2+} (Bio-Works) and pre-equilibrated with buffer A. After extensive washing with buffer 4 % buffer B (50 mM Tris pH 8, 300 mM NaCl, 500 mM Imidazole pH 8), the protein was eluted with 100 % buffer B, filtered with a 0.2 µm filter (Acrodisc LC 13 mm, Syringe filter Life Science) and applied to a Superdex 200 16/60 size-exclusion column (GE Life Sciences) equilibrated with 10 mM Tris pH 8, 100 mM NaCl, and 5 % glycerol. Pooled fractions corresponding to monomeric Sap^{AD} (column elution volume 68-78 mL) were filtered as before, adjusted to a concentration of 0.2 mg/mL and maintained at 30 °C until further use. Production and purification of $EA1^{AD}$ were performed as described elsewhere [22]. Monodispersity and polymerization state of Sap^{AD} or $EA1^{AD}$ preparations were evaluated by Dynamic Light scattering (DLS) or negative stain transmission electron microscopy (see below). *Selenomethionine labeled* Sap^{AD}: To produce selenomethionine labeled Sap^{AD} for structural studies, the methionine auxotrophic *E. coli* B843 strain was transformed with pAFSL1 and pre-cultured in M9-based minimal media, supplemented with SelenoMet Medium Base and SelenoMet Nutrient Mix as recommended by the manufacturer (Molecular Dimensions Ltd.), 40 µg/mL L-methionine and 100 µg/mL of ampicillin. O/N culture was washed in phosphate buffered saline (PBS, 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl) prior inoculation in the above minimal medium (1:100 dilution), now supplemented with 40 µg/mL L-selenomethionine (Acros Organics) instead of L-methionine. Protein expression and purification were performed as described for the non-labelled protein with the exception that all buffers used for the purification were supplemented with 1mM DTT to prevent selenomethionine oxidation.

Cloning, production and purification of Sap^{AD} domains. Coding sequences corresponding to individual Sap^{AD} domains (D1 (residues 216–295), D2 (residues 296-384), D3 (residues 384-490), D4 (residues 493-594), D5 (residues 596-706) and D6 (residues 707-814), each supplemented with a C-terminal 6-His tag) were PCR amplified from pAFSLP1 using primers listed in Supplementary Table 2. The generated gene fragments were cloned into the pDEST14 expression vector, creating pAFSLP2, pAFSLP3, pAFSLP4, pAFSLP5, pAFSLP6 and pAFSLP7 for Sap^{D1} to Sap^{D6}, respectively. Expression and purification of the recombinant Sap domains were performed as described for Sap^{AD}, with the exception that size exclusion was performed with a sephacryl S100 16/60 column (GE Life Sciences) in the following optimized storage buffers: Tris pH 8 (Sap^{D1}, Sap^{D2}, Sap^{D3} and Sap^{D5}; Tris pH 7,5 (Sap^{D4}); Hepes pH 7 (Sap^{D6}).

Sap^{AD} nanobody® (Nb) isolation, production and purification. A female llama (*Lama glama*) was weekly immunized with 6 subcutaneous injections of adjuvant (Gerbu LQ, GERBU

biotechnik) emulsified Sap^{AD} (0,14 mg per injection), each within 15 h from purification to ensure maximal monomeric Sap^{AD}. Four days after the final boost, total RNA was extracted from peripheral blood mononuclear cells in a 50 mL blood sample according to Domanska and coworkers [23]. llama immunization was performed in accordance with institutional guidelines, following experimental protocol reviewed and approved by the Vrije Universiteit Brussel Ethical Committee for Animals experiments (Project number 16-601-3). Starting from total RNA, cDNA was synthesized and the Nbs repertoire was amplified and cloned as described previously [24], except that phagemid pMESy4 was used as the display vector allowing the expression of C-terminal His6-EPEA tagged Nbs. The resulting phage library consisted of approximately $4.6x10^9$ independent clones containing an insert corresponding to the size of a Nb. To identify Sap^{AD}-specific binders, 1 µg of the monomeric Sap^{AD} antigen was solid phase immobilized in sodium bicarbonate buffer pH 8.2 in a 96-well Maxisorp plate (Nunc). Microwells were subsequently blocked with PBS containing 2 % skimmed milk powder prior to incubation with the phage library. Unspecific phages were removed by extensive washing with PBS, 0.05 % Tween-20 and bound phages were eluted after trypsin treatment. Two such selection rounds were performed and 94 single clones were randomly picked from the first and second round outputs. *E. coli WK6* was transformed with purified plasmids from the selected clones, grown on LB medium and induced with IPTG for periplasmic expression of monoclonal Nbs as described elsewhere [25]. Specificity of the Nbs was verified via ELISA by coating 1 µg of the monomeric Sap^{AD} in Maxisorp plates. Bound Nbs were detected via the "EPEA" tag using a the CaptureSelect Biotin anti-C-tag Conjugate (Life technologies) mixed with alkaline phosphatase (Promega) for revelation. Sap^{AD}-specific Nbs were sequenced resulting in a library of 20 unique monoclonal nanobodies. Sequences for Nbs used in this study are found in Supplementary Table 3. Selected Nbs were expressed and purified as described previously [25], with the exception that size exclusion chromatography (Biorad enrich SEC70 column) was performed as final step of purification for each Nb with optimized buffers (10 mM Hepes pH 7: Nb^{AF692} & Nb^{AF707}; 10 mM Tris pH 7.5: Nbs^{AF684},Nb^{AF687},Nb^{AF695} & Nb^{AF688}; 10 mM Tris pH 8: Nb^{AF679},Nbs^{AF683}, Nb^{AF694}, Nb^{AF702},Nb^{AF703},Nb^{AF704} & Nb11,) each supplemented with 100 mM NaCl and 5 % glycerol. Nb^{AF688} showed poor solubility and was not further used in the study or included in Nbs^{SAI}. Purified Nbs were buffer exchanged to PBS for bacterial or animal experiments.

