THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 52, pp. 31126–31137, December 25, 2015

Streptococcal 5′-Nucleotidase A (S5nA), a Novel *Streptococcus pyogenes* Virulence Factor That Facilitates Immune Evasion*

Received for publication, July 8, 2015, and in revised form, October 11, 2015 Published, JBC Papers in Press, November 2, 2015, DOI 10.1074/jbc.M115.677443

Lisa Zheng[‡1], **Adrina Khemlani**[‡1], **Natalie Lorenz**[‡§1,2], **Jacelyn M. S. Loh**[‡§2], **Ries J. Langley**[‡§], and **Thomas Proft**[‡§3]

From the [‡]*Department of Molecular Medicine and Pathology, School of Medical Sciences, and* [§]*the Maurice Wilkins Center, University of Auckland, Auckland, New Zealand*

Background: 5′-Nucleotidases are important virulence factors found in several bacterial pathogens.
Results: Streptococcal 5′-nucleotidase A (S5nA) generated immunomodulatory molecules adenosine and deoxyadenosine and rescued *Lactococcus lactis* in a blood killing assay.
Conclusion: S5nA is a novel *Streptococcus pyogenes* virulence factor that facilitates immune evasion from the host.
Significance: S5nA might be a target for developing new therapeutics or vaccines.

***Streptococcus pyogenes* is an important human pathogen that causes a wide range of diseases. Using bioinformatics analysis of the complete *S. pyogenes* strain SF370 genome, we have identified a novel *S. pyogenes* virulence factor, which we termed streptococcal 5′-nucleotidase A (S5nA). A recombinant form of S5nA hydrolyzed AMP and ADP, but not ATP, to generate the immunomodulatory molecule adenosine. Michaelis-Menten kinetics revealed a K_m of 169 μM and a V_{max} of 7550 nmol/mg/min for the substrate AMP. Furthermore, recombinant S5nA acted synergistically with *S. pyogenes* nuclease A to generate macrophage-toxic deoxyadenosine from DNA. The enzyme showed optimal activity between pH 5 and pH 6.5 and between 37 and 47 °C. Like other 5′-nucleotidases, S5nA requires divalent cations and was active in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+}. However, Zn^{2+} inhibited the enzymatic activity. Structural modeling combined with mutational analysis revealed a highly conserved catalytic dyad as well as conserved substrate and cation-binding sites. Recombinant S5nA significantly increased the survival of the non-pathogenic bacterium *Lactococcus lactis* during a human whole blood killing assay in a dose-dependent manner, suggesting a role as an *S. pyogenes* virulence factor. In conclusion, we have identified a novel *S. pyogenes* enzyme with 5′-nucleotidase activity and immune evasion properties.**

Streptococcus pyogenes (also known as group A *Streptococcus*) is a human opportunistic pathogen that is responsible for a number of diseases, ranging from skin and soft tissue infections, such as pharyngitis and impetigo, to severe invasive diseases, such as necrotizing fasciitis and streptococcal toxic shock syndrome (1, 2). Untreated pharyngitis can develop into acute rheumatic fever and rheumatic heart disease (1). Acute rheumatic fever and rheumatic heart disease are a major cause of acquired heart disease in the developing world, particular in school age children (3). Strikingly, the rates of acute rheumatic fever in New Zealand are among the highest in the world, with reported incidences of 150–380/100,000 for Maori and Pacific children 5–14 years old (4, 5).

Pathogenic bacteria have developed a variety of mechanisms to evade the host immune response. One evasive strategy is to interfere with the balance between pro- and anti-inflammatory immune responses by targeting the purinergic signaling pathway of the host (6, 7). Mammals express two nucleotidases that sequentially convert extracellular ATP into adenosine (Ado).[4] NTPDase/CD39 is an ecto-nucleoside triphosphate diphosphohydrolase that converts NTPs and NDPs to NMPs, whereas ecto-5′-nucleotidase/CD73 hydrolyzes NMPs to generate nucleosides (8). In response to infection or cell damage, the host cells produce the "danger signal" ATP, which results in the activation of the innate immune response and the secretion of pro-inflammatory cytokines after stimulation of the purinergic PY receptors (9). As a control mechanism to prevent overstimulation of this response, Ado antagonizes the effect of ATP by stimulation of adenosine receptors suppressing the pro-inflammatory response (10–13). Ado decreases the phagocytic activity of macrophages by suppressing the generation of nitric oxide (14), superoxide (15, 16), and pro-inflammatory cytokines (17). In addition, Ado inhibits neutrophil degranulation (18).

Over recent years, several extracellular nucleotidases have been identified in pathogenic bacteria and shown to play an

* This work was supported by a research grant from the Faculty Research Development Fund from the Faculty of Medical and Health Sciences, University of Auckland. The authors declare that they have no conflicts of interest with the contents of this article.
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AAK33792.
[1] These authors contributed equally to this work.
[2] Supported by the Health Research Council New Zealand.
[3] To whom correspondence should be addressed: Dept. of Molecular Medicine and Pathology, School of Medical Sciences, University of Auckland, Private Bag 92019, Auckland Mail Centre, New Zealand. Tel.: 64-9-373-7599 (ext. 86213); E-mail: t.proft@auckland.ac.nz.

[4] The abbreviations used are: Ado, adenosine; dAdo, deoxyadenosine; AdsA, adenosine synthase A; NudP, ecto-5′-nucleoside diphosphate phosphohydrolase; Ssads, *S. suis* adenosine synthase; S5nA, streptococcal 5′-nucleotidase A; rS5nA, recombinant S5nA; SpnA, *S. pyogenes* nuclease A; rSpnA, recombinant rSpnA; SSL7, staphylococcal superantigen-like protein 7.

important role in human disease, in particular evasion of the host immune response. *Staphylococcus aureus*, a Gram-positive pathogen and a major cause of hospital-acquired blood, skin, and soft tissue infections (19), produces adenosine synthase A (AdsA). AdsA is a cell wall-anchored enzyme that hydrolyzes AMP, ADP, and ATP to produce Ado. In addition, AdsA hydrolyzes the guanosine derivatives GMP, GDP, and GTP, although at a reduced rate, but not cytosine nucleotides (20, 21). AdsA was shown to be responsible for staphylococcal synthesis of adenosine in blood, escape from phagocytic clearance, and the formation of abscesses in organs of infected mice (20). Importantly, AdsA also converts deoxyadenosine monophosphate (dAMP) into deoxyadenosine (dAdo), which is capable of inducing caspase-3-mediated apoptosis in macrophages and monocytes (22). Another cell wall-anchored nucleotidase, ecto-5′-nucleotidase A, was identified in *Streptococcus sanguinis*. Ecto-5′-nucleotidase A was shown to hydrolyze ATP, ADP, and AMP and was able to inhibit platelet aggregation and contributed to experimental endocarditis in a rabbit model (23). A similar bacterial nucleotidase has also been described in *Streptococcus agalactiae* (group B *Streptococcus*), a leading cause of invasive infections in neonates (24). Ecto-5′-nucleoside diphosphate phosphohydrolase (NudP) is able to hydrolyze nucleoside mono- and diphosphates but not nucleoside triphosphates. Increased bacterial survival in mouse blood was reported in a process dependent on extracellular Ado (25). *Streptococcus suis* adenosine synthase (Ssads) is yet another recently identified bacterial nucleotidase (26). *S. suis* causes a range of invasive diseases in pigs (27) but has also been reported to infect humans (28). Ssads is a cell wall-anchored enzyme that is most closely related to AdsA from *S. aureus*. It was reported that Ssads impairs polymorphonuclear cell-mediated innate immunity and was important for virulence in a piglet infection model (26).

Here we report the identification of a novel extracellular nucleotidase in *Streptococcus pyogenes*. Streptococcal 5′-nucleotidase A (S5nA) hydrolyzes AMP and ADP, but not ATP, to generate Ado. In addition, we show conversion of dAMP into dAdo, a powerful inhibitor of macrophages and monocytes. Our results suggest a role of S5nA as a novel *S. pyogenes* virulence factor that deregulates the host immune response.

Experimental Procedures

Bacterial Strains and Growth Conditions—*Escherichia coli* BL21 cells (Novagen) were grown in LB medium (Oxoid) at 37 °C with aeration. *S. pyogenes* SF370 (serotype M1, ATCC 700294) was grown in Todd Hewitt medium (BD Biosciences) at 37 °C without aeration. *Lactococcus lactis* MG1363 (a kind gift from Nicholas Heng, Otago University, New Zealand) was grown in M17 medium (BD Biosciences) supplemented with 0.5% glucose (GM17) at 30 °C without aeration. Whenever appropriate, 50 μg/ml ampicillin or 30 μg/ml chloramphenicol was added to the medium for *E. coli*, and 100 μg/ml kanamycin was added for *L. lactis*.

Bioinformatic Analysis—The *s5nA* (*spy0872*) open reading frame was sequenced at Oklahoma University as part of the *S. pyogenes* SF370 genome project and deposited at GenBank™.

TABLE 1
PCR primers used in this study
Restriction sites are shown in bold.

