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

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**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 Mg2+, Ca2+, or Mn2+. However, Zn2+ 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 stimulating 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

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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 SpnA; SSL7, staphylococcal superantigen-like protein 7.
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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 blastn 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 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 Purification Kit (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 dyeoxy chain termination method using the DNA sequencing service at the School of Biological Science, University of Auckland. The 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 His6-tagged proteins were purified on Ni2+-iminodiacetic acid Sepharose (Sigma). The eluates containing the recombinant proteins were collected and analyzed on 12.5% SDS-polyacrylamide gels according to the

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**TABLE 1**

PCR primers used in this study

| Primer      | DNA sequence (5′–3′) |
|-------------|---------------------|
| S5nA-fw     | GCAGATTCGATTATGTGAGTGCAGATTTC |
| S5nA-rev    | GGATCCCTTAAGCTGAACTGAGATAGTAG |
| S5nA_D66A-fw| GCTGGAGCTATGGTCGGAGCCAGTCC |
| S5nA_D66A-rev| GTAATGGCTACTAAGCTGGAGATAGTAG |
| S5nA_H100A-fw| CTCTTGGTAATGCTGAATTTGACGAAGGACTAG |
| S5nA_H100A-rev| S5nA-fw |
| S5nA_F414A-rev| GCAGCTGGTTGTACAGCCTGTGC |
| S5nA_F414A-fw| CCAGCTGGTTGTACAGCCTGTGC |
| S5nA_F491A-fw| CCAAGCTTTTTCATGGTGCTGTTGAG |
| S5nA_F491A-rev| CCAAGCTTTTTCATGGTGCTGTTGAG |

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**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™.
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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 mM 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 MgCl₂, 2 mM CaCl₂ with 1 mM nucleotides (Sigma-Aldrich) and 0.1 mM 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.

For the quantification of inorganic phosphate (P₁) 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 P₁ was measured at 650 nm against a standard P₁ 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 MgCl₂, and 2 mM CaCl₂ was incubated at 37 °C for 1 h, before 0.1 μM S5nA and 10 mM MgCl₂ 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 A₄₅₀ = 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 NaHCO₃, 1.3 mM CaCl₂, 1 mM MgSO₄, 0.44 mM KH₂PO₄, 0.25 mM Na₂PO₄, 0.01 mM glucose) at a final concentration of 1 × 10⁶ 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 × 10⁶ 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/ml) 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 (Sero tec) 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 LPXTG 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 (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 LPXTG cell wall anchor domain (IPRO019931) between positions 637 and 670 (Fig. 1A). 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 equis EQI SEQUENCE 1278 (71% amino acid identity) and the S. agalactiae nucleotidase NudP (64%). Only limited sequence identity was found with S. sanguinis ecto-5’-nucleo-
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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, AKF32764), Ssads (S. suis, YP001197640), NudP (S. agalactiae, CDN6659), 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\(^{2+}\)-imidotriacetic 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. 1B).

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. 1C). 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 \( \mu \)M 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. 2A). In addition, we used a commercial kit to measure the release of Pi, during the enzymatic reaction. A time course showed that the reaction reached equilibrium after about 25 min when 1 mM AMP and 0.1 \( \mu \)M rS5nA were incubated at 37 °C (Fig. 2B). The initial release of Pi 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 \( \mu \)M and a \( V_{max} \) of 7550 ± 326 nmol of Pi/mg of S5nA/min (Fig. 2C). 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. 2D). However, Pi release from 1 mM ADP was ~3.5 times lower than from 1 mM AMP (Fig. 2E).

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

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 Pi were generated from 1 mM dAMP and AMP (Fig. 4B). Equilibrium of the reaction was reached after about 20 min (Fig. 4C) compared with 25 min with AMP (Fig. 2B). 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 DNA, the reaction reached equilibrium after about 25 min with dAMP, generating ~3.5 times more Pi than with AMP (Fig. 4D). Therefore, S5nA and SpnA form an enzyme combination with similar function. Indeed, when rSpnA and rS5nA were incubated with salmon sperm DNA, the reaction reached equilibrium after about 25 min with dAMP, generating ~3.5 times more Pi than with AMP (Fig. 4D). Therefore, S5nA and SpnA form an enzyme combination with similar function.
DNA, Pi production was observed (Fig. 5). Minimal Pi 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 Pi.

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 Pi 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. 6A). We observed that rS5nA functioned at a broad temperature range with optimal activity between 32 and 47 °C. No Pi 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 Mg²⁺, Mn²⁺, Ca²⁺, or Zn²⁺ (Fig. 6C and D). S5nA activity was slightly higher with Mg²⁺ compared with Ca²⁺ at concentrations of 0.1, 1, or 10 mM in a dose-dependent manner. The enzyme was also fully active with Mn²⁺ at 0.1 mM and 1 mM but less active at 10 mM. In contrast, S5nA activity in the presence of Zn²⁺ 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 Zn²⁺ (Fig. 6D). The inhibitory effect of 1 mM Zn²⁺ on S5nA activity could only slightly be compensated for by the addition of Mg²⁺ (~20% activity at 1 mM and 30% activity at 10 mM Mg²⁺) (Fig. 6E).

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 (data not shown).

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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 Pi from AMP. The enzymatic activity was significantly reduced with S5nA F414A ($K_m=275 \mu M$ and $V_{max}=3461 \text{ nmol P/mg/min}$). Very weak activity was seen with S5nA R392A ($K_m>800 \mu M$), and no enzymatic activity was detected with S5nA N99A and S5nA H100A ($K_m>2000 \mu M$) (Fig. 7D).