Sap^{AD} polymerization assays and dynamic light scattering (DLS). Intensity correlation functions of freshly purified Sap^{AD} solutions were collected at 25 °C in 4 µL Cyclic Olefin Copolymer (COC) disposable cuvettes at an angle of 90° using a Dynapro NanoStar DLS machine (Wyatt technology). The shown graphs display the regularization graphs obtained with Dynamics software and represent the calculated size distribution of Sap^{AD} in solution, alone or in presence of single Nbs, Nbs^{SAI}, mice or llama sera for the auto-correlation curve associated with a group of 10 measurements per experiment. The % mass represents the estimated total mass of the particles in solution corresponding to the user-specified peak divided by the estimated total mass of all particles in solution from the regularization data. Fresh Sap^{AD} preparations at a concentration of 0.22-0.3 mg/mL, maintained at 30 °C, show a monodisperse size distribution around a 4 nm particle diameter, corresponding to a folded monomeric state of the protein. Incubation of monomeric Sap^{AD} at RT results in polydisperse profiles over a 24h period (or near instantaneously when increasing protein concentration), with high particle diameter (1000 nm

and more) corresponding to polymeric Sap^{AD}. Sap^{AD} polymerization inhibition activity of Nbs or llama and mice sera was evaluated by DLS over time by addition of a 1.5-fold molar excess Nbs or a 1:1000 dilution of mice or llama sera to fresh monomeric Sap^{AD} preparations at 3,5 µM. Samples where then concentrated 10 fold at incubated at RT prior DLS measurement. The ration monomeric / polymeric Sap^{AD} was calculated by plotting the % mass of particles with size distribution below and above 10 nm diameter, respectively.

Sap^{AD} depolymerization assays and electron microscopy. Sap^{AD} assembly into 2D lattices and tubules was allowed to proceed by prolonged incubation at 25°C of 2 mg/mL freshly purified Sap^{AD} in PBS or PBS supplemented with 50mM EDTA in those experiment performed to determine if Sap^{AD} polymerization are dependent on divalent cations. The Sap^{AD} polymerization state was monitored by DLS (see above) and negative stain EM. To verify *in vitro* depolymerization activity of Nbs or mice sera on Sap^{AD} S-layer lattices, Sap^{AD} tubules were incubated with indicated concentrations of single Nbs (Nb11 or Nb^{AF692}), Nbs^{SAI}, a 1:1000 dilution of mice or llama sera or PBS buffer as negative control. Reactions were incubated for 24 hours and samples were taken for monitoring by TEM at 1, 5, 10 and 60 minutes post incubation (PI) in case of Nbs^{SAI} and Nb^{AF692}, or at 60 minutes PI for Nb11, PBS and mice or llama sera samples (Fig. 2d, e, f and Supplementary Figs. 5c, d). All samples were prepared for negative stain electron microscopy by applying 5 µl sample to a non-glow discharged formvar copper 400 mesh grid (EMS), rinsing with 10 µl PBS and staining for 30 seconds in 10 µl 1% uranyl formate. Negatively stained samples were imaged at 1200x magnification (pixel size 9.55Å) using an in-house 120 kV JEM 1400 (JEOL) microscope equipped with a LaB6 filament and CMOS camera (TVIPS TemCam F-416). Per biological replicate (n=2), five squares (15.3 μm^2 each) were randomly selected as technical replicates for tubule counting. Normalized tubule densities per grid square (i.e. tubule count / mean tubule count of the buffer control) were plotted (Fig. 2e) and tested for statistical significance against the buffer control using unpaired student *t* tests. The experiments shown in Fig. 2f and Supplementary Fig. 5d plots the number (labeled "c") and the length (ImageJ) of all individual Sap^{AD} tubules in the accumulated 5 grid squares of a representative biological replicate as scatter plot using the GraphPad software, with the sample mean ± standard deviation. Time-points were tested for statistical significance against the buffer control using the non-parametric Mann-Whitney *U* test.

