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Contamination of DNase Preparations Confounds Analysis of the Role of DNA in Alum-Adjuvanted Vaccines

Laura E. Noges,^{*,†} Janice White,^{*} John C. Cambier,^{*,†} John W. Kappler,^{*,†,‡} and Philippa Marrack^{*,†,‡}

Aluminum salt (alum) adjuvants have been used for many years as adjuvants for human vaccines because they are safe and effective. Despite its widespread use, the means by which alum acts as an adjuvant remains poorly understood. Recently, it was shown that injected alum is rapidly coated with host chromatin within mice. Experiments suggested that the host DNA in the coating chromatin contributed to alum's adjuvant activity. Some of the experiments used commercially purchased DNase and showed that coinjection of these DNase preparations with alum and Ag reduced the host's immune response to the vaccine. In this study, we report that some commercial DNase preparations are contaminated with proteases. These proteases are responsible for most of the ability of DNase preparations to inhibit alum's adjuvant activity. Nevertheless, DNase somewhat reduces responses to some Ags with alum. The effect of DNase is independent of its ability to cleave DNA, suggesting that alum improves CD4 responses to Ag via a pathway other than host DNA sensing. *The Journal of Immunology*, 2016, 197: 1221–1230.

For more than 80 years, insoluble aluminum salts (alum) have been safely used in vaccines to generate protective immunity in hundreds of millions of people. Nevertheless, the immune mechanisms triggered by alum are controversial or still unknown. Our group has previously reported that alum particles become entrapped by host chromatin upon i.m. injection, the route of administration for most human vaccines (1). Since this finding, another group and we have suggested that DNA released by host cells at the site of injection contributes to alum's adjuvant activity. Others and we reported that alum-associated DNA acted as an endogenous immunostimulatory signal for inflammatory monocyte-derived dendritic cells that mediate CD4 T cell priming and subsequent Ab responses (2, 3). The studies were based, in part, on transgenic CD4 T cell adoptive transfer experiments as well as experiments that combined alum vaccines with DNase I enzymes purchased from Roche Diagnostics. These experiments showed that coinjection of DNase inhibited the ability of alum to adjuvant CD4 but not CD8 T cell responses.

Our objective in this study was to reconcile the differences observed between the effects of DNA on CD4 versus CD8 T cell responses. We began by scrutinizing the quality of the DNase I reagents that had been used in past studies. In this study, we report that several of the commercial DNase reagents were and are contaminated with active proteases and that much of the effect of these DNases is due to these proteases, not the DNase activity itself. Nevertheless, we found that administration of pure DNase with Ag and alum i.m. did reduce CD4 T cell responses to some, but not all, Ags. The DNase inhibition of responses occurred even when the DNase preparation had no ability to cleave DNA. Our results suggest that the pathways whereby alum acts as an adjuvant depend on the nature of the Ag. Additionally, for those Ags that are susceptible to DNase inhibition, alum pathways are inhibited by DNase in an unexpected way that is independent of its enzymatic function.

Materials and Methods

Mice

C57/BL6J female mice were purchased from The Jackson Laboratory. STING-deficient mice were generated and provided by the laboratory of one of us (J.C.C.) at National Jewish Health. STING-deficient mice had their genotypes reconfirmed at the time of sacrifice. In all experiments, mice were age matched and between 6 and 18 wk of age at the time of first immunization. All animals were housed and maintained in the Biological Resource Center within National Jewish Health in accordance with the research guidelines of the National Jewish Health Institutional Animal Care and Use Committee.

Abs and reagents

Alhydrogel aluminum hydroxide (Brenntag) was purchased from Accurate Chemical. The following mAbs were purchased from eBioscience: allophycocyanin-eFluor 780 anti-CD4 (RM4-5), PE-Cy7 anti-CD8a (53-6.7), PerCP-Cy5.5 anti-CD44 (IM7), eFluor 450 anti-B220 (RA3-6B2), eFluor 450 anti-F4/80 (BM8), and FITC anti-PD1 (J43). Biotin anti-CXCR5 Abs against MHC class II (MHC II; Y3P) were purified from hybridoma supernatants. PE-IA^b/nucleoprotein (NP)_{311–325} (QVYSLIRPNENPAHK) and allophycocyanin-D^b/NP_{366–374} (ASNENMETM) tetramers were obtained from the National Institutes of Health Tetramer Core Facility. PE-IA^b/3K (FEAQKAKANKAVD) and allophycocyanin-K^b/OVA_{257–264} (SIINFEKL) tetramers were produced as described previously (4). OVA coupled to the influenza nucleoprotein peptide NP_{311–325} (OVA-NP) was generated using an Inject maleimide-activated OVA kit (Pierce Biotechnology)

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Abbreviations used in this article: alum, aluminum salt; MHC II, MHC class II; mutNP, mutant nucleoprotein; NP, nucleoprotein; Roche-DNaseGII, Roche DNase I grade II; Roche-rDNase, Roche DNase I recombinant; Sig-DNase, Sigma-Aldrich DNase I; Worth-DNase, Worthington Biochemical protease- and RNase-free DNase I; wtDNase, wild-type DNase.

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and cysteine-linked influenza A nucleoprotein peptide NP_{311–325} (QVYSLIRPNENPAHKGGGC) purchased from CHI Scientific. OVA-3K was created in the same manner using cysteine-linked 3K peptide (FEAQKAKANKAVDGGGC). The following reagents were purchased from Sigma-Aldrich: BSA, TPCK trypsin, chymotrypsin, and LPS (from *Escherichia coli*). Polyinosinic-polycytidylic acid was purchased from GE Healthcare, and anti-CD40 Ab (FGK-45) was purchased from Bio X Cell. PR8 influenza A NP was produced as described previously (5). Additionally, mutant NP (mutNP) containing five arginine residues mutated to alanines at positions 74, 75, 174, 175, and 221 was cloned and produced in the same manner using a baculovirus expression vector.

DNase reagents and protease inhibition

For DNase reagents and protease inhibition, see Table I. Two rounds of PMSF treatment (2 mM, 10 min incubation on ice) of Roche DNase I grade II (Roche-DNaseGII; 13 mg/ml in PBS) were used to inhibit proteases.

Immunizations

Mice were anesthetized with 2.5% (v/v) isoflurane and injected i.m. in each calf muscle with a total vol of 50 μ l per calf. All vaccines consisted of 10 μ g protein Ag (OVA-3K, OVA-NP, NP, or mutNP) that was fully adsorbed to 200 μ g alum (or combined with other adjuvants, as indicated) and suspended in endotoxin-free PBS (CellGro). When indicated, additional reagents (BSA, various DNases, trypsin, and/or chymotrypsin) were added as treatments to vaccines immediately before injection. The endotoxin content of each vaccine component was <1 endotoxin unit/injection, as measured by *Limulus* amebocyte lysate assay (Lonza).

Assessment of Ag-specific T cell priming

Popliteal lymph nodes were harvested into ice-cold balanced salt solution and disrupted through nylon mesh to create single-cell suspensions. Cells were stained with tetramers (IA^b/NP_{311–325} or IA^b/3K, and K^b/SIINFEKL or D^p/NP_{366–374}) for 2 h at 37°C. Abs for CD4, CD44, CD8a, B220, MHC II, and F4/80 were added to cells and further incubated for at least 30 min on ice or at 4°C. Cells were then washed and analyzed on a CyAn ADP analyzer (Beckman Coulter) flow cytometer using Summit software (DakoCytomation). Data were analyzed using FlowJo software (Tree Star). IA^b/NP_{311–325} or IA^b/3K tetramer⁺ cells were defined after gating on live CD4⁺CD44^{hi} cells that were negative for CD8a, B220, MHC II, and F4/80. K^b/SIINFEKL or D^p/NP_{366–374} tetramer⁺ cells were similarly defined, but were gated on CD8a⁺ and CD4⁺. When required, tetramer⁺ CD4 T cells were examined for T follicular helper cell phenotype by cell surface staining of PD1 and CXCR5.