Primer	DNA sequence (5′–3′)
S5nA.fw	GC**GGATCC**GATCAAGTTGATGTGCAATTC
S5nA.rev	GG**GAATTC**CTAAGTGGAACTAGAGATAG
S5nA_D66A.fw	GCTGGAGCTATGGTCGGAGCCAGTCC
S5nA_D66A.rev	GACCATAGCTCCAGCTTGAACACG
S5nA_N99A.fw	CTTGGTGCTCATGAATTTGACGAAGGAC
S5nA_N99A.rev	TCATGAGCACCAAGAGTGCCATATTC
S5nA_H100A.fw	CTCTTGGTAATGCTGAATTTGACGAAGGACTAG
S5nA_H100A.rev	CAGCATTACCAAGAGTGCCATATTC
S5nA_R392A.fw	GGTATTGCAAGTGACCTAGTTGTC
S5nA_R392A.rev	CACTTGCAATACCACCATTATTGGTC
S5nA_F414A.fw	GTACAACCAGCTGGTAATATCCTTCAAGTC
S5nA_F414A.rev	CCAGCTGGTTGTACAGCCTGTGC
S5nA_F491A.fw	CGACGCTCTTTATGGTGGTGGTGATG
S5nA_F491A.rev	CCATAAAGAGCGTCGTTGACAACAACGGTG

The Signal P 4.0 server at was used to predict the presence and length of the N-terminal signal peptide. For functional predictions of Spy0872/S5nA, we searched the InterProScan software at EMBL-EBI. BLAST searches were performed using the tblastn program. Sequence alignments and the phylogenetic tree were generated using the ClustalW server.

The structural model of S5nA was generated using the Swiss PDB modeling server at the Swiss Institute of Bioinformatics with automatic template search (29). The structural images were created with the PDB Swiss Viewer version 4.1.0.

The protein structure coordinates for the mammalian nucleotidase CD73 (4H1S) were downloaded from the Protein Data Bank.

Cloning of s5nA and s5nA Mutants—The *spy0872/s5nA* open reading frame (ORF) without the regions encoding for the predicted N-terminal signal sequence and the C-terminal sortase domain (nucleotide positions 82–1909) was amplified from 50 ng of genomic DNA of *S. pyogenes* SF370 (ATCC 700294) by 30 cycles of PCR with iproof polymerase (Bio-Rad) at an annealing temperature of 53 °C using primers S5nA.fw and S5nA.rev (see Table 1). A TAG stop codon was introduced with the reverse primer at nucleotide position 1909.

The PCR products were enriched using the Wizard PCR DNA purification system (Promega) and cloned into the BamHI/EcoRI restriction sites of pBlueScript SKII vectors (Stratagene) followed by transformation into *Escherichia coli* DH5α (Sigma). The s5nA mutants were generated by overlap PCR using primers listed in Table 1.

The cloned DNA fragments were analyzed by the dideoxy chain termination method using the DNA sequencing service at the School of Biological Science, University of Auckland. The *spy0872/s5nA* fragments were transferred from pBlueScript into the BamHI/EcoRI cloning site of pPROEX-Htb expression vector (Life Technologies).

Expression and Purification of Recombinant S5nA and S5nA Mutants—The recombinant pPROEX-Htb plasmids were introduced into *E. coli* BL21 (Novagen) by heat-shock transformation. Cultures were grown at 30 °C, and protein expression was induced for 3–4 h after adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). The His_6-tagged proteins were purified on Ni^{2+}-iminodiacetic acid Sepharose (Sigma). The eluates containing the recombinant proteins were collected and analyzed on 12.5% SDS-polyacrylamide gels according to the

procedure of Laemmli. The eluates with the highest protein amounts were pooled and further purified by anion exchange chromatography using a Mono Q 4.6/100 PE column (GE Healthcare). The column was equilibrated with 5 ml of 10 mM Tris-HCl (pH 7.0), and the proteins were eluted using a gradient of 0–50% of 10 mM Tris-HCl (pH 7.0), 1 M NaCl at a flow rate of 2 ml/min. Finally, rS5nA was subjected to size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 50 mM phosphate buffer (pH 7.0) and 150 mM NaCl. Recombinant *S. pyogenes* nuclease A (SpnA) was produced as described previously (30).

Enzymatic Activity Assays—The generation of nucleosides was determined by reverse-phase fast protein liquid chromatography using a Waters C18 Spherisorb® reverse-phase column (250 mm × 4.6 mm, 5-μm particle size) and an AKTA Explorer system (Amersham Biosciences). Reactions were carried out in 50 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ with 1 mM nucleotides (Sigma-Aldrich) and 0.1 μM recombinant S5nA in a total volume of 1 ml at 37 °C for 45 min. The enzymatic reaction was stopped by adding EDTA to a final concentration of 50 mM. Reaction samples (40 μl) were loaded in 20 mM KH_2PO_4 (pH 5.65), and compounds were eluted in a linear solvent gradient with 60% methanol running from 0 to 22.5% at a flow rate of 1 ml/min. Nucleosides were detected at an absorbance of 254 nm. Commercial nucleosides (Sigma-Aldrich) were used as standards.

For the quantification of inorganic phosphate (P_i) release, reactions were carried out in 50 mM Tris-HCl adjusted to different pH values with selected metal cations, 1 mM nucleotides (Sigma-Aldrich), and 0.1 μM recombinant S5nA in a total volume of 20 μl and incubated at various temperatures. The enzymatic reaction was stopped by adding EDTA to a final concentration of 50 mM.

The enzyme kinetics were analyzed by incubation of increasing amounts of AMP with a fixed concentration of 0.1 μM S5nA WT or mutant at 37 °C in a total volume of 50 μl. Reactions were stopped as described above. The release of inorganic phosphate was measured using a malachite green phosphate colorimetric assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Release of P_i was measured at 650 nm against a standard P_i curve. Michaelis-Menten curve fitting using nonlinear regression was performed using GraphPad Prism version 6 software.

To test the synergistic activity of S5nA and SpnA on DNA, a 200 μl reaction mix containing 50 μg of UltraPure™ salmon sperm DNA (Invitrogen), 12.5 μM SpnA, 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ was incubated at 37 °C for 1 h, before 0.1 μM S5nA and 10 mM $MgCl_2$ were added, and the reaction was incubated for a further 2 h. Generation of dAdo and inorganic phosphate was analyzed as described above.

Whole Blood Killing Assay—L. lactis MG1363 was cultured until reaching A_{600} = 0.7–0.8. Bacterial culture was centrifuged at 4415 × *g* for 5 min and suspended in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 0.44 KH_2PO_4, 0.25 mM Na_2PO_4, 0.01 mM glucose) at a final concentration of 1×10^7 cfu/ml. A dilution series of the *L. lactis* inoculate was drop-plated in triplicate on GM17 agar to confirm the inoculation cfu. Blood was taken from healthy volunteers after informed consent. Heparinized blood (350 μl) was combined with 1×10^5 cfu of *L. lactis* and the indicated concentrations of rS5nA and made up to a final volume of 500 μl with Hanks' balanced salt solution. Following incubation for 3 h at 37 °C with slow rotation, samples were serially diluted and drop-plated in triplicates on GM17 agar, and bacterial survival was enumerated by colony count the following day. Purified recombinant staphylococcal superantigen-like protein 7 (SSL7; a kind gift from Fiona Clow, University of Auckland) was used as a positive control, and rS5nA N99A was used as a negative control. Statistical significance was calculated using a two-way analysis of variance and Dunns's multiple comparison test.

Seroconversion—Recombinant S5nA (2 μg/lane) was run on a 12.5% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Pall Life Sciences) using a Semi Phor Western blot chamber (Hoefer Scientific Instruments). The membrane was blocked with 5% skimmed milk powder in TBST (10 mM Tris/HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature and incubated with human serum samples (1:500 in TBST) for 1 h. After washing with TBS, the membranes were incubated with goat anti-human Fc/HRP antibody (Serotec) for 1 h. The Western blots were developed using an ECL Western blotting detection kit (Amersham Biosciences), and bands were visualized with a Fujifilm LAS-3000 Intelligent Dark Box scanner (Alphatech). Serum samples were collected at Middlemore Hospital (Auckland, New Zealand) between July 2001 and January 2003 with approval by the Auckland Human Ethics Committee.

Results

Identification of S5nA—All previously reported bacterial nucleotidases produced by Gram-positive bacteria are cell wall-anchored proteins with a characteristic C-terminal cell wall anchoring domain and a LP*X*TG sorting motif that is recognized by the housekeeping sortase A enzyme (21, 23, 25). Using the *S. pyogenes* SF370 (serotype M1) genome sequence (31), we searched for all proteins with typical Gram-positive cell wall anchor domains and found an open reading frame (*spy0872*) that was annotated as putative secreted 5′-nucleotidase (AAK33792). Bioinformatic analysis of the translated 670-amino acid sequence showed significant homologies to members of the 5′-nucleotidase/apyrase protein family (IPRO006179) with two distinct domains: a metallophosphatase-like protein domain (IPRO29052) between position 26 and 363 and a 5′-nucleotidase C-terminal domain (IPRO008334) between positions 366 and 564. In addition, the protein sequence contains a predicted signal peptide sequence (positions 1–27) and a LPITG cell wall anchor domain (IPRO019931) between positions 637 and 670) (Fig. 1*A*). A BLAST search against the NCBI database revealed related putative nucleotidases in other pathogenic streptococcal species, like *Streptococcus dysgalactiae* UshA (75% amino acid identity) and *Streptococcus equi* SEQ1278 (71% amino acid identity) and the *S. agalactiae* nucleotidase NudP (64%). Only limited sequence identity was found with *S. sanguinis* ecto-5′-nucleo-