Sap^{AD} crystallization and structure determination. A fresh monomeric preparation of native or selenomethionine-labelled Sap^{AD} (3 µM) was incubated at 30°C with a purified Nb^{AF683} and Nb^{AF694} or Nb^{AF684} and Nb^{AF694} respectively, each at 1.5 fold molar excess. The Sap^{AD}- Nbs complexes were mix and filtered with a 0.2 µm filter prior to 40 fold concentration by AMICON® 10 KDa MWCO centrifugal filter unit. After 3 weeks at 20°C crystals of selenomethionine labeled Sap^{AD} in complex with Nb^{AF684}-Nb^{AF694}, non-labeled Sap^{AD} in complex with Nb^{AF683} and Nb^{AF694} were obtained using sitting-drop vapour-diffusion and a crystallization solution containing 0.1 M SPG (2-amino-2-(hydroxymethyl) propane-1,3-diol) buffer at pH 6.0, and 25 % w/v polyethylene glycol 1500.The crystallization buffer was supplemented with 10 % glycerol and crystals were mounted in nylon loops and flash-cooled in liquid nitrogen. Diffraction data for Sap^{AD}-$NbAF^{684}$-Nb^{AF694} and Sap^{AD}-$NbAF^{683}$-Nb^{AF694}were collected at Diamond Light Source (Didcot, UK) on beamlines I03 and I24 under experiments MX12718-10 and MX17150-10, respectively. Single crystal diffraction data for Sap^{AD}-$NbAF^{684}$-

Nb^{AF694} were collected at a wavelength of 0.9795 Å, corresponding to the Se K-edge absorption peak, integrated and scaled using XDS[26] and Autoproc[27] into space group $C222_1$ with unit cell parameters a= 107.89 Å, b= 115.35 Å and c= 151.05 Å and truncated to 2.7 Å resolution. Heavy atom sites were determined and refined using the programs ShelXD [28] and Sharp [29], respectively. Experimental phases were determined according the Single Anomalous Dispersion (SAD) method resulting in initial phases with an R_{cullis} of 0.57 and Figure of Merit (FOM) of 0.46 to 4.17 Å resolution, which were solvent modified and extended to 2.95 Å resolution (FOM = 0.89) using the CCP4 programs DM and Solomon [30-32] , yielding good quality maps that allowed unambiguous tracing of the Sap^{AD} structure. The Sap^{AD} model was built manually using Coot [33] and refined to 2.7 Å resolution using Refmac5 [34] and Buster [35], yielding a final model with a R and freeR factor of 18.7% and 25.0%, respectively, and 0.2% of residues marked as Ramachandran outliers. Data for Sap^{AD}-Nb^{AF683}-Nb^{AF694} were collected to 2.73 Å resolution at 0.96858 Å wavelength, and processed using Autoproc [27], in space group P1 with unit-cell dimensions *a* = 74.23 Å, *b* = 79.91 Å, *c* = 81.23 Å, α=88.7 β=82.0 γ=85.6 encompassing two Sap^{AD}-Nb^{AF683}-Nb^{AF694} copies in the asymmetric unit. The structure was solved by molecular replacement using Phaser [36] using the experimental structure of Sap^{AD}, Nb^{AF683} and Nb^{AF694} as search models. The structure was built manually using Coot [33] and refined using Buster [35] to a R and freeR factor of 17.5% and 21.8%, respectively, and 1,2% of residues labelled as Ramachandran outliers. See Supplementary Table 1 for data collection and refinement statistics. Figures were made using Pymol.