Ab detection by ELISA

For OVA- and NP-specific IgG1 or IgE detection, we incubated serially diluted sera from immunized mice on 96-well Immulon plates (Thermo Scientific) coated with OVA (grade VII, Sigma-Aldrich) at 100 μ g/ml or NP at 10 μ g/ml. We detected bound IgG1 using alkaline phosphatase-conjugated anti-mouse IgG1 Abs (BD Pharmingen) followed by incubation with *p*-nitrophenyl phosphate and measurement by spectrophotometry. Bound IgE was detected using biotin-conjugated anti-mouse IgE Abs (BD Pharmingen), followed by streptavidin-conjugated HRP and tetramethylbenzidine substrate. To determine relative units, we used positive control serum samples from C57BL/6 mice that contained OVA-specific or NP-specific Abs.

SDS-PAGE

Samples (>8 mg/ml) were reduced, heat denatured, and applied to a PhastGel homogeneous 12.5% polyacrylamide gel (GE Healthcare) as recommended by the manufacturer. Low molecular mass standards (GE Healthcare) were applied alongside samples. Gels were stained with Coomassie brilliant blue G-250 (Bio-Rad).

Protease activity assay

A Pierce protease assay kit (Thermo Scientific) was used according to the manufacturer's instructions. TPCK trypsin standard was serially diluted by 5-fold whereas samples were serially diluted by 2-fold.

DNase activity assay

DNase reagents were serially diluted in 96-well round-bottomed tissue culture plates and exposed to 800 ng genomic mouse DNA within PBS plus 25 mM MgCl₂ and 5 mM CaCl₂ for 30 min at 37°C. Enzymatic activity was stopped by heat denaturation at 95°C for 5 min and samples were immediately placed on ice and assessed for dsDNA content using a Qubit dsDNA broad range assay kit.

Mass spectrometry

Samples were prepared in PBS and reduced, denatured, alkylated, and digested overnight at 37°C with Trypsin Gold (Promega). Peptides within samples were chromatographically resolved on-line using a C18 column and 1260 series HPLC (Agilent Technologies, Palo Alto, CA) and analyzed using a 6550 liquid chromatography/mass spectroscopy quadrupole time-of-flight mass spectrometer (Agilent Technologies) in the National Jewish Health Proteomics Facility. Raw data were extracted and searched using the Spectrum Mill search engine (rev. B.04.00.127, Agilent Technologies). "Peak picking" is performed within Spectrum Mill with the following parameters: signal-to-noise set at 15, maximum charge state of 4 was allowed ($z = 4$), and the program was directed to find a precursor charge state. During searching the following parameters were applied: searched the Swiss-Prot bovine database, carbamidomethylation as a fixed modification, oxidized methionine and deamidated asparagine as variable modifications, collected spectra were compared with tryptic peptides in the database, maximum of two missed cleavages, precursor mass tolerance ± 20 ppm, product mass tolerance ± 50 ppm, and maximum ambiguous precursor charge of 3. Data were evaluated and protein identifications were considered significant when the following confidence thresholds were met: minimum of three peptides per protein, protein score of >10, individual peptide scores of at least 6, and scored percent intensity of at least 60%. The scored percent intensity provides an indication of the percentage of the total ion intensity that matches the peptide's tandem mass spectroscopy spectrum. Standards were run at the beginning of each day of analyses for quality control purposes.

Ag destruction assay

Protein Ags (OVA-NP or NP) were prepared at 200 μ g/ml in PBS plus 25 mM MgCl₂ and exposed to treatments for 0, 1, or 21–23 h at 37°C. To mimic vaccine preparations, treatments were as follows: BSA (20 mg/ml), Roche-DNaseGII (20 mg/ml), trypsin (200 μ g/ml), or chymotrypsin (200 μ g/ml). OVA-NP, OVA-3K, or NP mixtures were then added in duplicate to tissue culture wells containing Chb-2.4.4 Ag-presenting B cells and '3NP311-2' NP_{311–325}-specific T cell hybridomas or 'B3K0508' 3K-specific T cell hybridomas. Cells were incubated for 24 h at 37°C then supernatants were tested for IL-2 by sandwich ELISA. Supernatants were serially diluted on 96-well Immulon plates (Thermo Scientific) coated with anti-IL-2 (eBioscience). Bound IL-2 was detected with biotin-conjugated anti-IL-2 (eBioscience) followed by HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and subsequent incubation with 1-Step ultra tetramethylbenzidine substrate (Thermo Scientific) and 2 M H₂SO₄ stopping solution. Plates were measured by spectrophotometry.

Statistical analyses

Data are presented as means $\pm$ SEM. We estimated the differences between mean values by using two-tailed pairwise Student *t* tests (GraphPad Prism software). A *p* value <0.05 was considered significant.

Results

Roche-DNaseGII does not affect adaptive immune responses to epitopes within intact protein Ags

Others and we recently reported that coinjected DNase I treatment impairs naive CD4 T cell priming following immunization with alum adjuvant perhaps because the adjuvant activity of alum depends on endogenous DNA danger signals, and these are eliminated by DNase (2, 3). To follow up on these findings, we first analyzed T cell responses in mice to i.m. vaccinations containing alum, with and without DNase treatment and two model Ags selected to stimulate CD4 or CD8 T cells in C57BL/6 mice: chicken OVA chemically conjugated to influenza nucleoprotein peptide NP_{311–325} (QVYSLIRPNENPAHK) (OVA-NP) and intact influenza A NP, respectively. We used the NP peptide rather than a peptide endogenous to OVA as a CD4 T cell stimulator because OVA-specific CD4 T cell responses are inconsistent among individual C57BL/6 mice, whereas C57BL/6 CD4 T cell responses to NP_{311–325} are very consistent and easily detected 5–9 d after immunization with the appropriate peptide/MHC tetramer (5, 6).

Mice were given OVA-NP adsorbed to alum with or without Roche-DNaseGII enzyme that had been isolated from bovine pancreas. To control for the addition of Roche-DNaseGII, which could potentially compete with OVA-NP either as a foreign Ag or for binding to alum, we added BSA instead of DNase to the immunizations administered to control mice. The addition of BSA to

the alum vaccines did not significantly affect Ag-specific responses by comparison with immunization with alum (Supplemental Fig. 1). T cell responses were quantified in immunized mice 7 d after injection. Coinjection of Roche-DNaseGII with alum plus OVA-NP resulted in a 4-fold reduction in numbers of NP_{311–325}-specific CD4 T cells in the draining nodes (Fig. 1A). However, Roche-DNaseGII did not affect SIINFEKL-specific CD8 T cell or IgG1 anti-OVA responses to OVA-NP plus alum (Fig. 1B, 1C).

To extend our studies, we tested the effects of Roche-DNaseGII on vaccines containing OVA-NP and other adjuvants: LPS or anti-CD40 plus polyinosinic-polycytidylic acid. Roche-DNaseGII reduced the CD4 T cell responses to the NP_{311–325} peptide in these vaccines just as well as it affected the alum-containing vaccine (Fig. 1D). This was a surprise because neither adjuvant has been thought to act via host DNA. Thus, our findings suggested that the Roche-DNaseGII preparation might affect immunity in some unsuspected way.