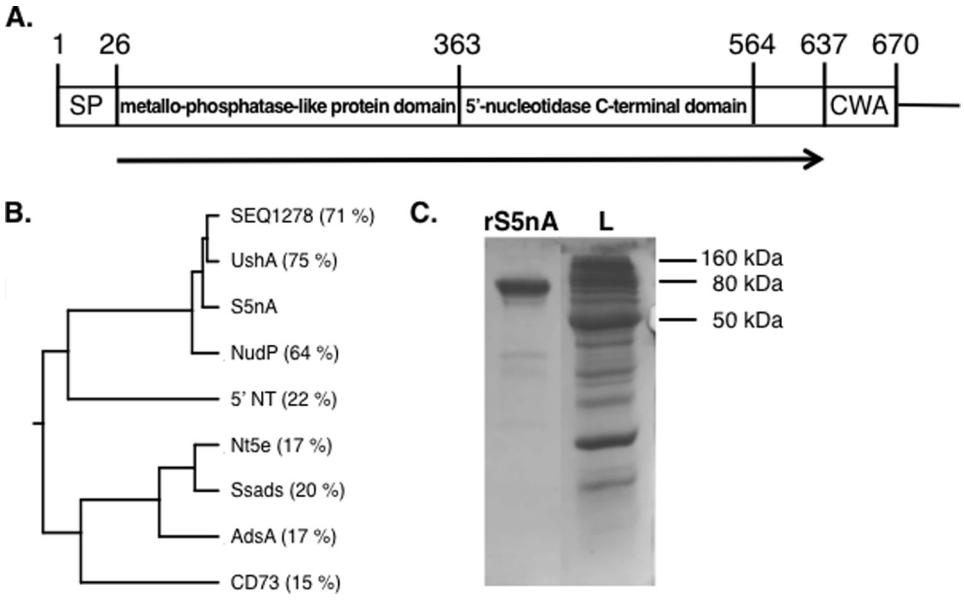

FIGURE 1. *A*, schematic presentation of S5nA based on predictions with InterProScan software at EMBL-EBI. The *numbers* on *top* indicate amino acid positions. *SP*, signal peptide sequence; *CWA*, cell wall anchor domain. The *arrow below* indicates the region that was generated as recombinant protein in *E. coli*. *B*, rooted phylogenetic tree (UPGMA (UPGMA (unweighted pair group method with arithmetic mean)) of 5′-nucleotidases. The tree was generated with ClustalW using complete protein sequences of S5nA (*S. pyogenes*, AAK33792), AdsA (*S. aureus*, ESR29110), ecto-5′-nucleotidase A (*S. sanguinis*, AFK32764), Ssads (*S. suis*, YP001197640), NudP (*S. agalactiae*, CDN66659), UshA (*S. dysgalactiae*, ADX24386), SEQ1278 (*S. equi*, CAW94038), 5′-nucleotidase (*5′NT*) (*E. coli*, AJM76137), and CD73 (*Homo sapiens*, AAH65937). The *numbers* in *parentheses* show amino acid identities with S5nA. UshA and SEQ1278 are putative 5′-nucleotidases that have not yet been characterized. *C*, recombinant S5nA was expressed and purified from *E. coli* by immobilized metal chelate chromatography (Ni^{2+}-iminodiacetic acid Sepharose), anion exchange chromatography (Mono Q 4.6/100 PE), and size exclusion chromatography (Superdex 200 10/300 GL). Protein purity was ~95%, as estimated from a 12.5% SDS-polyacrylamide gel. *L*, molecular weight marker.

tidase A (17%), *S. suis* Ssads (20%), *S. aureus* AdsA (17%), *E. coli* 5′-nucleotidase (22%), and human CD73 (15%) (Fig. 1*B*).

Substrate Specificity of S5nA—Because S5nA is predicted to be anchored to the bacterial cell wall, we expressed a truncated version of the protein that lacks the N-terminal signal peptide sequence and the C-terminal sortase domain, which is responsible for cell wall anchoring. The protein was generated in *E. coli* with an N-terminal histidine tag for affinity chromatography and was further purified by anion exchange chromatography to yield rS5nA with a purity of ~95%, as estimated from an SDS-polyacrylamide gel (Fig. 1*C*). rS5nA migrated at ~70 kDa, which is in agreement with its calculated molecular mass of 69,869.88 Da (including N-terminal histidine tag).

To analyze the enzymatic activity of S5nA, we initially focused on AMP as substrate, because the predicted reaction product adenosine has been shown to modulate the host immune response (8, 11, 13, 15, 17, 18). Adenosine production was analyzed by separating the compounds on a reverse-phase column after 45 min at standard reaction conditions (1 mM of AMP, 0.1 μM rS5nA, 37 °C, pH 7.4, in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$). Adenosine eluted from the column at 45 ml, equivalent to the elution profile of commercial adenosine, which was used as a control (Fig. 2*A*). In addition, we used a commercial kit to measure the release of P_i during the enzymatic reaction. A time course showed that the reaction reached equilibrium after about 25 min when 1 mM AMP and 0.1 μM rS5nA were incubated at 37 °C (Fig. 2*B*). The initial release of P_i varied with the AMP concentration, and the reaction kinetics followed the Michaelis-Menten model for a single substrate with a K_m of 168.3 ± 38 μM and a V_{max} of 7550 ± 326 nmol of P_i/mg of S5nA/min (Fig. 2*C*). Because other Gram-positive nucleotidases, such as AdsA, NudP, and Ssads, are also able to hydrolyze ADP and ATP, we additionally tested these two substrates. We found that ADP was also hydrolyzed by S5nA but not ATP (Fig. 2*D*). However, P_i release from 1 mM ADP was ~3.5 times lower than from 1 mM AMP (Fig. 2*E*).

Other nucleotide monophosphates (TMP, GMP, and CMP) were also hydrolyzed by rS5nA and eluted at 30, 25, and 10 ml, respectively (Fig. 3*A*). At equal substrate molarities (1 mM), the release of P_i from CMP was comparable with that of AMP, whereas GMP and TMP were inferior substrates, generating ~2–3 times less P_i (Fig. 3*B*).

S5nA Works with SpnA to Generate dAdo from DNA—Schneewind and colleagues (22) have recently shown that *S. aureus* AdsA is capable of converting dAMP into dAdo, which can induce caspase-3-mediated apoptosis in macrophages and monocytes. We therefore analyzed whether S5nA can also generate dAdo from dAMP. As shown in Fig. 4, S5nA indeed hydrolyzes dAMP. Under the same reaction conditions, equivalent amounts of P_i were generated from 1 mM dAMP and AMP (Fig. 4*B*). Equilibrium of the reaction was reached after about 20 min (Fig. 4*C*) compared with 25 min with AMP (Fig. 2*B*). Schneewind and colleagues (22) also reported that AdsA works together with a nuclease, Nuc, to produce dAdo from neutrophil extracellular traps (NETs). Nuc first degrades the DNA backbone of the NET structure to generate dNMPs, including dAMP, which then serves as a substrate for AdsA (22). We have recently demonstrated that SpnA, an *S. pyogenes* cell wall-anchored nuclease, is capable of degrading NETs (30). We were therefore interested in whether SpnA and S5nA could form an enzyme combination with similar function. Indeed, when rSpnA and rS5nA were incubated with salmon sperm