Small angle X-ray scattering (SAXS). SAXS data for monomeric Sap^{AD} in complex with Nb^{AF683} were collected at home source using a Rigaku BioSAXS-2000 instrument. Monomeric Sap^{AD} was preincubated with a 5 fold excess of Nb^{AF683} prior to sample concentration. Sample was then loaded on a Superdex 200 16/60 (GE Life sciences) equilibrated with Sap^{AD} storage buffer and eluted fractions were subjected to the data collection. Scattering intensities were collected on 70 µL samples of Sap^{AD}-Nb^{AF683} at 1, 3 and 5 mg/mL. The radial averaging and the buffer subtraction were performed using the Rigaku SAXSLab software and averaged data were analysed using the ATSAS software package [37]. SAXS profiles of the three sample concentrations superposed well and showed linear Guinier plots with an estimated Rg of 39.5 Å (±1.5) (Supplementary Fig. 3b) supporting the monodispersity of the sample. For further analysis, SAXS data of the 3.0 mg/mL sample were used. The molecular mass of the scattering particle was derived using the QR method. Calculation of the probability distribution curve was done using the ATSAS program GNOM [38] and CRYSOL [39] was used for calculation of the theoretical scattering profile of the Sap^{AD}-Nb^{AF683} complex extracted from the Sap^{AD}-Nb^{AF683}-Nb^{AF694} crystal structure and used for fitting into the experimental SAXS volume. *Ab initio* shape information of the particle in the experimental scattering data was calculated using the probability distribution curve (P(R) curve) information using the DAMMIN software of the ATSAS package. The resulting dummy atom models from 10 independent DAMMIN runs were averaged and filtered with DAMAVER resulting in the final *ab initio* model. The Situs module pdb2vol was used to convert the averaged, filtered models into volumetric map and SUPCOMB was used to superimpose the Sap^{AD}-Nb^{AF683} structure with the dummy atom models [40].

Sap^{AD} 2D crystallization and cryo-electron microscopy. Sap^{AD} assembly into 2D lattices and tubules was achieved by prolonged incubation of 2 mg/mL freshly purified Sap^{AD} in PBS at 25°C. Tubules and 2D sheets were left to polymerize for 2 weeks before being plunged for cryo-electron

microscopy. Grids were prepared by spotting 3 µl of undiluted solution on quantifoil R2/1 mesh 400 grids (Quantifoil), blotting manually for 1" and plunging in liquid ethane with a home-made plunging device. Data was collected at NeCEN (Leiden, The Netherlands) on a FEI Titan Krios (Thermo Fisher) equipped with XFEG, Cs-corrector and Falcon 3 camera. A total of 2331 images were collected in linear mode with exposure of 1.03 s and a dose fractionation scheme of 55.5 e/Å^2 spread out over 40 frames. Nominal recording magnification was 47.000x corresponding to a pixel size of 1.44 Å. Images were drift- and beam-induced motion corrected without weighting using MotionCor2 [41] with 3 x 3 patches, whilst grouping frames by 3. Contrast transfer function (CTF) fitting was done with CtfFind4.1 [42]. Lattice maps were calculated in real space using RELION2.0 [43] for manual picking, particle extraction and 2D classification. A total of 52 images were picked for 2D crystal reconstruction based on 2-dimensional sheets, whilst multiple single tubules were used separately for calculating the lattice of the tubules. In addition, unit cell parameters of 36 well-diffracting 2D sheets based on the diffraction pattern were calculated with FOCUS 1.1 using the 2dx software package [44]. A ~10% difference in vector length for *in vitro* grown Sap^{AD} lattices versus *in vivo* Sap S-layers may represent a shrinkage of the unit cell in negative stained TEM of surface bound S-layer on deflated cells versus cryoTEM on solution dispersed Sap^{AD} S-layer, or a small rearrangement due to the missing attachment domain in the latter.

***B. anthracis* 34F2 S-layer composition and Protective antigen (PA) production in BHI or RM$^+$ media.** *B. anthracis* 34F2 O/N culture was spun down, washed and diluted in Brain Heart Infusion broth (BHI) or RM$^+$ medium (R medium supplemented with Foetal Calf Serum [45]) to have a starting inoculum with OD_{600} of 0.05. Cells were grown at 37°C on BHI or 35°C on RM$^+$ with shaking and harvested at the indicated time points (Supplementary Figs. 7b,c, 9a,b). Harvested cells were normalized to OD_{600} 0.1 in 1x Laemmli buffer and incubated during 1h at 95°C. Whole cell extract was diluted (1:20 in water) and spotted on nitrocellulose membrane prior blocking. Blocking, incubation with antibody and washing of the membrane were done in PBS supplemented with 0.05% Tween-20 (v/v) and 4% (w/v) non-fat dry milk in case of blocking step. Membranes were then incubated with mice α-Sap or α-EA1 serum (1:1000). After an extensive wash with water, membranes were incubated with alkaline phosphatase conjugated goat α-mouse antibody (AQ3562 Sigma-Aldrich). Following a last wash step, proteins presence was revealed by incubation with detection reagent (alkaline phosphatase buffer supplemented with NBT/BCIP; Roche). Purified recombinant Sap^{AD} or EA1^{AD} proteins (0.3 mg/mL), were spotted on a nitrocellulose membrane as controls. For the detection of protective antigen expression, *B. anthracis* 34F2 cultures grown O/N in BHI or RM$^+$ were spun down and prepared as described above. Samples were loaded and separated by 8% SDS-PAGE before transfer onto polyvinylidene difluoride (PVDF) membrane by western blotting. Blocking, incubation with antibody and washing of the membrane were done as described above with a blocking step containing 3% (w/v) non-fat dry milk. Immunoblots were incubated overnight with monoclonal mouse α-PA primary antibody (Bei Resources; Ref: NR-4488, clone PA 2II 14B7-1-1). Recombinant PA protein (made in house) was used as positive control. Horseradish peroxidase-conjugated goat anti-mouse (Cat. No 115-035-146, Jackson Immunoresearch Laboratories) secondary antibody was used to detect proteins by enhanced chemiluminescence.