Unexpectedly, when intact NP was used as an alum vaccine Ag, we found that CD4 and CD8 T cell responses were both unaffected by Roche-DNaseGII (Fig. 2A, 2B) even though the CD4 T cell response we measured was, in both cases, against the same NP peptide. In the virus, influenza NP binds viral RNA. To ensure that the NP in our experiments was not bound to RNA that could act as a confounding adjuvant that might be unaffected by DNase, we produced mutNP that cannot bind RNA (7). This alteration did not, however, affect the ability of mutNP to generate T cell or Ab responses (Fig. 2C–F), the latter even with the addition of DNase (Fig. 2G), suggesting that any nucleic acid bound to the NP in our experiments was not acting as an additional adjuvant. These experiments indicated that the T cell response to intact NP adjuvanted by alum was independent of host nucleic acids.

Some commercial DNase I reagents are contaminated with active proteases

We were concerned about the fact that the Roche-DNaseGII inhibited only CD4 T cell responses to OVA-NP. To find out whether other commercial DNase products behaved in the same way as Roche-DNaseGII, we tested two other DNase preparations: Sigma-Aldrich DNase I (Sig-DNase, from bovine pancreas) and Roche DNase I recombinant (Roche-rDNase, from *Pichia pastoris*) (Table I). DNases were given as 2000 Kunitz units/mouse, based on vendor-reported Kunitz units of activity.

The three DNases had different effects on CD4 T cell responses to alum plus OVA-NP (Fig. 3A). Sig-DNase had the same inhibitory effects as did Roche-DNaseGII. Roche-rDNase, alternatively, did not

significantly impair CD4 T cell responses, although there was a trend toward a reduction. This DNase was produced by yeast, however, and is probably glycosylated with high mannose sugars that might have adjuvant properties in mice. Therefore, we could not conclude that Roche-rDNase had no effect on CD4 T cell priming, because it might have possessed confounding adjuvant properties.

The protein content of the DNases was analyzed on SDS-PAGE gels. Many bands appeared in both the Roche-DNaseGII and Sig-DNase lanes. The lane containing Roche-rDNase, alternatively, had only two heterogeneous bands at the estimated molecular mass for glycosylated DNase, that is, 31–34 kDa (8) (Fig. 3B). In agreement with the gel analyses, mass spectrometry showed that the Roche-rDNase mainly contained DNase. In contrast, mass spectrometry of the Roche-DNaseGII and Sig-DNase revealed that, in addition to DNase, the preparations contained trypsin, chymotrypsin, and other contaminants (Fig. 3C).

The DNase samples were tested for protease activity. As predicted from the mass spectrometry results, the Roche-DNaseGII and Sig-DNase samples were active (Fig. 3D) but the Roche-rDNase was not (Fig. 3E). These differences were probably due to the source of the reagents, because both Roche-DNaseGII and Sig-DNase are isolated from bovine pancreas, a rich source of protein digestive enzymes.

We also tested whether the proteases contaminating the Roche-DNaseGII could destroy the NP_{311–325} antigenic epitope we had been using, either conjugated to OVA or present within intact NP protein. OVA-NP or NP Ag was incubated for 0, 1, or 21 h with or without Roche-DNaseGII or control BSA. The Ag mixtures were then added to Ag-presenting Chb cells, which bear IAb, and 3NP311-2 T hybridoma cells specific for IAb/NP_{311–325} for 24 h. Subsequent production of IL-2 indicated the presence of intact peptide ligand. The Roche-DNaseGII preparation rapidly destroyed NP_{311–325} when it was conjugated to OVA (Fig. 3F) but destroyed the NP_{311–325} epitope much more slowly when it was contained in its natural context, intact NP protein (Fig. 3G). Notably, Roche-DNaseGII destroyed the epitope even at the 0 h time point, when it was mixed with OVA-NP and immediately added to cells. Peptides within intact, native proteins might be more resistant to destruction than are peptides that have been conjugated to the carrier or that are exposed and thus available to protease attack, as witnessed by the results in Fig. 3F and 3G.

In conclusion, none of these three commercial DNase reagents can be used as unequivocal measurements of the effects of DNase, either because they contain contaminating proteases or because

FIGURE 1. DNase treatment inhibits CD4 T cell responses to OVA-NP. Mice were immunized with the indicated combinations of Ag with and without adjuvant with and without 1 mg of Roche-DNaseGII or BSA. Ag-specific CD4 (A) and CD8 (B) T cells were quantified on day 7 after immunization with tetramer and Abs as described in *Materials and Methods*. (C) Serum samples were taken 21 d after immunization and tested for the presence of anti-OVA IgG1 Abs by ELISA. RU, relative units. Data in (A) and (B) were combined from four to six independent experiments each with $n = 3$ –4, data in (C) were combined from two independent experiments each with $n = 8$, and data in (D) were combined from two to three independent experiments each with $n = 3$ –4. Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant ($p > 0.05$).

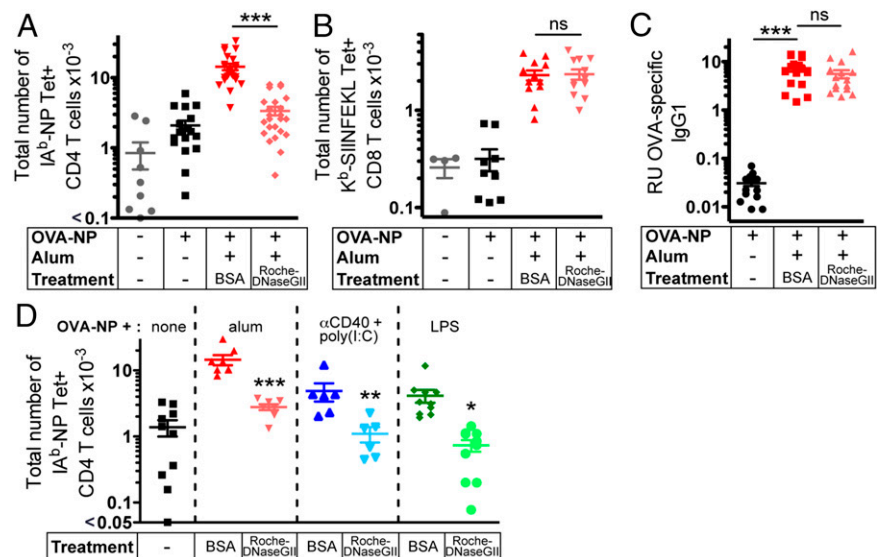
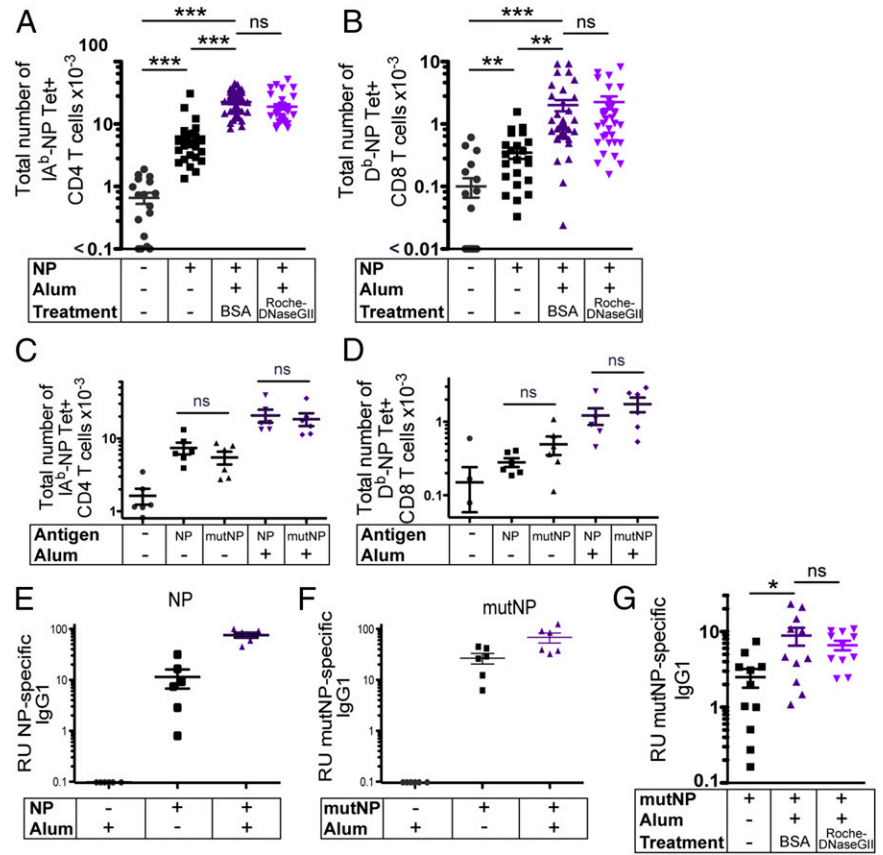