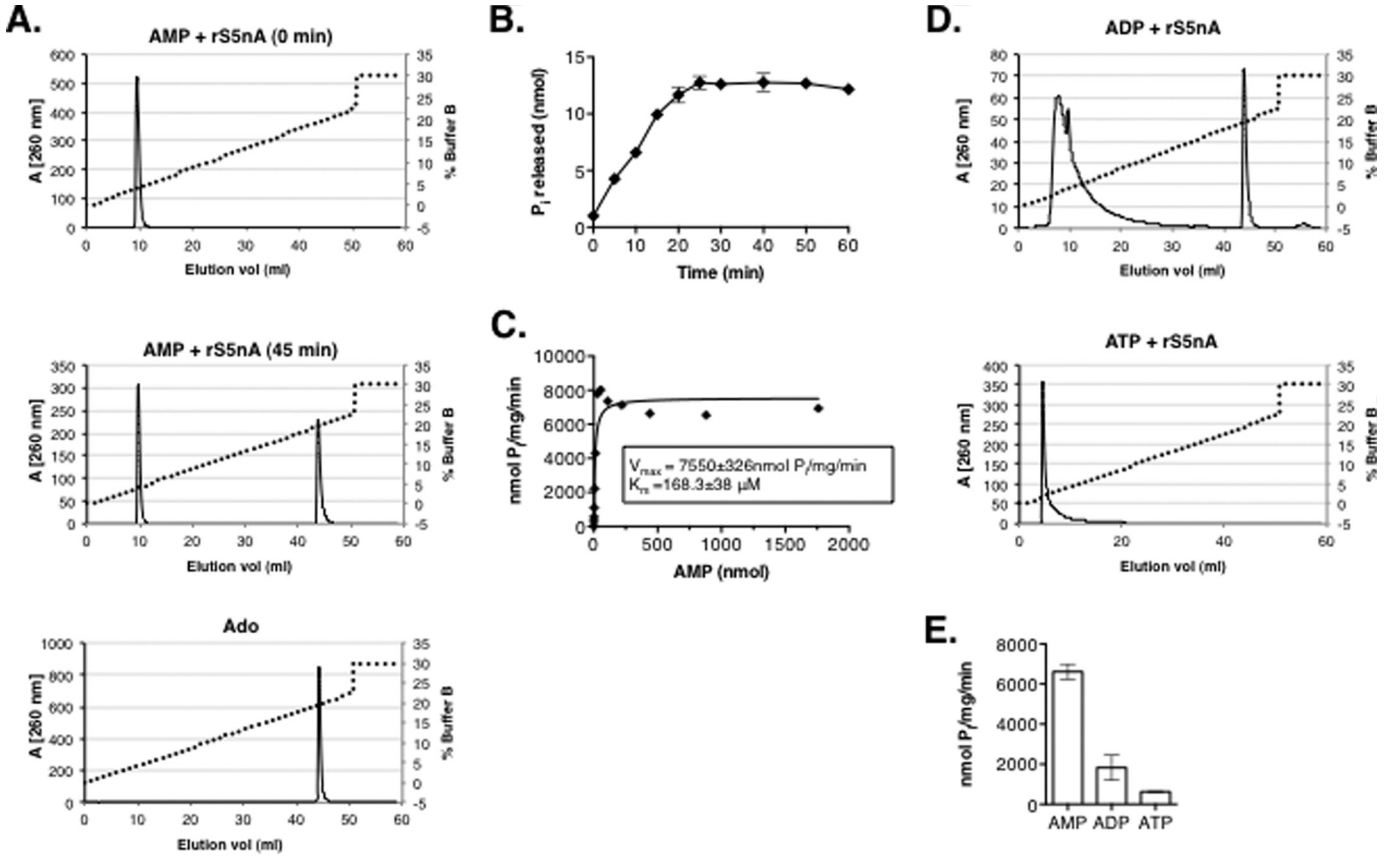

FIGURE 2. **S5nA hydrolyzes AMP and ADP but not ATP.** *A*, reaction mixes with 1 mM AMP and 0.1 μM rS5nA were separated by reverse-phase HPLC after 0 min (*top*) and 45 min (*middle*) incubation at 37 °C. Commercial adenosine was used as a control to determine the elution volume (*bottom*). *B*, reaction mixes with 1 mM AMP and 0.1 μM rS5nA were incubated at various time points, and the release of P_i was measured using a malachite green phosphate colorimetric assay kit. *C*, the V_{max} and K_m values for hydrolysis of AMP were obtained by velocity measurements with different concentrations of substrate using GraphPad Prism version 6 software. *D*, reaction mixes with 1 mM ADP (*top*) or 1 mM ATP (*bottom*) and 0.1 μM rS5nA were separated by reverse-phase HPLC after a 45-min incubation at 37 °C. *E*, reaction mixes with 1 mM AMP, 1 mM ADP, or 1 mM ATP and 0.1 μM rS5nA were incubated at 37 °C for 15 min, and the release of P_i was measured. In *B* and *E*, *error bars* represent the S.D. from two individual experiments.

DNA, P_i production was observed (Fig. 5). Minimal P_i release was observed when DNA was incubated with rSpnA alone, but the amount was ~20 times less than from the reaction with both enzymes. Similarly, incubation of DNA with rS5nA alone or a combination of rSpnA with inactive rS5nA mutants did not result in significant amounts of P_i.

Effect of pH, Temperature, and Metal Co-factors on rS5nA Activity—The enzymatic activity of rS5nA was analyzed for a range of different conditions by measuring the release of P_i from AMP. The optimal enzymatic activity was found to be between pH 5 and pH 6.5. The activity decreased at higher pH but was still at ~25% at pH 9 (Fig. 6*A*). We observed that rS5nA functioned at a broad temperature range with optimal activity between 32 and 47 °C. No P_i was released in the absence of rS5nA, indicating that AMP did not spontaneously hydrolyze at higher temperatures (data not shown).

Nucleotidases require divalent cations as co-factors for enzymatic activity. We analyzed the activity of rS5nA at 37 °C and pH 7 with either Mg^{2+}, Mn^{2+}, Ca^{2+}, or Zn^{2+} (Fig. 6, *C* and *D*). S5nA activity was slightly higher with Mg^{2+} compared with Ca^{2+} at concentrations of 0.1, 1, or 10 mM in a dose-dependent manner. The enzyme was also fully active with Mn^{2+} at 0.1 mM and 1 mM but less active at 10 mM. In contrast, S5nA activity in the presence of Zn^{2+} strongly depended on the cation concentration. Only about 30% enzymatic activity was achieved at 0.05 mM, and the activity dropped further to ~15% for 0.5 mM or higher concentrations of Zn^{2+} (Fig. 6*D*). The inhibitory effect of 1 mM Zn^{2+} on S5nA activity could only slightly be compensated for by the addition of Mg^{2+} (~20% activity at 1 mM and 30% activity at 10 mM Mg^{2+}) (Fig. 6*E*).

Computer-generated Model of S5nA and Mutation Analysis—To gain insight into the S5nA protein structure, a computer-generated model was generated using the PDB Swiss modeler with automatic template search. Surprisingly, the optimal template was identified as the zinc-dependent mammalian nucleotidase CD73 (32), followed by the zinc-dependent *E. coli* nucleotidase (33). S5nA is predicted to consist of two domains connected by an α-helix. The N-terminal domain binds two divalent cations, and the C-terminal domain is responsible for substrate binding. The active site is located at the interface between the two domains (Fig. 7*A*). Although the quality of the model must be regarded as below average (QMEAN4 = −10.11), the important core residues are highly conserved and superimpose well with CD73 and also with the structure of the *E. coli* 5′-nucleotidase (Fig. 7*B* and Table 2). Structure superposition (Fig. 7*C*) shows alignment of Phe-414 and Phe-491 with the CD73 residues Phe-417 and Phe-500, respectively (Phe-429 and Phe-498 in *E. coli* nucleotidase). These two residues build a

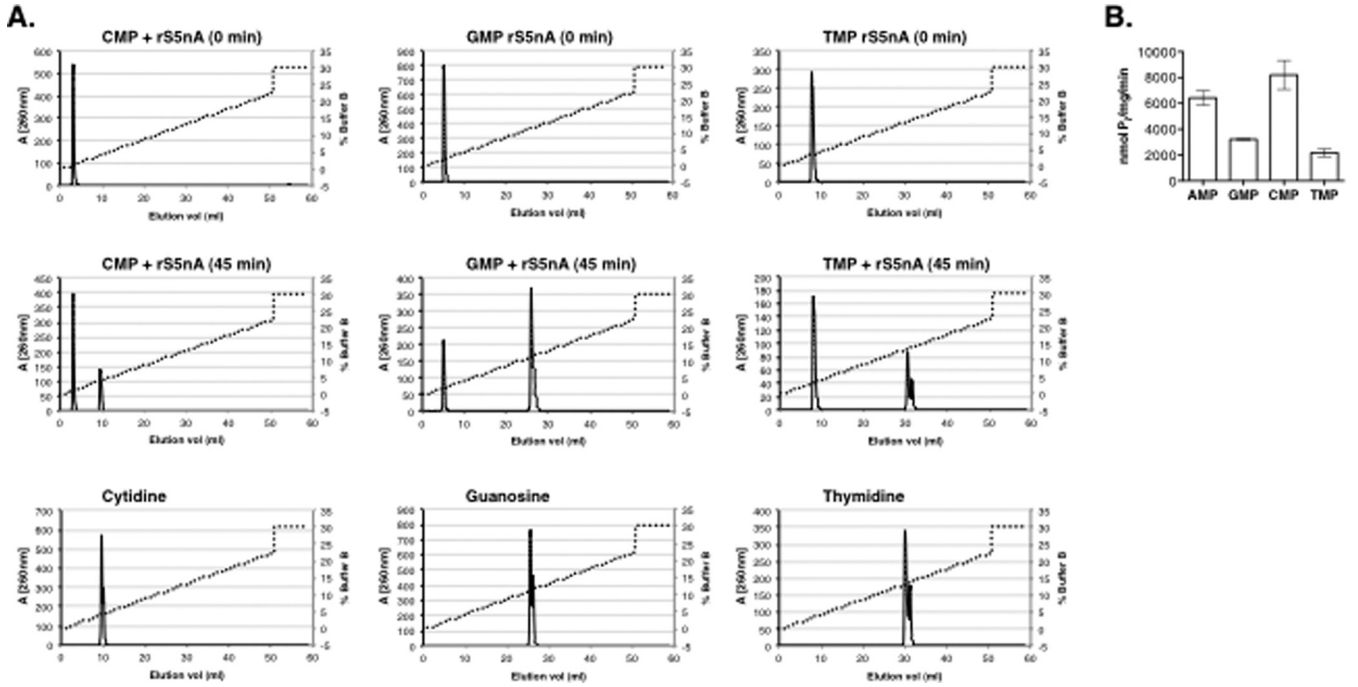

FIGURE 3. **S5nA also hydrolyzes other NMPs.** *A*, reaction mixes with 1 mM CMP (*left*), 1 mM GMP (*middle*), or 1 mM TMP (*right*) and 0.1 μM rS5nA were separated by reverse-phase HPLC after 0-min (*top*) and 45-min (*middle*) incubation at 37 °C at pH 7.4 in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Individual nucleosides were used as controls to determine the elution volume (*bottom*). *B*, reaction mixes with various 1 mM NMPs and 0.1 μM rS5nA were incubated as above, and the release of P_i was measured using a malachite green phosphate colorimetric assay kit. *Error bars*, S.D. from two individual experiments.

pocket that stacks the aromatic purine moiety of the substrate. Asn-99, His-100, and Arg-392 superimpose with CD73 phosphate-binding residues Asn-117, His-118, and Arg-395, respectively (Asn-116, His-117, and Arg-410 in *E. coli* nucleotidase). The putative catalytic dyad is formed by His-100 and Asp-103, which superimpose well with His-118 and Asp-121 in CD73 and His-117 and Asp-120 in *E. coli* nucleotidase. The zinc-binding sites are also highly conserved in S5nA. Residues Asp-12, His-14, Asp-66, His-225, and His-262 superimpose with CD73 zinc-binding residues Asp-36, His-38, Asp-85, His-220, and His-243, respectively (Asp-41, His-43, Asp-84, His-217, and His-252 in *E. coli* nucleotidase). Only one core residue is not conserved; the CD73 zinc-binding residue Asn-245 is replaced by His-264 in S5nA and Gln-245 in *E. coli* nucleotidase.