Light and fluorescent microscopy. Cells samples subjected to microscopic analysis, except for the time lapse experiments, were fixed with PBS supplemented with 4% paraformaldehyde (PFA) prior their observation in glass slide and coverslip. DIC and Fluorescent microscopy

images were acquired with Zeiss LSM 880 airyscan confocal microscope with a magnification of 200x. Time-lapse acquisition of Nbs treated cells growth in BHI were performed in an Incucyte^{TM} Zoom system (Essen BioScience), acquiring phase contrast images using a 20x objective every 15 min in 4 different zones of the well in order to cover the entire well surface. Cell confluency on single images was estimated with the Incucyte^{TM} Zoom software by calculating the ratio of pixels defined as corresponding to bacterial cells over background.

Electron microscopy on cells. Cells samples subjected to electron microscopy analysis were fixed for 30minutes in 2% PFA, 2.5% Glutaraldehyde in 0.1M Na-Cacodylate buffer pH7.4. In case of scanning electron microscopy (SEM) analysis, fixed cells were first were washed in 0.1M Na-Cacodylate buffer pH7.4 and then incubated in 2% OsO4 in 0.1M Na-Cacodylate buffer, pH7.4. Osmicated samples were washed twice in ultrapure water prior to a stepwise ethanol dehydration performed with various concentrations of the latter (50%, 70%, 85%, 100%). A last step of dehydration was performed in hexamethyldisilazane (HMDS) solution (Sigma, Australia). Samples in HMDS were spotted on silicon grids (Ted Pella) and air-dried over night at room temperature. Samples were next coated with 5 nm Platinum (Pt) in a Quorum Q 150T ES sputter coater (Quorum Technologies).The air-dried samples coated with Pt were placed in a Gemini 2 Cross beam 540 Zeiss SEM microscope for imaging at 1.50 kV with a SE2 detector. In case of transmission electron microscopy (TEM) analysis, fixed cells were first washed with 0.1M Na-cacodylate pH7.4 buffer and then deposited onto a non-glow discharged formvar copper 400 mesh grid (EMS), before staining with 1% uranyl formate. Negatively stained cells were then imaged using an in-house 120 kV JEM 1400 (JEOL) microscope equipped with a LaB6 filament and CMOS camera (TVIPS TemCam F-416).

Cell morphology and viability studies. *B. anthracis* 34F2, RBA91 (Δ*sap*) or SM91[12] (Δ*eag*) cells were grown in BHI as described above and harvested in mid exponential growth phase (2-3 h post inoculation) or, in case of cells grown in RM^{+}, from an O/N culture. The chromosomes of harvested cells (corresponding approximately to 2x10^{6} CFU), following a PBS washing step, was stained with Syto9 (Invitrogen; 1,169 µM in 0,85% NaCl). Cells were then incubated during 20 min at RT with PBS supplemented with 100 µM single Nb or Nbs^{SAI} mix labelled with DyLight 650 (Thermo Scientific; Fig. 3a, Supplementary Fig. 8a) or DyLight 594 (Thermo sientific; Fig. 3e, Supplementary Figs. 7e, 8b and 9e) or unlabeled 200 µM Nbs^{SAI} mix (Fig. 3b).For Sap and EA1cell surface, bacteria were additionally immunostained with mouse antiserum raised against purified recombinant Sap^{AD} or EA1^{AD} (see below) followed by incubation with Dylight 633 labelled monoclonal goat-anti-mouse (1:1000; QL222838; Thermo Scientific). Between every labelling step the excess dye, Nb or serum was removed by centrifugation. For cell morphology scoring and cell length measurements, bacteria were randomly selected from samples treated with PBS, Nbs^{SAI} or Nb^{AF692} and analysed with the ImageJ software. The cell lengths of all counted cells (originating from slides from four independent experiments, each containing at least 40 cells) and the ratio of unaffected versus scoured cells were plotted as scatter plots with sample mean and bar graph respectively using the GraphPad software (Fig. 3b, c). Cells with collapsed morphology were not counted in the analysis because of the difficulty in visually determining the number of cells included in the collapsed cell masses. To determine membrane integrity and cell viability in NbS^{SAI}, PBS or Triton 10% treated cells, bacteria were grown on BHI and harvested at mid log and treated with NbS^{SAI}, PBS or Triton 10% as described above.