FIGURE 2. DNase treatment does not affect immune responses to NP. Mice were immunized with the indicated combinations of Ag with and without adjuvant with and without 1 mg of Roche-DNaseGII or BSA. Ag-specific CD4 (**A** and **C**) and CD8 (**B** and **D**) T cells were quantified on day 7 (**A** and **B**) or day 9 (**C** and **D**) after immunization with tetramer and Abs as described in *Materials and Methods*. (**C**) Serum samples were taken 14 (**E** and **F**) or 21 d (**G**) after immunization and tested for the presence of anti-mutNP or anti-NP IgG1 Abs by ELISA. RU, relative units. Data in (**A**) and (**B**) were combined from 10 independent experiments each with $n = 3$ –4 or from two independent experiments with $n = 3$ (**C** and **D**) or $n = 5$ –6 (**G**). (**E** and **F**) Data are representative of two independent experiments with $n = 6$. Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant ($p > 0.05$).



they include potentially confounding high mannose glycosylations. Thus, careful examination of such reagents should be done prior to their use in certain experiments.

The proteases contaminating some commercial DNase preparations are major contributors to the inhibitory effects of DNases

We tested, in two ways, the idea that the proteases contaminating the Roche-DNaseGII and Sig-DNase contributed to their inhibitory effects. First, we inhibited proteases within the Roche-DNaseGII preparation by PMSF pretreatment. This treatment reduced the protease activity in the DNase preparation ~ 10 -fold without affecting its DNase activity (Fig. 4A, 4B). In these experiments no DNase preparation affected CD8 T cell responses (Fig. 4D). As in

previous experiments, the untreated Roche-DNaseGII dramatically inhibited CD4 T cell responses to OVA-NP. The PMSF pretreated Roche-DNaseGII also significantly reduced CD4 T cell responses, but much less profoundly than did the untreated enzyme (Fig. 4C). The continued but lessened inhibitory effect of the PMSF-treated DNase could have been due to the fact that PMSF did not completely eliminate the activity of the proteases in the preparation (Fig. 4A). Alternatively, this reduced inhibitory effect could have been caused by the DNase activity itself of the PMSF-treated Roche-DNaseGII. In this regard, the magnitude of inhibition by the PMSF-treated DNase is reminiscent of that observed with Roche rDNase (Fig. 3A).

The plasma of mice contains high levels of proteins, including some that might inhibit the activity of proteolytic enzymes such as

Table I. DNase reagents and protease inhibition

Alias	Product Name	Vendor	Source	Catalog No.	Lots Used
Roche-DNaseGII	DNase I, grade II	Roche	Bovine pancreas	10104159001	10172600, 13814800
Roche-rDNase	DNase I recombinant	Roche	From bovine pancreas, expressed in <i>Pichia pastoris</i> grade I	04 536 282 001	10392800
Sig-DNase	DNase I, from bovine pancreas	Sigma-Aldrich	Bovine pancreas	D4513-1VL	040M7012, 070M7008V
Worth-DNase	DNase I, RNase and protease free	Worthington Biochemical	Bovine pancreas	LS006334	52N13773
wtDNase	—	—	Wild-type DNase I from bovine pancreas plus 6 $\times$ His tag, expressed in 293F cells	—	—
mutDNase	—	—	Mutant DNase I (R111A, D212A, H252A) from bovine pancreas plus 6 $\times$ His tag, expressed in 293F cells	—	—

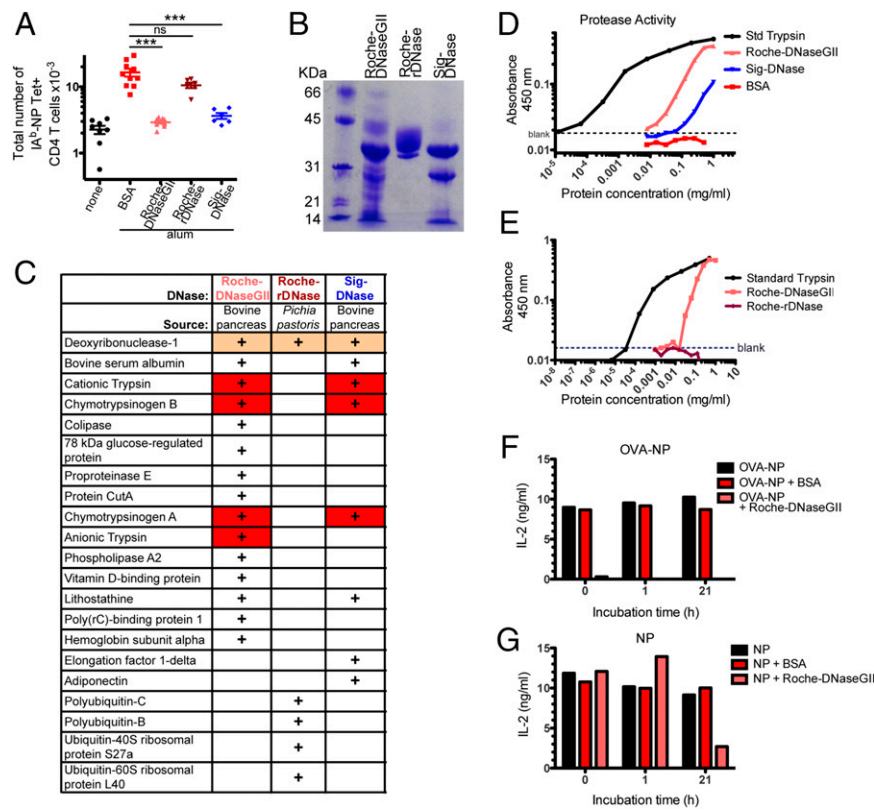


FIGURE 3. Commercial DNases are contaminated with active proteases. **(A)** Mice were immunized with OVA-NP with and without alum with and without 2000 Kunitz units DNase I reagent from indicated sources. Ag-specific CD4 T cells were quantified on day 7 as in Fig. 1. Data were combined from two to three independent experiments each with $n = 3$ –4. Error bars show means $\pm$ SEM. Statistical differences are with respect to the positive control group (treated with OVA-NP plus alum plus BSA) and were determined using an unpaired t test. *** $p \leq 0.001$. ns, not significant ($p > 0.05$). **(B)** SDS-PAGE was performed on the indicated DNase preparations. Gels were stained with Coomassie brilliant blue dye and the final image was cropped for clarity. **(C)** DNase products were analyzed by mass spectrometry, as described in *Materials and Methods*. Spectra were compared with Swiss-Prot databases to identify protein contents within samples (tan, DNase I; red, protease). Database matches (+) were listed when a sample contained at least three distinct peptides from the protein. **(D and E)** DNase samples were assessed for protease activity at indicated concentrations using the Pierce protease assay kit. The dotted line indicates the lowest level of detection. Per milligram of protein, Roche-DNaseGII and Sig-DNase contained protease activities equal to ~ 10 μ g and 1 μ g of trypsin, respectively. **(F and G)** OVA-NP or NP were treated with or without BSA or Roche-DNaseGII for 0, 1, or 21 h at 37°C and added in duplicate to Chb cells plus 3NP311-2 T cell hybridomas. Supernatant was assessed for IL-2 by ELISA 24 h after adding Ag. Data are representative of (C) one or (B and D) two independent experiments or (F and G) grouped from two experiments. (E) This assay was only performed once with one sample per reagent due to limited supply of lot 10392800 of Roche-rDNase.