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 rS5nA on *L. lactis* survival in human 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. 9A) and 20 control sera from healthy blood donors (Fig. 9B). Antibodies against S5nA...
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. 9C). 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...
C-terminal cell wall anchor domain including an LPXTG-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 $\mu$M for AMP as substrate, which is comparable with that of NudP (35 $\mu$M) (25) but higher than for Ssads (117.8 $\mu$M) (26) and lower than AdsA ($K_m = 930 \mu$M) (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

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**FIGURE 6. Kinetic activity of rS5nA.** Hydrolysis of 1 mM AMP by 0.1 $\mu$m 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 Pi using a malachite green phosphate colorimetric assay kit. 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

TABLE 2

| Nucleoside binding pocket | CD73 | E. coli 5’-nucleotidase | S5nA |
|---------------------------|------|-------------------------|------|
| Phe-417                   | Phe-429 | Phe-414                |
| Phe-500                   | Phe-498 | Phe-491                |

| Phosphate binding         | CD73 | E. coli 5’-nucleotidase | S5nA |
|---------------------------|------|-------------------------|------|
| Asn-117                   | Asn-116 | Asn-99                |
| His-118                   | His-117 | His-100                |
| Arg-395                   | Arg-410 | Arg-392               |

| Zinc binding              | CD73 | E. coli 5’-nucleotidase | S5nA |
|---------------------------|------|-------------------------|------|
| 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 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 +++. 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 Mg2+ and Mn2+ at concentrations between 0.1 and 10 mM and a slightly lower activity with Ca2+. S5nA is also active in the presence of Zn2+ but only at low concentrations. Nucleotidases appear to vary in their cation requirements. Whereas AdsA shows a similar profile (active with Mg2+, Mn2+, or low concentrations of Zn2+) (21), NudP specifically requires Mn2+ (25).

Although several 5’-nucleotidases have been crystallized in the presence of Zn2+ (e.g. CD73 (32), E. coli nucleotidase (33), and Hemophilus influenzae NAD nucleotidase (40)), the biological role of Zn2+ appears to vary. The bacterial enzymes are only active at very low Zn2+ 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, Zn2+ reconstitution resulted in the highest enzymatic activity when compared with other divalent cations. Furthermore, it was shown that CD73 is inactive with Mg2+ as metal cofactor (32). Notably, the Zn2+ 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.
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. Edwards, C. K., 3rd, Watts, L. M., Parmely, M. J., Linnik, M. D., Long, R. E., Hasko, G., and Cronstein, B. N. (2004) Adenosine: an endogenous regulator of innate immunity. *J. Immunol.* 173, 5479–5489.
5. Z. Vitiello, L., Gorini, S., Rosano, G., and la Sala, A. (2012) Immunoregulation in human neutrophils. *BMC Biochem.* 13, 6.
6. Junger, W. G. (2011) Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* 11, 201–212.
7. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J., Eniyoji, 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.
8. Di Virgilio, F. (2007) Purinergic signalling in the immune system: a brief update. *Purinergic Signal.* 3, 1–3.
9. Hasko, G., and Cronstein, B. N. (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol.* 25, 33–39.
10. Vitiello, L., Gorini, S., Rosano, G., and la Sala, A. (2012) Immunoregulation through extracellular nucleotides. *BMC Biol.* 12, 511–518.
11. Ikeda, M., Ferrari, D., and Eltzschig, H. K. (2014) Nucleotide signalling during inflammation. *Nature* 509, 310–317.
12. Krone, N., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., and Celada, A. (1999) FIM-y-up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J. Immunol.* 162, 3607–3614.
13. 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.
14. Edwards, C. K., 3rd, Watts, L. M., Parmely, M. J., Linnik, M. D., Long, R. E., and Borchardt, D. R. (1994) Effect of the carbocyclic nucleoside analogue MDL 20,112 on inhibition of interferon-γ-induced priming of Lewis (LEW/N) rat macrophages for enhanced respiratory burst and MHC class II antigen expression. *J. Leukoc. Biol.* 56, 133–144.
15. Hasko, G., Szabo, 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 endothotoxic mice. *J. Immunol.* 157, 4634–4640.
16. 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.
17. Taylor, A. R. (2013) Methicillin-resistant *Staphylococcus aureus* aureus infections. *Prim. Care* 40, 637–654.
18. 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.
19. Thammavongsa, V., Schneewind, O., and Missiakas, D. M. (2011) Enzymatic properties of *Staphylococcus aureus* adenosine synthase (AdA). *BMC Biochem.* 12, 56.
20. Thammavongsa, V., Missiakas, D. M., and Schneewind, O. (2013) *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science* 342, 863–866.
21. Fan, J., Zhang, Y., Chaung-Smith, O. N., Frank, K. L., Guenther, B. D., Kern, M., Schilvert, P. M., and Herzberg, M. C. (2012) Ecto-5′-nucleotidase: a candidate virulence factor in *Streptococcus sanguinis* experimental endocarditis. *PLoS One* 7, e38059.
22. Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K., Cousins, 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.
23. 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.
24. 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.
25. Lu, 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.
26. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2009) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195–201.
27. Chang, A., Khemlani, A., Kang, H., and Proft, T. (2011) Functional analysis of *Streptococcus pyogenes* nucleoside A (SpnA), a novel group A streptococcal virulence factor. *Mol. Microbiol.* 79, 1629–1642.
28. Heuts, D. P., Weissenborn, M. J., Olkov, 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.
29. Lorenz, N., Clow, F., Radcliff, F. J., and Fraser, J. D. (2013) Full functional characterization of SSL7, a novel *Streptococcus pyogenes* virulence factor. *J. Infect. Dis.* 210, 35–45.
30. Watson, L., Hendry, J., and Tschudin, S. C. (2012) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiologic significance. *Purinergic Signal.* 2, 409–430.
31. 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.
32. 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. Derre´-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