To experimentally confirm the predicted function of the identified residues in S5nA, we generated alanine conversion mutants of four selected S5nA residues: one nucleoside-binding residue (F414A), two phosphate-binding residues (N99A and R392A), and one catalytic dyad residue (H100A). Recombinant S5nA mutants were purified, and the enzymatic activity was measured by the release of P_i from AMP. The enzymatic activity was significantly reduced with S5nA F414A (K_m = 275 μM and V_{max} = 3461 nmol P_i/mg/min). Very weak activity was seen with S5nA R392A ($K_m > 800$ μM), and no enzymatic activity was detected with S5nA N99A and S5nA H100A (K_m>2000 μM) (Fig. 7*D*).

Recombinant S5nA Increases the Survival of L. lactis in Human Whole Blood—To evaluate a potential role of S5nA in immune evasion, we examined the effect of S5nA on *L. lactis* survival in human whole blood. *L. lactis* is a non-pathogenic, food grade bacterium that is rapidly killed when incubated with whole blood. Increased survival of the bacteria in blood has been used to analyze potential virulence factors (30, 34). The addition of rS5nA to *L. lactis* in human whole blood significantly increased bacterial survival in a dose-dependent manner (Fig. 8). *L. lactis* was almost completely killed after 3 h (2.8 ± 1% survival). However, the addition of 1 μM rS5nA almost completely rescued the bacteria (83.4 ± 24.5% survival rate, $p <$ 0.001). A significantly increased survival rate (63%, $p < 0.001$) was also observed with equimolar amounts of the control protein SSL7. SSL7 is an *S. aureus* immune evasion factor previously shown to increase the survival of *L. lactis* in human blood (34). The addition of 0.01 and 0.1 μM rS5nA also increased bacterial survival in a concentration-dependent manner (8.9 and 18.1%, respectively), but this was not statistically significant ($p > 0.1$). The addition of the catalytic site mutant rS5nA N99A had no significant effect on bacterial survival, suggesting that survival can be attributed to the enzymatic activity of S5nA.

Because the production of the immunomodulatory components Ado and dAdo depends on the availability of their respective precursors, we repeated the experiments but this time preincubated the rS5nA with AMP or dAMP. There were no significant differences in bacterial survival with or without the addition of substrate (data not shown), suggesting that there are sufficient amounts of substrate in human blood.

S5nA Is Expressed during S. pyogenes Infection—The presence of specific antibodies against a protein produced by a pathogen is a useful indicator of protein expression during infection. Using Western blot analysis, we tested activity against rS5nA in convalescent serum samples from 21 patients with invasive *S. pyogenes* disease (Fig. 9*A*) and 20 control sera from healthy blood donors (Fig. 9*B*). Antibodies against S5nA

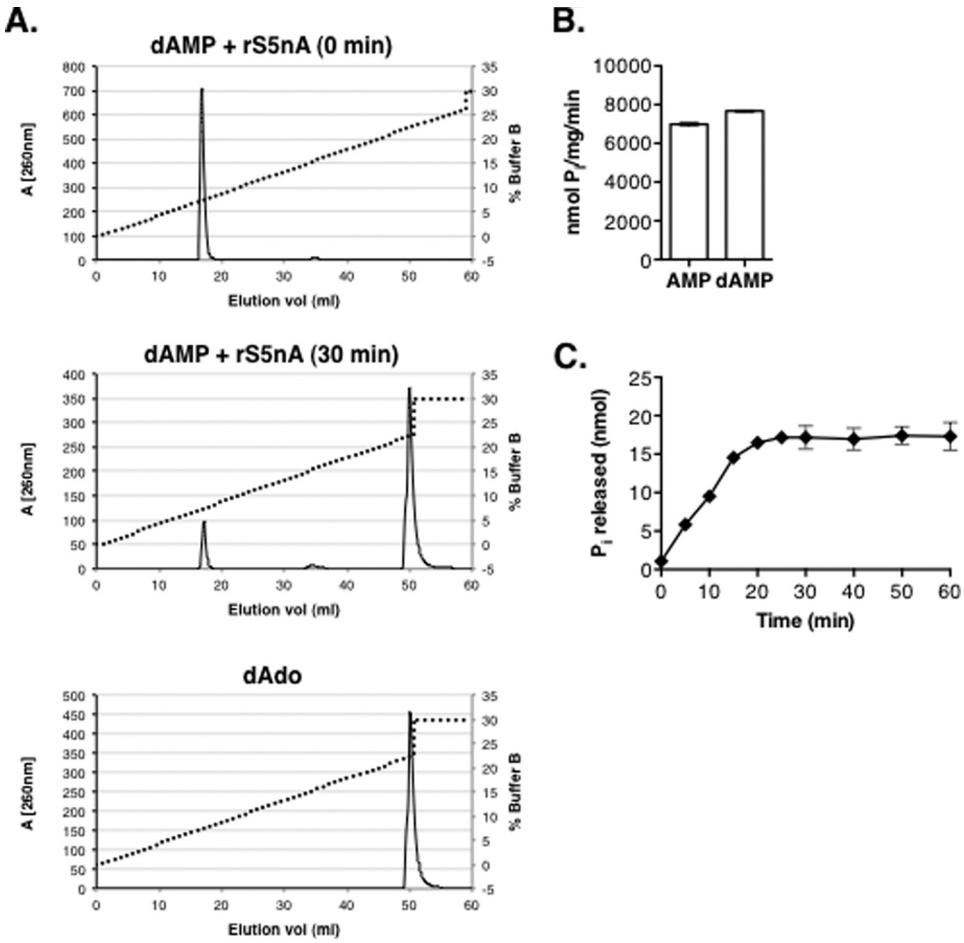

FIGURE 4. **dAMP is a better substrate for S5nA than AMP.** *A*, reaction mixes with 1 mM AMP (*top*) or 1 mM dAMP (middle) and 0. 1 μM rS5nA were separated by reverse-phase HPLC after 0-min (*top*) and 30-min (*middle*) incubation at 37 °C. Commercial dAdo was used as a control to determine the elution volume (*bottom*). *B*, reaction mixes with 1 mM AMP or 1 mM dAMP and 0.1 μM rS5nA were incubated at 37 °C at pH 7.4 in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$, and the release of P_i was measured using a malachite green phosphate colorimetric assay kit. *C*, reaction mixes with 1 mM dAMP and 0.1 μM rS5nA were incubated at various time points, and the release of P_i was measured. For *B* and *C*, *error bars* represent the S.D. from two individual experiments.

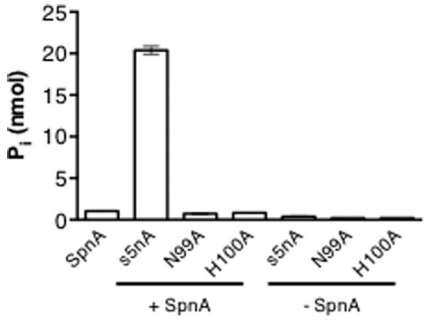

FIGURE 5. **SpnA and S5nA work together to generate dAdo from DNA.** Salmon sperm DNA was incubated with rSpnA for 1 h at 37 °C at pH 7.0, followed by a 2-h incubation with or without rS5nA WT or mutants. P_i release was measured using a malachite green phosphate colorimetric assay kit. *Error bars*, S.D. from two individual experiments.

were more frequently found in serum from patients and in higher titers compared with serum from healthy donors. Of the 21 disease patients, five had strong titers (3+ or 4+), nine had moderate titers (2+), and three patients had weak titers (+). Only one patient did not have detectable antibodies against S5nA. In contrast, the healthy cohort included only two moderate titers (2+) and 10 weak titers (+), whereas eight donors had no detectable antibodies against S5nA.

We also analyzed three serum samples from a patient with streptococcal toxic shock syndrome (35) taken over a period of 2 weeks (Fig. 9*C*). The first sample was taken during the acute phase of the disease, 2 days after hospital admission, and lacked any detectable anti-S5nA antibodies. During convalescence, the patient seroconverted and had a weak but detectable anti-S5nA antibody titer after 6 days and a strong titer after 16 days after admission. These results strongly indicate that S5nA is produced during *S. pyogenes* disease.

Discussion

Over the last 4 years, several cell wall-anchored enzymes with nucleotidase activity have been discovered in pathogenic Gram-positive cocci. Each of these enzymes was shown to convert AMP into the immunomodulator adenosine, facilitating the evasion of the bacteria from the host immune response (20–23, 25, 26). In addition, the *S. aureus* AdsA and NudP from *S. agalactiae* are also able to convert dAMP into deoxyadenosine, which has cytotoxic effects on macrophages and monocytes (22, 25). Here, we report the discovery of a novel nucleotidase from the human pathogen *S. pyogenes*, which we name S5nA. S5nA was discovered by bioinformatic analysis of predicted cell wall-anchored proteins (proteins that contain a