trypsin. Therefore, we tested whether trypsin or chymotrypsin, in the amounts known to be present in Roche-DNaseGII, would affect CD4 T cell responses, first in vitro and then in vivo in the absence of DNase itself. We established that each milligram of Roche-DNaseGII was contaminated with proteases that have cumulative activities equivalent to either 10 μ g of trypsin or chymotrypsin (Fig. 3D and data not shown). In vitro Ag-destruction studies confirmed that trypsin treatment was less efficient than chymotrypsin at destroying the NP_{311–325} epitope within OVA-NP (Fig. 4E). Chymotrypsin could destroy the NP_{311–325} epitope (QVYSLIRPNENPAHK) by cleaving after Y313, a putative anchor residue for binding the peptide to IA^b, and/or after L315. Trypsin treatment would completely sever NP_{311–325} peptides from OVA molecules by cleaving on the C-terminal side of the K325. Trypsin might destroy the NP_{311–325} epitope by cleaving after R317, but this cleavage might be less efficient because the relevant bond may be protected by the following proline residue (9, 10). Such a result is confirmed by the fact that, in vitro, chymotrypsin inhibited CD4 T cell responses to NP_{311–325} much more rapidly than did trypsin (Fig. 4E).

To find out whether contaminating proteases could reduce CD4 T cell responses in vivo, OVA-NP plus alum vaccines were given without or with 10 μ g of trypsin or chymotrypsin, the amount of protease that would be present in the Roche-DNaseGII given in previous experiments. We found that both of these proteases could

independently reduce NP_{311–325}-specific CD4 T cell responses (Fig. 4F). Notably, chymotrypsin was the more potent treatment. None of the preparations affected CD8 T cell responses (Fig. 4G). These experiments suggested that the profound inhibitory effects of Roche-DNaseGII were mostly due to its contaminating proteases. The lack of effect of the proteases on CD8 T cell responses is probably due to the fact that proteases act inefficiently on natively folded proteins, including OVA, and therefore the CD8 T cell epitopes may be functionally protected from destruction (11, 12).

DNase, independently of its enzyme activity, can inhibit CD4 T cell responses to Ag plus alum

Noticing that the *Pichia*-produced Roche-rDNase (marginally) and the PMSF-treated Roche-DNaseGII (significantly) reduced responses to OVA-NP plus alum (Figs. 3A, 4C), we tested whether other protease-free preparations of DNase might have similar activities. Therefore, we obtained or prepared three such preparations. The first to be tested was the commercially available mammalian-produced Worthington Biochemical protease- and RNase-free DNase I (Worh-DNase, from bovine pancreas). This preparation is satisfactorily pure as established by SDS-PAGE (Supplemental Fig. 2A), mass spectrometry (Supplemental Fig.

2B), and protease activity (Fig. 5A), while still having DNase activity comparable to that of Roche-DNaseGII (Fig. 5B). We compared the effects of equivalent doses (DNase activity) of Worth-DNase and Roche-DNaseGII on the vaccine adjuvant activity of alum in T cell responses to OVA-NP. The protease-contaminated Roche-DNaseGII reduced the CD4 T cell response 5-fold. The Worth-DNase reduced the response less profoundly, but nevertheless the reduction it induced, by comparison with the response to OVA-NP plus alum only, was significant (Fig. 5C). Furthermore, Roche-DNaseGII impaired Ag-specific T follicular helper cell differentiation, whereas Worth-DNase did so marginally, but not significantly (Supplemental Fig. 3). Neither treatment impaired CD8 T cell priming, as expected (Fig. 5D).

To check the effect of pure DNase on alum responses in another way, we produced enzymatically active (wild-type DNase [wtDNase]) and inactive (mutDNase) recombinant bovine DNase I proteins using a mammalian expression system. mutDNase differed from wtDNase by three substitutions: R111A, D212A, and H252A (13). We confirmed that the wtDNase enzymatic activity per milligram protein was equivalent to that of Roche-DNaseGII and that the mutDNase had no DNase enzymatic activity at 100 $\mu\text{g/ml}$ (Fig. 5E) and 1 mg/ml (unpublished data). As before, we assessed the purity of these homemade DNases by SDS-PAGE (Supplemental Fig. 2A), mass spectrometry (Supplemental Fig. 2B), and protease activity (Fig. 5F). The mammalian-expressed recombinant wtDNase and mutDNase both had higher molecular masses than did bovine pancreas-sourced

DNases, probably because mammalian cell lines can create different protein glycosylation patterns (14).

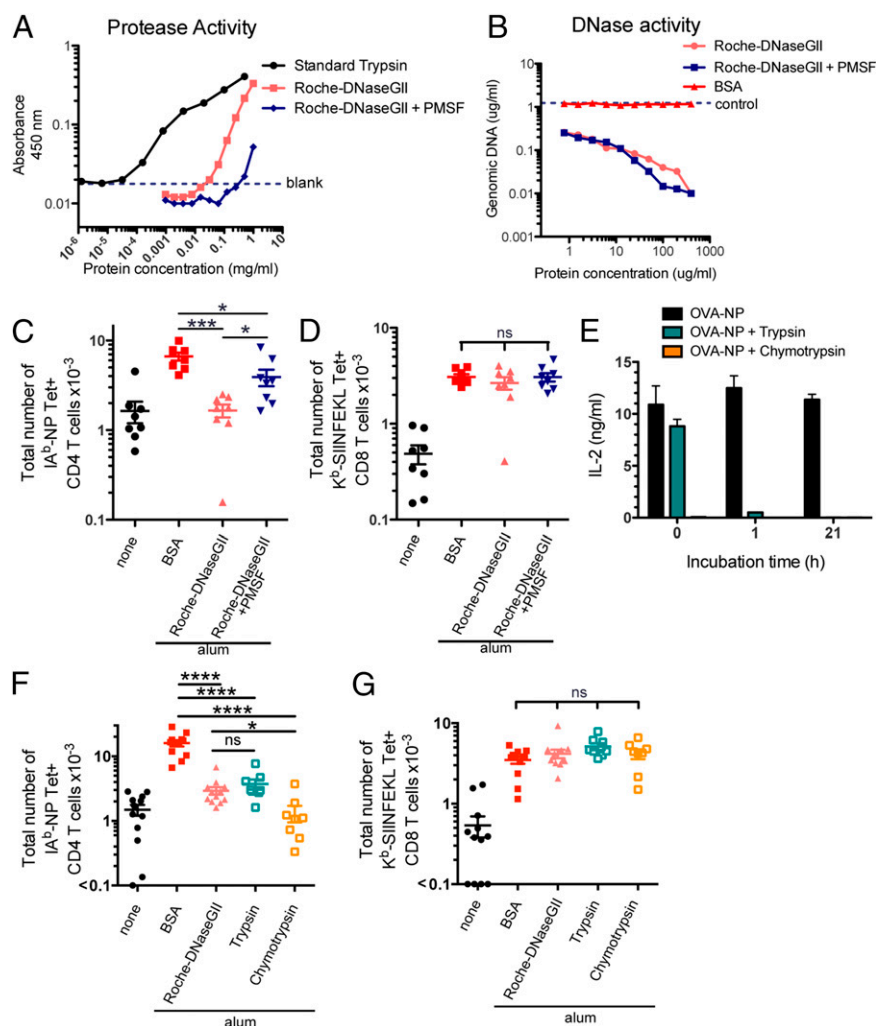
Coinjection of wtDNase or mutDNase with alum vaccines yielded the same numbers of Ag-specific CD4 or CD8 T cells (Fig. 5G, 5H). Curiously, both the control mutDNase and the active wtDNase treatments reduced CD4 T cell responses significantly, by ~ 2 -fold, when compared with those induced in the absence of added protein or with added BSA. This dampening effect was consistent between wtDNase, mutDNase, and Worth-DNase (Fig. 5C) and is similar in magnitude to that seen with the PMSF-treated Roche-DNaseGII and, to a lesser extent, the Roche-rDNase (Figs. 3A, 4C).