Excess of treatment was then removed by centrifugation and cells were stained with the cell membrane permeable Syto9 (green) and impermeable propidium iodide (red) nucleic acid strains as suggested by the manufacturer (LIVE/DEAD™ BacLight™ assay). Excess DNA staining was then removed by centrifugation and wash in PBS, before cells were fixation and visualization.

Nbs effect on growth. Cell confluency estimation over time by Incucyte™ Zoom system: *B. anthracis* 34F2 cells grown on BHI and harvested in mid log phase as described above (~$2x10^3$ CFU) were incubated with PBS, 200 µM Nbs^{SAI} mix or 200 µM single Nb during 20 min at RT. Cells were then vortexed and inoculated in fresh BHI with a dilution of 1:10. Static growth in liquid BHI at 37°C in 96 flat bottom wells plates was recorded every 15 min during the first 5 h post inoculation as described above. Cell OD_{600} estimation over time: O/N cultured *B. anthracis* 34F2 (~$2x10^6$ CFU), grown and harvested as described above, were incubated with indicated concentrations of PBS, Nbs^{SAI} mix or single Nb during 20 min at RT. Cells were then gently vortexed and inoculated in fresh BHI with a 1:5 dilution and cultured at 37°C with shaking. Prior OD_{600} measurements at indicated points, cells were harvested and vortexed. In case of evaluation of Nbs effect on the growth of an early exponential phase of growth culture, 40 µM Nbs^{SAI}, single Nb or the equivalent volume of PBS were added to a *B. anthracis* 34F2 culture at 2h post inoculum (Supplementary Fig. 6c). Confluency and OD_{600} results are the mean of three biological replicates.

Nbs^{SAI} therapy in *B. anthracis* infected mice. *B. anthracis* 34F2 cells were grown O/N in RM^+ media as described previously [45]. Cells were harvested by centrifugation, washed three times in PBS and resuspended in PBS supplemented or not with 200 µM Nbs^{SAI} or single Nbs, so that 100 µl of suspension would contain ~100.000 CFUs. Bacteria were incubated 45 min at RT prior to injection in the mice. 7-12 week-old female C57BL/6 mice (purchased from Charles River Laboratories) were randomly divided into different cages upon arrival. One week after, each cage received an individual treatment. No blinding was performed. Mice were injected subcutaneously with 100µl *B. anthracis* 34F2 suspension. Mice received 100 µl injections of 200 µM Nbs^{SAI} (40 µM each), 200 µM of a single Nb or PBS after 7, 24, 31, 48, 55, 72, 79, 96 and 120 h post infection (Fig. 4 a, b). In case of the mice group that received a single Nbs^{SAI} dose at the time of infection, animals received their first treatment only (together with the bacterial inoculum; Fig. 4b). In case of the post-infection treatment experiment (Fig. 4b), mice received their first Nbs^{SAI} treatment 15 min post infection rather than as part of the infection inoculum; in this case bacteria were resuspended in PBS as for the mock experiment. Mice condition and survival was monitored twice daily during the experiment and in the first 10 days following the last treatment dose. All animal experiments were performed in accordance with institutional guidelines, following experimental protocol review and approval by the Ghent University Committee on the Use and Care of Animals (Bioethical file number EC2018-009).

Mice immunization with Sap^{AD}, Sap^{D1}, Sap^{D4} and Sap^{D6} and protective effect against anthrax. Antigens preparation: Monomeric Sap^{AD} was freshly prepared for each immunization using protein production and purification protocol described above. Sap^{AD} single domains (D1, D4 or D6), prepared as described above, were stored in aliquots at -20 °C and thawed prior to

each immunization. Immunization: 16 week-old female C57BL/6 mice (purchased from Janvier Laboratories) (8 mice per group), were also randomly divided into different cages upon arrival. One weeks after, each cage received an individual immunization. No blinding was performed. Mice were injected subcutaneously with 10 μg of antigen (vaccinated group) or PBS (the mock control group), ones weekly over three consecutive weeks. Antigens or buffer were injected in presence of Complete or Incomplete Freund's Adjuvant (Sigma®), for the first and last two injections, respectively. Immunoassays for α-Sap^{AD} IgG detection: Blood samples were collected pre- and one week post immunization. Serum samples were assayed for the presence of IgG antibodies specific to Sap^{AD}, Sap^{D1}, Sap^{D4} and Sap^{D6} in three independent ELISA experiments, with three technical replicates each (Fig. 4d). To determine the presence of Sap^{AD} or single domains-specific IgG antibodies in the sera, plates were coated with 10 μg antigens (Sap^{AD} in the case of the PBS group to establish any non-specific binding) in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Serum samples were prior serially diluted for determination of the point titer. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgG antibodies (AQ3562 Sigma-Aldrich). Mice infections: *B. anthracis* 34F2 cells were grown over night in RM+ medium and prepared for mice infection as described above. Mice were challenged with 100 μl of bacterial suspension in PBS containing ~100.000 CFUs, 10 days after the last immunization. Mice survival was monitored twice a day up to 14 days after challenge (Fig. 4c). All animal experiments were performed in accordance with institutional guidelines, following experimental protocols review and approval by the Ghent University Committee on the Use and Care of Animals (Bioethical file number EC2017-06).