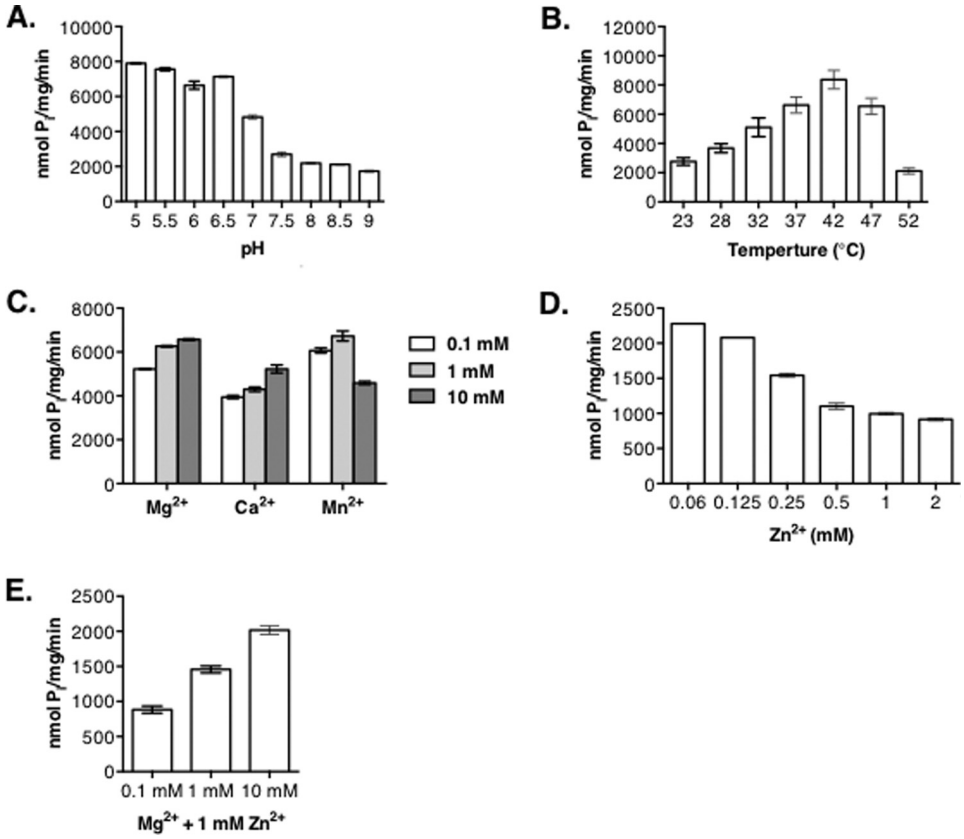

FIGURE 6. **Kinetic activity of rS5nA.** Hydrolysis of 1 mM AMP by 0.1 μM rS5nA over 15 min was analyzed at varying conditions. *A*, AMP hydrolysis at a pH range of 5–9 at 37 °C in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$. *B*, AMP hydrolysis at temperatures ranging from 23 to 52 °C at pH 7.0 in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$. *C*, AMP hydrolysis in the presence of different metal cations at 37 °C and pH 7.0. *D*, Zn^{2+} showed an inhibitory effect on rS5nA activity at concentrations >0.05 mM, which could only partially be restored with an excess of Mg^{2+} (*E*). The enzymatic activity was quantified by measuring the release of P_i using a malachite green phosphate colorimetric assay kit. *Error bars*, S.D. from two individual experiments.

C-terminal cell wall anchor domain including an LP*X*TG-sorting motif). S5nA belongs to the family of ecto-5′-nucleotidases but shows only low sequence homology to previously characterized nucleotidases. Analysis of recombinant S5nA produced in *E. coli* confirmed the predicted nucleosidase activity and showed both similarities and differences compared with the nucleotidases found in other Gram-positive cocci. S5nA hydrolyzes AMP and, with reduced efficiency, ADP but not ATP. NudP from *S. agalactiae* shows similar substrate specificity, whereas ecto-5′-nucleotidase A from *S. sanguinis*, Ssads from *S. suis*, and AdsA from *S. aureus* also hydrolyze ATP. In fact, ADP and ATP were found to be better substrates than AMP for both Ssads and AdsA (21, 26). Michaelis-Menten kinetics revealed a K_m value of 168 μM for AMP as substrate, which is comparable with that of NudP (35 μM) (25) but higher than for Ssads (117.8 nM) (26) and lower than AdsA (K_m = 930 μM) (21).

There were also differences in substrate preference compared with other NMPs. S5nA hydrolyzes AMP and CMP with similar efficiency but shows lower efficiency for hydrolyzing GMP and TMP. In contrast, CMP was shown to be a poor substrate for NudP (23) and was not hydrolyzed by AdsA (20, 21). AdsA also hydrolyzed GMP and TMP with low efficiency (20, 21).

Structural modeling of S5nA using automated template search resulted in a protein structure model based on the mammalian CD73 protein crystal structure. CD73 is one of two mammalian enzymes that sequentially convert ATP to adenosine. First, ATP and ADP are hydrolyzed by the ecto-NTPDase CD39 (NTPDase 1), followed by cleavage of AMP by the 5′-nucleotidase CD73. It is believed that structural differences in the substrate binding pockets are responsible for substrate specificity (36). Superposition of the S5nA protein model with the CD73 structure revealed strong conservation of core residues involved in substrate and metal ion binding, which might explain the preference for AMP seen in S5nA. The catalytic Asp-His dyad is also fully conserved. The role of the predicted residues was confirmed by mutation analysis. Identified residues for phosphate binding (Asn-99 and Arg-392) and the predicted catalytic dyad residue His-100 were found to be critical for AMP hydrolysis. This suggests that the enzymatic mechanism is similar to what has been described for CD73 and *E. coli* 5′-nucleotidase (32, 33).

S5nA is also capable to convert dAMP into dAdo. This has also been described for AdsA and NudP, and in all three cases, the efficiency is higher for dAMP as substrate compared with AMP (21, 25). The ability to hydrolyze dAMP has not been analyzed yet for Ssads or Nt5A and might be a common feature of all nucleotidases from pathogenic Gram-positive cocci. As shown recently, dAdo triggers the caspase-3-mediated death of macrophages and monocytes restricting macrophage influx into staphylococcal abscesses. Furthermore, it was shown that AdsA works in combination with the staphylococcal nuclease

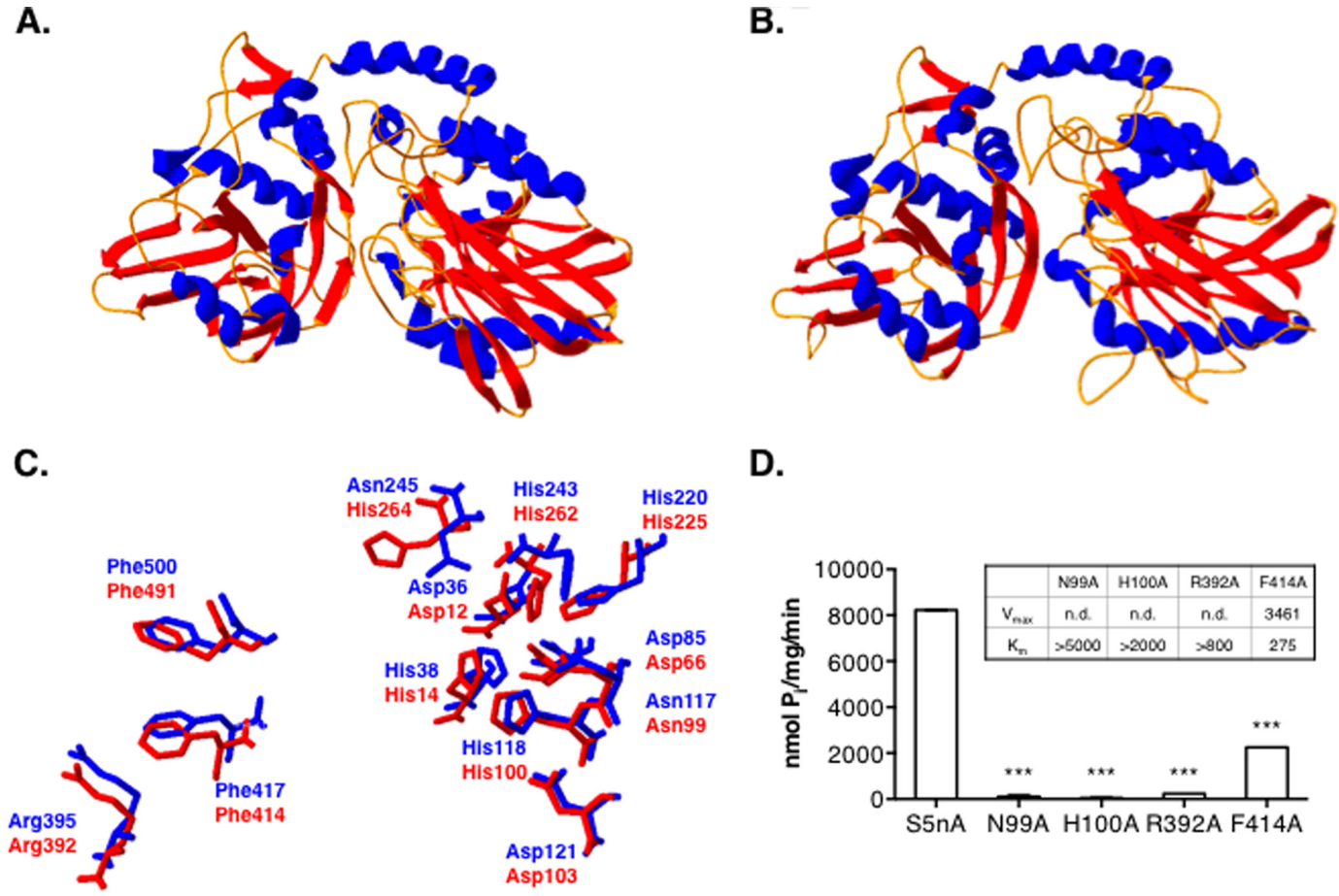

FIGURE 7. **S5nA has a conserved 5′-nucleotidase protein structure.** *A*, protein structure of the mammalian nucleotidase CD73 (Protein Data Bank code 4H1S). *B*, computer-generated protein structure of S5nA. The model was created using the Swiss PDB modeler with automated template search. The protein structure images were produced with the PDB Swiss Viewer version 4.1.0. *C*, superposition of the S5nA model structure with the CD73 protein structure revealed highly conserved key residues, which include amino acids involved in substrate binding, the catalytic triad, and metal binding. S5nA residues are shown in *red*, and CD73 residues are shown in *blue*. *D*, four selected key residues were targeted for alanine substitutions, and the recombinant mutant proteins were compared with WT S5nA under standard reaction conditions. The enzymatic activity was quantified by measuring the release of P_i using a malachite green phosphate colorimetric assay kit. *Error bars*, S.D. from two individual experiments. Statistical significance when compared with WT S5nA was calculated using one-way analysis of variance with Tukey's multiple comparison test. ***, $p < 0.001$. The V_{max} (nmol of P_i/mg/min) and K_m (μM) values for hydrolysis of AMP were obtained by velocity measurements with different concentrations of substrate using GraphPad Prism version 6 software.