This consistent reduction caused by DNase, in the absence of proteases and even in the absence of enzymic activity of DNase, suggested that DNase might be affecting responses adjuvanted by alum in an unexpected way, as discussed below.

STING is dispensable in alum vaccine responses

In another approach to measure the effects of host DNA on the adjuvant activity of alum, we evaluated the role of STING, a cytoplasmic DNA-sensing molecule, on alum vaccination. Previous experiments had suggested that defects in the STING signaling pathway reduced the ability of alum to prime T cell responses to OVA or the 3K peptide (2, 3), perhaps because alum allows host DNA to transport into cell cytoplasm where STING would be activated (15–18). We repeated these experiments with STING-deficient mice and OVA-NP plus alum. We found that CD4 and

FIGURE 4. Proteases impair CD4 T cell responses to alum plus OVA-NP. (A and B) Roche-DNaseGII was treated with PMSF and assessed for (A) protease activity at indicated concentrations using a Pierce protease assay kit and (B) DNase enzymatic activity as described in *Materials and Methods*. Dotted lines indicate (A) lowest level of detection or (B) highest level of DNase activity. Data are representative of two independent experiments each with one sample per reagent. (C, D, F, and G) Mice were immunized with OVA-NP with and without alum with and without 1 mg of BSA or indicated treatment: 1 mg of Roche-DNaseGII with and without PMSF or 10 μg of trypsin or chymotrypsin. Ag-specific CD4 (C and F) and CD8 (D and G) T cells were quantified on day 7 as described in Fig. 1. Data in (C), (D), (F), and (G) were combined from two to three independent experiments depending on the group, each with $n = 4$. (E) OVA-NP was treated with trypsin or chymotrypsin for 0, 1, or 21 h at 37°C and added in duplicate to Chb cells plus 3NP311-2 T cell hybridomas. Supernatant was assessed for IL-2 by ELISA 24 h after adding Ag. Data are representative of two independent experiments. Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$. ns, not significant ($p > 0.05$).



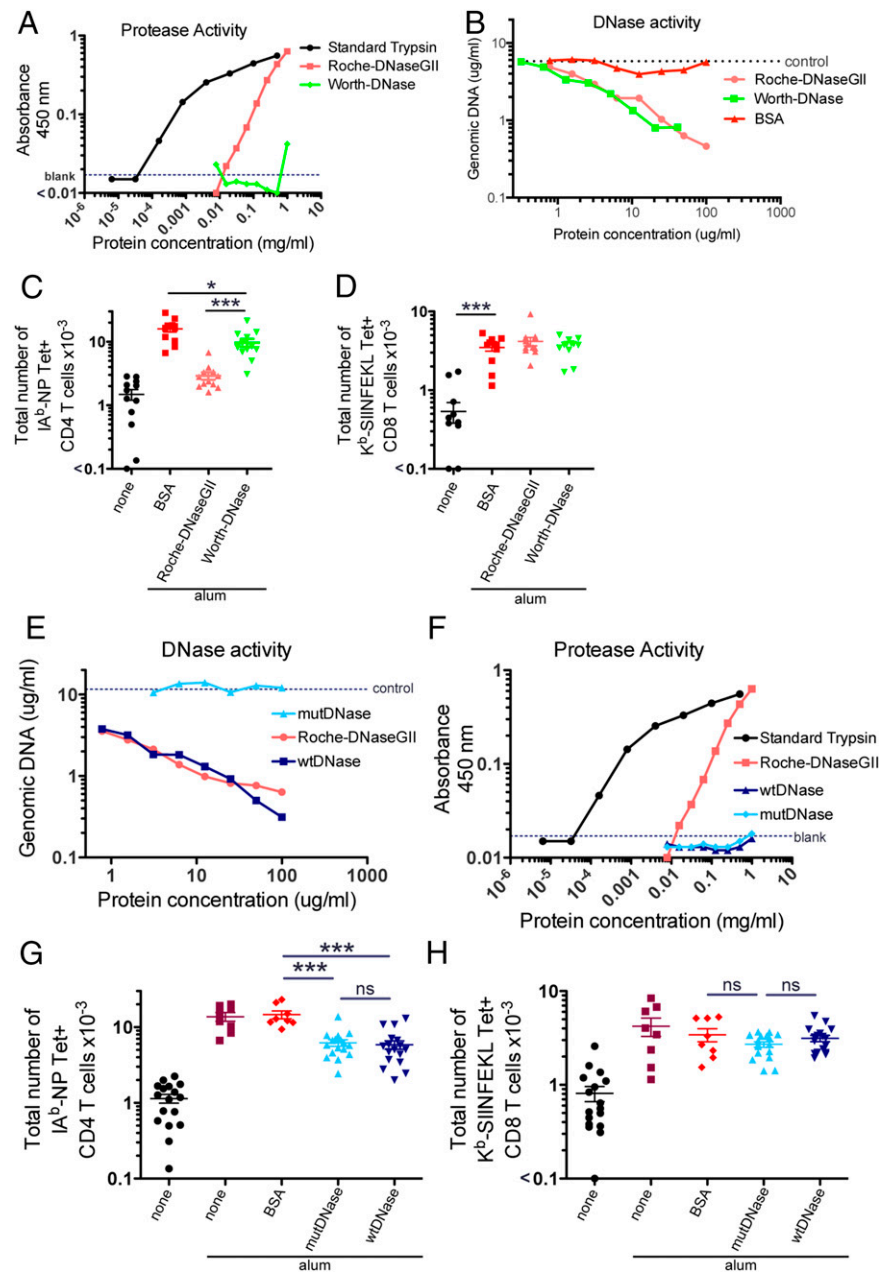


FIGURE 5. DNase activity does not impair T cell responses to alum plus Ag. DNase samples were assessed for protease activity (**A** and **F**) at indicated concentrations using a Pierce protease assay kit and their DNase enzymatic activity (**B** and **E**) as described in *Materials and Methods*. Dotted line indicates lowest level of detection. Data are representative of two independent experiments each with one sample per reagent. (**C**, **D**, **G**, and **H**) Mice were immunized with OVA-NP with and without alum with and without 1 mg of the indicated treatment. Ag-specific CD4 (**C** and **G**) and CD8 (**D** and **H**) T cells were quantified on day 7 as described in Fig. 1. Data for no treatment, BSA, and Roche-DNaseGII groups in (**C**) and (**D**) were already reported in Fig. 4F and 4G. Data in (**C**), (**D**), (**G**), and (**H**) were combined from two to four independent experiments each with $n = 4$ – 5 . Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. * $p \leq 0.05$, *** $p \leq 0.001$. ns, not significant ($p > 0.05$).