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Acknowledgements We thank Dr. Pierre Wattiau (CODA_CERVA Brussels) for providing the *Bacillus anthracis* 34F2 strain as well as Dr. Pierre Goossens (Institut Pasteur, Paris) for providing the S-layer knockout mutants RBA91 and SM91. We also thank Peter Borghgraef for the assistance during SEM acquisition. We are grateful to BEI Resources, NIAID, NIH for providing us αPA monoclonal antibodies. We thank Alison E. Pirro Lundqvist for assistance during Nbs selection and identification. We thank Dr. Ranjan Kumar Singh for assistance during SAXS data analysis. This research was supported by VIB and FWO Flanders through project grant G028113N. We are grateful to the beamline staff at I03, Diamond Light Source, England for support with the data collection under proposal MX12718-10.

Author contributions A.F. and H.R. conceived the project and wrote the manuscript; H.D.G performed gene assembly; A.F. performed cloning, protein production, functional and biophysical analysis, bacterial work as well as Nbs identification.; E.P. and JS supervised Ilama immunization and Nbs identification; W.J. assisted in protein production; A.F. and H.R performed structural studies; S.V.d.V. performed and analyzed TEM experiments, supervised by A.F and H.R.; A.F. performed all microscopy experiments, with assistance of A. G. for fluorescent microscopy; F. V. H. performed mice experiments with the assistance of A. F. and supervised by M.L. and H.R.

Competing interests A priority application on compounds used to inhibit bacterial S-layer protein assembly has been filed by VIB and Vrije Universiteit Brussel at the European Patent Office stating AF and HR as inventors. The other authors declare no competing financial interests.

Data and materials availability Coordinates and structure factors of the Sap^{AD}-Nbs^{AF684}-Nbs^{AF694} and Sap^{AD}-Nbs^{AF683}-Nbs^{AF694} complexes have been deposited in PDB under accession codes 6HHU and 6QX4 respectively. All other data are available in the manuscript or the supplementary materials.

Figure legends

Fig. 1 | X-ray structure of *B. anthracis* Sap^{AD}. (**a**) Schematic representation of the cell envelope organization of *B. anthracis* in the absence or presence of the polyglutamate (PGA) capsule (left- and right, respectively). (**b**) Schematic domain organization of *B. anthracis* Sap. The N-terminal ~215 residues consist of a signal peptide (SP) and a pseudorepeat of three SLH domains that form a cell wall anchoring domain. The Sap S-layer assembly region (Sap^{AD}) comprises six independent domains (labelled D1-D6) as revealed in this study. (**c**) Ribbon representation of the X-ray structure of Sap^{AD} (residues 216-814;) comprises six independent β-domains (D1-D6) that assembly into a flat, tile-like unit. (**d**) Surface representation of the Sap^{AD} X-ray structure with Nb^{AF694} and Nb^{AF684} shown as ribbon representation; these Nbs, used as crystallization aides, bind two independent epitopes in D1 (Nb^{AF694}) and the D1-D2 interface (Nb^{AF684}).

Fig. 2 | Single domain antibodies inhibit Sap S-layer formation and affect bacterial growth. (**a**) SDS-PAGE of purified *B. anthracis* Sap^{AD} and particle size distribution of fresh (<1h) and aged (24h) Sap^{AD} solutions measured by DLS. Shown data are representative for three independently repeated experiments (**b**) Negative stain TEM of Sap^{AD} 2D crystals and tubules, scale bars correspond to 200 nm (**c**) cryoEM class averages and unit cell parameters of Sap^{AD} sheets (left, *n*=1651, from 52 sheets), and tubules (right, *n*=109, from 3 tubules) (**d, e, f**) Sap^{AD} tubule depolymerization activity of Nbs^{SAI}. (d) representative negative stain TEM micrographs of Sap^{AD} tubules treated with 15 μM Nbs^{SAI} (t: time post treatment); (e) normalized Sap^{AD} tubule number density after exposure to 15 μM Nb11, Nbs^{SAI}, or 1:1000 mice sera (*n*=10 replicates, from 2 biologically independent experiments; sample mean ± sd); (f) Sap^{AD} tubule length distribution after treatment with 15 μM Nbs^{SAI} (c: tubule count per 5 TEM squares from an experiment representative of 3 biologically independent replicates, **---**: sample mean ± sd; ****:*P*<0.0001, two-tailed Mann-Whitney *U* test relative to buffer, with *n*=c) **(g)** S-layer assembly ratio assessed by DLS at 24h after addition of buffer, 15 μM Nb11 or Nbs^{SAI}, 1:1000 mice or llama sera to Sap^{AD} monomer (sample mean ± sd, ns: *P*>0.05, ****:*P*<0.0001, two-tailed T test relative to buffer, *n*=3 biologically independent experiments) scale bars correspond to 1 μm (**h**) Growth curves of *B. anthracis* cultured on BHI medium supplemented with buffer, 20 μM Nbs^{SAI} or Nbs^{S2}. (NI: not inoculated, sample mean ± sd, *n*=3 biologically independent experiments) (**i**) Phase contrast frames showing *B. anthracis* culture density 5h post inoculum in absence or presence of 20 μM Nbs^{SAI}; shown frames come from a representative experiment out of three independent biological replicates. Scale bars correspond to 200 μm.