TABLE 2

Conserved core residues among S5nA, mammalian CD73, and *E. coli* 5′-NT

CD73	*E. coli* 5′-nucleotidase	S5nA
Nucleoside binding pocket		
Phe-417	Phe-429	Phe-414
Phe-500	Phe-498	Phe-491
Phosphate binding		
Asn-117	Asn-116	Asn-99
His-118	His-117	His-100
Arg-395	Arg-410	Arg-392
Zinc binding		
Asp-36	Asp-41	Asp-12
His-38	His-43	His-14
Asp-85	Asp-84	Asp-66
Asn-117	Asn-116	Asn-99
His-220	His-217	His-225
His-243	His-252	His-262

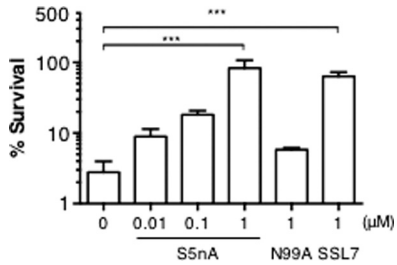

FIGURE 8. **Whole blood killing assay with *L. lactis* and recombinant s5nA.** *L. lactis* was grown to mid-logarithmic growth phase and mixed with fresh human whole blood taken from healthy volunteers and incubated at standard enzymatic assay conditions. Recombinant S5nA was added at various concentrations and increased bacterial survival in a dose-dependent manner. In contrast, the catalytic site mutant rS5nA N99A had no significant effect on bacterial survival. Recombinant SSL7 was used as a positive control. Statistical significance was calculated using a two-way analysis of variance with Dunn's multiple comparison test. ***, $p < 0.001$. *Error bars*, S.D. from two individual experiments.

Nuc, which degrades the DNA backbone of NETs to provide the dAMP substrate for AdsA (22). NETs are molecular structures comprising neutrophil DNA spiked with antimicrobial peptides that can trap and kill bacteria, including *S. pyogenes* (37). We have recently characterized SpnA as a cell wall-anchored nuclease in *S. pyogenes* that is also capable of degrading NETs (30). We show here that a combination of rS5nA and rSpnA generates inorganic phosphate from DNA, suggesting that, as in *S. aureus*, these two enzymes cause a "double-hit" to

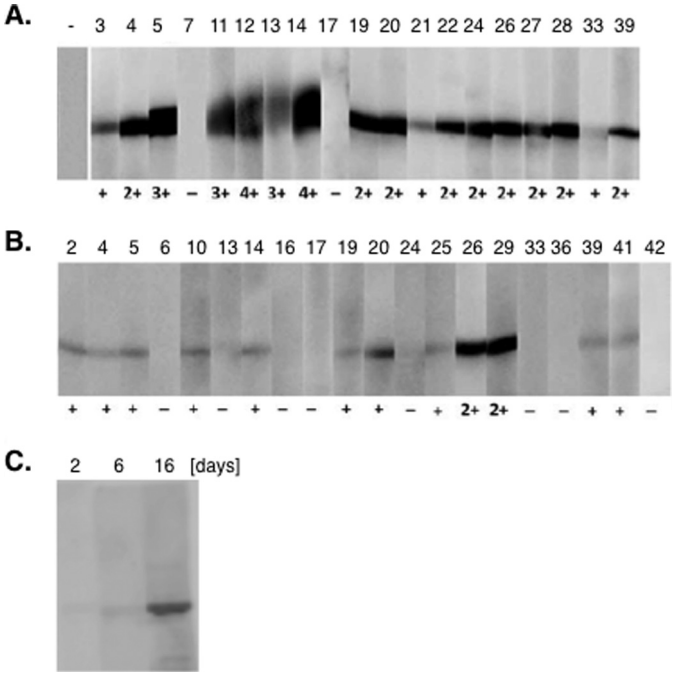

FIGURE 9. **Western blot analysis of rS5nA with human serum samples.** rS5nA (2 μg/lane) was run on SDS-PAGE and blotted onto a nitrocellulose membrane. Human serum samples were used (1:500). Western blots were developed using an ECL Western blotting detection kit, and bands were visualized with a Fujifilm LAS-3000 Intelligent Dark Box scanner. *A*, serum samples from patients with invasive *S. pyogenes* disease submitted to Middlemore Hospital, Auckland. *B*, serum samples from healthy blood donors. Serum reactivity was scored from + to 4+. *C*, serum samples from a single patient with streptococcal toxic shock syndrome taken 2, 6, and 16 days after hospital admission. The same patient has previously also shown seroconversion to SpnA (30).

the host's innate immune response by destroying NETs and killing macrophages via generation of dAdo. In fact, this might be a common strategy because NET-cleaving nucleases in addition to 5′-nucleotidases are also found in other pathogenic bacteria (*e.g.* in *S. sanguinis*) (23, 38). However, NudP from *S. agalactiae* is unable to hydrolyze dAMP, and, although this pathogen expresses a NET-cleaving nuclease, production of dAdo from NETs therefore appears unlikely (25, 39).

The enzymatic activity of S5nA was analyzed for a range of conditions, showing a good adaptation to the environment in the host, such as blood, extracellular fluids, and abscesses. S5nA is active at blood pH (pH 7.3–7.4) and shows optimal activity under acidic conditions found (*e.g.* in the purulent material of abscesses) (pH 6). In contrast to AdsA, S5nA is less active in alkaline environments. The pH range of NudP activity was reported to be even more restricted (between pH 6.5 and 7.7) (25). S5nA is active over a broad temperature range with a maximum activity at 42 °C. This might also reflect an adaptation to the environmental conditions in abscesses and inflamed tissue.

S5nA is also active with several different metal cofactors. Optimal activity was observed with Mg^{2+} and Mn^{2+} at concentrations between 0.1 and 10 mM and a slightly lower activity with Ca^{2+}. S5nA is also active in the presence of Zn^{2+} but only at low concentrations. Nucleotidases appear to vary in their cation requirements. Whereas AdsA shows a similar profile (active with Mg^{2+}, Mn^{2+}, or low concentrations of Zn^{2+}) (21), NudP specifically requires Mn^{2+} (25).

Although several 5′-nucleotidases have been crystalized in the presence of Zn^{2+} (*e.g.* CD73 (32), *E. coli* nucleotidase (33), and *Hemophilus influenza* NAD nucleotidase (40)), the biological role of Zn^{2+} appears to vary. The bacterial enzymes are only active at very low Zn^{2+} concentrations and inhibited at higher concentrations (21, 25, 41). In contrast, CD73 has been described as a zinc-dependent 5′-nucleosidase due to the fact that after stripping metal ions by EDTA treatment, Zn^{2+} reconstitution resulted in the highest enzymatic activity when compared with other divalent cations. Furthermore, it was shown that CD73 is inactive with Mg^{2+} as metal cofactor (32). Notably, the Zn^{2+} concentration in human blood is ~6.2 mg/liter (~0.1 mM) (42), which would allow optimal S5nA activity.

Extracellular nucleotides regulate the balance between pro- and anti-inflammatory responses in the blood (7, 12). A human whole blood killing assay with non-pathogenic *L. lactis* showed a dose-dependent increase in bacterial survival when rS5nA was added. Significantly reduced killing was observed with 1 and 0.1 μM recombinant enzyme, and this was due to the enzymatic activity of S5nA because the catalytic site mutant S5nA N99A had no detectable effect on bacterial cell survival. This is in line with a study by Firon *et al.* (25), who showed decreased survival of a *S. agalactiae* mutant with a *nudP* gene deletion. They also demonstrated that the mutant could be rescued by the addition of adenosine, further highlighting the role of extracellular nucleotides in the modulation of the host response to pathogens (25).

Serum antibodies to virulence factors in blood samples from convalescent disease patients can be used as a diagnostic tool to identify the infectious agent, and this also provides experimental proof that the virulence factor was expressed during the infection. We found moderate to strong antibody responses against S5nA in 14 of 19 serum samples from patients with invasive *S. pyogenes* disease but only in 2 of 20 serum samples from healthy donors. In addition, acute and convalescent serum samples obtained from a patient with streptococcal toxic shock syndrome (35) indicated significant seroconversion. Notably, we have previously shown seroconversion to the cell wall-anchored nuclease SpnA using the same panel of serum samples from this patient (30). This strongly suggests co-expression of SpnA and S5nA during disease and provides further evidence for a synergistic action to generate dAdo from NET DNA. Therefore, SpnA and S5nA might be suitable targets for vaccine development. Alternatively, a drug designed to inhibit SpnA and S5nA activity to maintain NET integrity and prevent the production of dAdo might be useful as an adjunctive therapy to conventional antibiotic treatment.

In summary, this study demonstrates that *S. pyogenes* produces an extracellular enzyme with 5′-nucleotidase activity, which is able to hydrolyze AMP, ADP, and dAMP to generate the immunomodulatory substrates adenosine and deoxyadenosine. S5nA shares some common features with other bacterial 5′-nucleotidases that have been shown to facilitate immune evasion in the host. Future research using isogenic *s5nA* gene deletion mutants in animal infection models might shed further light onto the physiological function of this novel *S. pyogenes* virulence factor.