CD8 T cells in STING-deficient mice responded normally to this vaccine in both quantity of Ag-specific T cell activation (Fig. 6A, 6B) and T follicular helper cell differentiation (Fig. 6C), although Ag-specific CD4 T cells were slightly but not statistically significantly reduced in number. One confounding factor that might account for the difference between the results presented in this study and previously (3) is the fact that, after administration of Ag plus alum, the lymph nodes from STING-deficient mice contained fewer cells ($p < 0.05$) than did those from wild-type mice, averaging, for the two popliteals, $1.51 \pm 0.16 \times 10^7$ for the STING-deficient lymph nodes, versus $1.96 \pm 0.12 \times 10^7$ for the wild-types. Ag-specific IgG1 responses were, however, significantly reduced in STING-deficient mice immunized with OVA-NP, although, contrary to the results from related previously published experiments, IgE Ab responses were normal (Fig. 6D, 6E). These findings suggest that the STING signaling pathway is dispensable for adaptive immune responses to certain Ags, such as OVA-NP, in the context of alum.

CD4 T cell responses to OVA-3K are affected by DNase

The experiments described so far suggest that much of the inhibitory effect of commercially produced DNases is due to contaminating proteases, a conclusion that confounds to some extent previous ideas of others and ourselves. However, in our case, previous experiments had studied the responses of C57BL/6 CD4 T cells to the 3K peptide, conjugated, similar to the NP peptide used above, to OVA (OVA-3K). To find out whether the results reported above, involving OVA-NP, applied also to OVA-3K, we immunized mice with OVA-3K without or with alum and with the various preparations of DNase. As before, alum-induced 3K-specific CD4 T cell responses were greatly reduced when coinjected with Roche-DNaseGII (Fig. 7A) and the 3K epitope was destroyed by Roche-DNaseGII when coincubated overnight (Fig. 7B). However, the CD4 T cell response to 3K in vaccines containing OVA-3K plus alum was also significantly reduced by coinjection of Worth-DNase, or either the mutDNase or wtDNase that we prepared

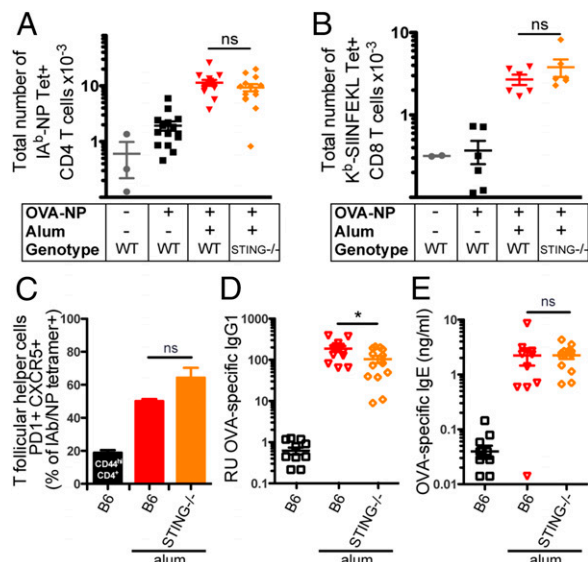


FIGURE 6. STING is dispensable in adaptive immune responses to alum. Wild-type or STING-deficient mice were immunized with OVA-NP with and without alum. Ag-specific CD4 (A) and CD8 (B) T cells were quantified on day 7 as described in Fig. 1 and T follicular helper cell phenotype was assessed in Ag-specific CD4 T cells (C). (D and E) Serum samples were taken 21 d after immunization and tested for the presence of anti-OVA IgG1 or IgE Abs by ELISA. RU, relative units. Data were combined from five (A) or two (B–E) independent experiments each with $n = 3$ –6. Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. * $p \leq 0.05$. ns, not significant ($p > 0.05$).

ourselves. The effect of STING deficiency on CD4 T cell responses to 3K bound to OVA was again not significant, although in this case there was a trend toward a reduction in overall numbers of T cells generated (Fig. 7C). As expected, STING deficiency did not affect CD8 T cell responses to OVA-3K (Fig. 7D). These results were very similar to those that occurred when OVA-NP was the Ag, indicating that the data reported in the present study are representative of responses to both of these Ags.

Taken together, our data suggest that the protease contaminants in impure Roche-DNaseGII and Sig-DNase affect CD4 T cell priming by alum because they destroy or remove the CD4 epitope of interest. Nevertheless, the data show that DNase given with alum vaccines does slightly inhibit CD4 T cell responses, even when proteases are absent, suggesting that there might be some small effect of DNase protein itself on the potency of alum-adjuncted vaccines. This effect does not depend on the ability of the DNase to digest DNA.

Discussion

Recently, a number of publications reported studies on the modes of action of alum in its widely used role as a vaccine adjuvant. Others and we have shown that alum activates inflammasomes and induces the production of what appear to be chromatin-rich neutrophil extracellular traps that envelop the alum particles (1–3, 19). Various experiments have suggested that IL-1 β produced by inflammasome activation and/or host chromatin and DNA contribute to the adjuvant effects of alum (17, 19–21). However, some experiments have shown that inflammasome activation is not always needed for alum to be effective in vaccines (20, 22, 23). In this study, we show that this same conclusion may apply to some extent to the contribution of host DNA to alum activity.

The immunostimulatory properties of host DNA have been appreciated for some time now. In 1999, Suzuki et al. (24) showed that exposure to calf thymus DNA, for example, increased levels

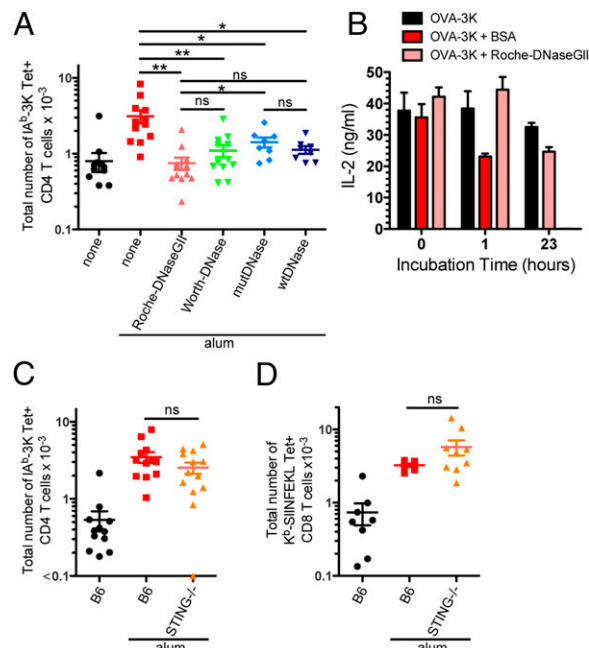


FIGURE 7. DNase treatment reduces CD4 T cell responses to OVA-3K plus alum although STING is dispensable. Wild-type (A, C, and D) or STING-deficient (C and D) mice were immunized with OVA-3K with and without alum (A, C, and D) with and without 1 mg of BSA or indicated DNase treatment (A). Ag-specific CD4 (A and C) and CD8 (D) T cells were quantified on day 7 after immunization with tetramer and Abs as described in *Materials and Methods*. Data were combined from two to three independent experiments, each with $n = 4$. (B) OVA-3K was treated with or without BSA or Roche-DNaseGII for 0, 1, or 23 h at 37°C and added in duplicate to Chb cells with B3K0508 T cell hybridomas. Supernatant was assessed for IL-2 by ELISA 24 h after adding Ag. Error bars show means $\pm$ SEM for each group. Statistical differences were determined using an unpaired Student t test. ** $p \leq 0.01$. ns, not significant ($p > 0.05$).

of MHC class I and MHC II on cells and that this induction was inhibited by DNase. Later Ishii et al. (25) showed that this phenomenon, manifested by increased production of mRNA for IFNs and other inflammatory proteins, was independent of TLR or RIG-I but partially dependent on Tbk1 and IKKi. Effects on different mRNAs varied depending on the recognition pathway involved. These results were bolstered by observations in mice lacking various forms of DNase. Mice deficient in DNase I or DNase II develop a lupus-like disease (26, 27), and animals or humans lacking TREX1 (DNase III) acquire a number of inflammatory diseases (28, 29). Additionally, others have shown that naked, isolated, host DNA can, if coinjected with Ag, act as an adjuvant on its own (2, 30, 31).