Fig. 3 | Nbs^{SAI} affect *B. anthracis* cell morphology. (a) Fluorescent and differential interference contrast (DIC) micrographs of exponential phase *B. anthracis* 34F2 (*sap*$^{+}$, *eag*$^{+}$) cells stained with Syto9 nucleic acid dye and treated with buffer or DyLight 650 labeled Nbs^{SAI}, Nb^{AF692} or Nb^{AF703}. Nb^{AF703} treated cells show a normal (labeled **n**) cell morphology as seen for buffer treated cells. Nbs^{SAI} or Nb^{AF692} treated cultures contain normal as well as affected cells, appearing as intact cells with a scoured cell surface and condensed DNA (labeled **s**) or as collapsed cell mass (labeled **c**). The scoured phenotype is also observed in buffer-treated RBA91 cells (*Δsap*; lower panels), also stained with mouse α-EA1 polyclonal and DyLight 633 conjugated goat α-mouse to reveal EA1 distribution at cell surface. (**b**) Scanning (upper) and transmission (lower) electron microscopy images of *B. anthracis* 34F2 cells treated with buffer or 200 μM Nbs^{SAI}, as well as buffer-treated RBA91 cells (labelled *Δsap*). RBA91 and Nbs^{SAI}-treated cells (3h post inoculation) present a scoured phenotype. At early exponential phase (1h post inoculation) all RBA91 cells present a scoured morphology (inset), whereas later time-points showed a mix scoured and unaffected cells. TEM images show the loss of the ordered surface monolayer in RBA91 and in Nbs^{SAI} treated cells; scale bars correspond to 2 μm and 10 nm for SEM and TEM images respectively. (**c**) Cell morphology ratio observed in buffer and Nbs^{SAI} or Nb^{AF692} treated samples. (—: mean ± sd, *n*=4 biologically independent experiments of 50 cells each). (**d**) Cell length in normal and scoured cells (mean ± 95%CI, ****:*P*<0.0001, two-tailed Mann-Whitney *U* test, with *n*=c, data points from 4 biologically independent experiments of 50 cell each). (**e**) Staining with mouse α-Sap polyclonal and DyLight 633 conjugated goat α-mouse antibodies to reveal localization of Sap S-layer in *B. anthracis* 34F2 cells treated with buffer or 100 μM Dylight 594 labeled Nbs^{SAI}. All experiments were repeated with independent biological samples at least three times, with similar results. Fluorescent microscopy images' scale bars correspond to 2 μm.

Fig. 4 | Clearance of *B. anthracis* infection *via* Nbs^{SAI} treatment. (**a-b**) Schematic diagram and survival curves for *B. anthracis* infection and Nbs treatment studies in mice. Treatment consists of 10 subcutaneous 100 μL doses of 200 μM Nbs or buffer over a 6 day course post infection. Except for buffer controls and panel b (post-Nbs^{SAI}), the *B. anthracis* inoculum (10^5 CFU) contains a first treatment dose of Nbs^{SAI}. **(a)** Survival curves of mice treated with 200 μM Nbs^{SAI} and individual nanobodies with (Nb^{AF692}) or without (Nb11) Sap S-layer assembly inhibitory activity. **(b)** Survival curves for mice treated with buffer, Nbs^{SAI} as a single dose or 10 doses, with first 15 min post-infection (post-Nbs^{SAI}) (**c**) Survival curves and *B. anthracis* infection scheme for mice immunized with monomeric Sap^{AD}, D1, D4 or D6. In all panels, *n*= number of mice per group. (**d**) Antigen-specific IgG responses as determined by ELISA for mice pre (black) and 7 days post (grey) immunization with monomeric Sap^{AD} or individual Sap domains (D1, D4 or D6). Bars represent individual animals (error bars = sd, *n*=3 independent experiments, data points are mean of three technical replicates each).