Author Contributions—L. Z. performed experiments shown in Figs. 2 (*A* and *D*), 3*A*, 4*A*, 7*D*, and 9 as part of her M.Sc. project. A. K. provided technical assistance and contributed to experiments shown in Figs. 2 (*B* and *E*), 3*B*, 4 (*B* and *C*), 5, 6, and 7*D*. N. L. designed and carried out the assay shown in Figs. 2*C* and 8. J. M. S. L. and R. J. L. contributed to the design of the study and student supervision. T. P. designed the overall study and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgment—We thank Fiona Clow for providing the purified recombinant SSL7 protein.

References

1. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* **5,** 685–694
2. Cunningham, M. W. (2008) Pathogenesis of group A streptococcal infections and their sequelae. *Adv. Exp. Med. Biol.* **609,** 29–42
3. Ralph, A. P., and Carapetis, J. R. (2013) Group a streptococcal diseases and their global burden. *Curr. Top. Microbiol. Immunol.* **368,** 1–27
4. Jaine, R., Baker, M., and Venugopal, K. (2008) Epidemiology of acute rheumatic fever in New Zealand 1996–2005. *J. Paediatr. Child Health* **44,** 564–571
5. Jaine, R., Baker, M., and Venugopal, K. (2011) Acute rheumatic fever associated with household crowding in a developed country. *Pediatr. Infect. Dis. J.* **30,** 315–319
6. Di Virgilio, F. (2007) Purinergic signalling in the immune system: a brief update. *Purinergic Signal.* **3,** 1–3
7. Junger, W. G. (2011) Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* **11,** 201–212
8. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., and Robson, S. C. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204,** 1257–1265
9. Trautmann, A. (2009) Extracellular ATP in the immune system: more than just a "danger signal". *Sci. Signal.* **2,** pe6
10. Gorini, S., Gatta, L., Pontecorvo, L., Vitiello, L., and la Sala, A. (2013) Regulation of innate immunity by extracellular nucleotides. *Am. J. Blood Res.* **3,** 14–28
11. Haskó, G., and Cronstein, B. N. (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol.* **25,** 33–39
12. Vitiello, L., Gorini, S., Rosano, G., and la Sala, A. (2012) Immunoregulation through extracellular nucleotides. *Blood* **120,** 511–518
13. Idzko, M., Ferrari, D., and Eltzschig, H. K. (2014) Nucleotide signalling during inflammation. *Nature* **509,** 310–317
14. Xaus, J., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., and Celada, A. (1999) IFN-γ up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J. Immunol.* **162,** 3607–3614
15. Cronstein, B. N., Kramer, S. B., Weissmann, G., and Hirschhorn, R. (1983) Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. *J. Exp. Med.* **158,** 1160–1177
16. Edwards, C. K., 3rd, Watts, L. M., Parmely, M. J., Linnik, M. D., Long, R. E., and Borcherding, D. R. (1994) Effect of the carbocyclic nucleoside analogue MDL 201,112 on inhibition of interferon-γ-induced priming of Lewis (LEW/N) rat macrophages for enhanced respiratory burst and MHC class II Ia+ antigen expression. *J. Leukoc. Biol.* **56,** 133–144
17. Haskó, G., Szabó, C., Németh, Z. H., Kvetan, V., Pastores, S. M., and Vizi, E. S. (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-α, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J. Immunol.* **157,** 4634–4640
18. Bouma, M. G., Jeunhomme, T. M., Boyle, D. L., Dentener, M. A., Voitenok, N. N., van den Wildenberg, F. A., and Buurman, W. A. (1997) Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A2 and A3 receptors. *J. Immunol.* **158,** 5400–5408
19. Taylor, A. R. (2013) Methicillin-resistant *Staphylococcus aureus* infections. *Prim. Care* **40,** 637–654
20. Thammavongsa, V., Kern, J. W., Missiakas, D. M., and Schneewind, O. (2009) *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J. Exp. Med.* **206,** 2417–2427
21. Thammavongsa, V., Schneewind, O., and Missiakas, D. M. (2011) Enzymatic properties of *Staphylococcus aureus* adenosine synthase (AdsA). *BMC Biochem.* **12,** 56
22. Thammavongsa, V., Missiakas, D. M., and Schneewind, O. (2013) *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science* **342,** 863–866
23. Fan, J., Zhang, Y., Chuang-Smith, O. N., Frank, K. L., Guenther, B. D., Kern, M., Schlievert, P. M., and Herzberg, M. C. (2012) Ecto-5′-nucleotidase: a candidate virulence factor in *Streptococcus sanguinis* experimental endocarditis. *PLoS One* **7,** e38059
24. Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K., Cousens, S., and Heath, P. T. (2012) Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. *Lancet* **379,** 547–556
25. Firon, A., Dinis, M., Raynal, B., Poyart, C., Trieu-Cuot, P., and Kaminski, P. A. (2014) Extracellular nucleotide catabolism by the Group B *Streptococcus* ectonucleotidase NudP increases bacterial survival in blood. *J. Biol. Chem.* **289,** 5479–5489
26. Liu, P., Pian, Y., Li, X., Liu, R., Xie, W., Zhang, C., Zheng, Y., Jiang, Y., and Yuan, Y. (2014) *Streptococcus suis* adenosine synthase functions as an effector in evasion of PMN-mediated innate immunity. *J. Infect. Dis.* **210,** 35–45
27. Lun, Z. R., Wang, Q. P., Chen, X. G., Li, A. X., and Zhu, X. Q. (2007) *Streptococcus suis*: an emerging zoonotic pathogen. *Lancet Infect. Dis.* **7,** 201–209
28. Wertheim, H. F., Nghia, H. D., Taylor, W., and Schultsz, C. (2009) *Streptococcus suis*: an emerging human pathogen. *Clin. Infect. Dis.* **48,** 617–625
29. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22,** 195–201
30. Chang, A., Khemlani, A., Kang, H., and Proft, T. (2011) Functional analysis of *Streptococcus pyogenes* nuclease A (SpnA), a novel group A streptococcal virulence factor. *Mol. Microbiol.* **79,** 1629–1642
31. Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N., Kenton, S., Lai, H. S., Lin, S. P., Qian, Y., Jia, H. G., Najar, F. Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S. W., Roe, B. A., and McLaughlin, R. (2001) Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4658–4663
32. Heuts, D. P., Weissenborn, M. J., Olkhov, R. V., Shaw, A. M., Gummadova, J., Levy, C., and Scrutton, N. S. (2012) Crystal structure of a soluble form of human CD73 with ecto-5′-nucleotidase activity. *Chembiochem* **13,** 2384–2391
33. Knöfel, T., and Sträter, N. (1999) X-ray structure of the *Escherichia coli* periplasmic 5′-nucleotidase containing a dimetal catalytic site. *Nat. Struct. Biol.* **6,** 448–453
34. Lorenz, N., Clow, F., Radcliff, F. J., and Fraser, J. D. (2013) Full functional activity of SSL7 requires binding of both complement C5 and IgA. *Immunol. Cell Biol.* **91,** 469–476
35. Yang, L., Thomas, M., Woodhouse, A., Martin, D., Fraser, J. D., and Proft, T. (2005) Involvement of streptococcal mitogenic exotoxin Z in streptococcal toxic shock syndrome. *J. Clin. Microbiol.* **43,** 3570–3573
36. Robson, S. C., Sévigny, J., and Zimmermann, H. (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signal.* **2,** 409–430
37. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303,** 1532–1535
38. Morita, C., Sumioka, R., Nakata, M., Okahashi, N., Wada, S., Yamashiro, T., Hayashi, M., Hamada, S., Sumitomo, T., and Kawabata, S. (2014) Cell

wall-anchored nuclease of *Streptococcus sanguinis* contributes to escape from neutrophil extracellular trap-mediated bacteriocidal activity. *PLoS One* **9,** e103125

39. Derré-Bobillot, A., Cortes-Perez, N. G., Yamamoto, Y., Kharrat, P., Couvé, E., Da Cunha, V., Decker, P., Boissier, M. C., Escartin, F., Cesselin, B., Langella, P., Bermúdez-Humarán, L. G., and Gaudu, P. (2013) Nuclease A (Gbs0661), an extracellular nuclease of *Streptococcus agalactiae*, attacks the neutrophil extracellular traps and is needed for full virulence. *Mol. Microbiol.* **89,** 518–531
40. Garavaglia, S., Bruzzone, S., Cassani, C., Canella, L., Allegrone, G., Sturla, L., Mannino, E., Millo, E., De Flora, A., and Rizzi, M. (2012) The high-resolution crystal structure of periplasmic *Haemophilus influenzae* NAD nucleotidase reveals a novel enzymatic function of human CD73 related to NAD metabolism. *Biochem. J.* **441,** 131–141
41. Neu, H. C. (1967) The 5′-nucleotidase of *Escherichia coli.* II. Surface localization and purification of the *Escherichia coli* 5′-nucleotidase inhibitor. *J. Biol. Chem.* **242,** 3905–3911
42. Xu, B., Chia, S. E., and Ong, C. N. (1994) Concentrations of cadmium, lead, selenium, and zinc in human blood and seminal plasma. *Biol. Trace Elem. Res.* **40,** 49–57

Microbiology:

Streptococcal 5′-Nucleotidase A (S5nA), a Novel *Streptococcus pyogenes* Virulence Factor That Facilitates Immune Evasion

Lisa Zheng, Adrina Khemlani, Natalie Lorenz, Jacelyn M. S. Loh, Ries J. Langley and Thomas Proft

J. Biol. Chem. 2015, 290:31126-31137.
doi: 10.1074/jbc.M115.677443 originally published online November 2, 2015