As far as the interaction of DNA with alum adjuvants, host chromatin is rapidly deposited on alum particles upon injection into animals, leading to alum particle aggregation (1). Given this observation, it was perhaps not surprising when two groups reported that the adjuvant effects of alum were dramatically inhibited by DNase. These results suggested that the host DNA on the alum particles was an important contributor to the adjuvanticity of alum (2, 3). In the present study, we show that at least our experiments were to some extent misinterpreted because much of the inhibitory effects of the DNase preparations turn out to be due to contaminating proteases that probably act by destroying the Ag. Even so, host DNA probably plays a role in the effects of alum. Marichal et al. (2) showed that injection of host DNA itself had some adjuvant effects. Moreover, both Marichal et al. (2) and McKee et al.

(3) showed that the adjuvant effects of alum were reduced in mice lacking STING (3) or its downstream, signaling partners Tbk1 and Irf3 (2). In our hands, the reduction caused by lack of STING was much less than that caused by DNase. Therefore, alum still had some stimulatory effects in STING-deficient animals (3), a result that was also apparent in the Tbk1^{-/-} or Irf3^{-/-} animals. Taken together, these experiments indicated that DNA-recognizing pathways involving STING, Irf3, or Tbk1 contributed to the adjuvant effects of alum, but were not the only important components. Perhaps this is why our current experiments showed only slight and usually not statistically significant effects in STING^{-/-} mice. The animals in this study were raised in a different location than those of our previous experiments, so due to reasons beyond our current understanding, such as possible genetic drift, other alum-induced pathways may more effectively replace the STING pathway in these subjects.

Nevertheless, it appears that coinjection of DNase has some direct effect on alum's stimulation of CD4 T cell responses: DNase (lacking proteases) consistently reduced CD4 T cell responses to NP or 3K peptides covalently linked to OVA. DNases, whether they were contaminated with proteases, did not reduce alum-adjuvanted CD8 T cell responses to intact OVA or CD4 responses to intact NP. The fact that natively folded proteins are relatively resistant to proteases has long been known (11, 12). Therefore, the native proteins likely progress to the late endosomal compartment before they can be cleaved and their immunostimulatory peptides released. The relatively unstructured NP and 3K peptides that are chemically coupled to the surface of OVA are much more easily digested, as shown in the present study, and may therefore be made available to bind to MHC II proteins in early endosomes. It is possible that the inhibitory effects of DNase apply only to the adjuvant effects of alum in early endosomes and that other DNase-resistant alum-induced effects apply to the late endosomal compartment.

Finally, there is the surprising result that DNase that lacked the ability to digest DNA had the same moderately inhibitory effect on CD4 T cell responses to the NP and 3K peptides as the wild-type enzyme. However, CD8 T cell and Ab responses to intact proteins were not affected by DNase. Two possible explanations for this phenomenon come to mind. The contact area between DNase I and DNA is very large (32). Our mutation of the three amino acids at the active site of DNase probably does not inhibit binding of the enzyme to its substrate. Therefore, the mutated DNase may, similar to its wild-type counterpart, partially inhibit alum's adjuvant activity by sequestering DNA and associated chromatin proteins, including the high mobility group box 1 proteins (33) and other damage-associated molecular patterns that can act as adjuvants (reviewed in Ref. 34). Alternatively, DNase may be partially inhibitory because of its long known, but still mysterious, very high affinity for G-actin (35). Actin itself has immunostimulatory effects (36–38) and thus perhaps engagement by DNase inhibits these properties of actin.

In summary, the adjuvant activities of alum remain confusing, because the material has been reported to act in so many ways. Briefly, alum is known to induce many inflammatory pathways including the creation of an Ag depot within the body (39–41), activation of APCs (reviewed in Ref. 42), inflammasome activation (17, 19–22), and release of endogenous danger signals such as uric acid and host DNA (1, 2, 43). Many of these pathways have been implicated in or dismissed as contributors to the adjuvant activities of alum, depending on the vaccine scenario. It is likely that the biochemical properties of Ags, Ag dose, immunization schedule, type of APC exposed to alum, and differences in precursor T cell frequencies for given epitopes all

affect which types of immunostimulatory pathways triggered by alum vaccines ultimately contribute to the immune response (44–48). Thus, the many reported mediators of the effects of alum may simply reflect the many pathways by which these insoluble salts may act.

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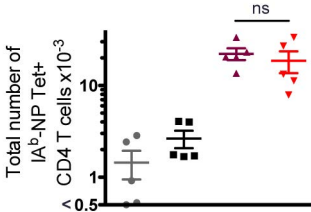
Disclosures

The authors have no financial conflicts of interest.

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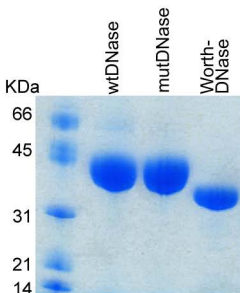


OVA-NP	-	+	+	+
Alum	-	-	+	+
Treatment	-	-	-	BSA

Supplemental Fig. 1. Added BSA has no effect on the magnitude of NP311-325-specific CD4 T cell responses to alum immunization.

Mice were immunized with OVA-NP {plus minus} alum {plus minus} 1 mg BSA or Roche-DNaseGII. Antigen-specific CD4 T cells were quantified on d7 after immunization with tetramer and antibodies as described in Materials and Methods. Data is representative of 2 independent experiments each with n = 3 or 5. Error bars show means {plus minus} SEM for each group. Statistical differences were determined using an unpaired Student's t test; ns indicates $p > 0.05$.

A



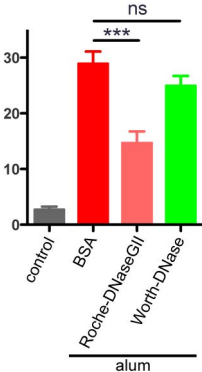
B

DNase:	Worth-DNase	wtDNase
Source:	Bovine pancreas	293F cells (human)
Deoxyribonuclease-1	+	+
Bovine serum albumin	+	
Actin, cytoplasmic 2		+
Actin, cytoplasmic 1		+
Beta-actin-like protein 2		+
POTE ankyrin domain family member E		+
POTE ankyrin domain family member F		+
POTE ankyrin domain family member I		+
Putative beta-actin-like protein 3		+
POTE ankyrin domain family member J		+
Actin, alpha skeletal muscle		+
Actin, alpha cardiac muscle 1		+
Actin, aortic smooth muscle		+
Actin, gamma-enteric smooth muscle		+
Gelsolin		+
Adenylyl cyclase-associated protein 1		+
Tenascin		+
Adenylyl cyclase-associated protein 2		+
Granulins		+
Profilin-2		+
Selenoprotein P		+
Splicing factor, proline- and glutamine-rich		+
Dipeptidyl peptidase 9		+

Supplemental Figure 2. DNase purchased from Worthington or prepared in house are not contaminated with proteases

Preparations of DNase purchased from Worthington Corp or prepared in house from transfected 293F cells were subjected to mass spectrometric analysis. Shown are the proteins, fragments of which were found in the analyses.

T follicular helper cells
PD1+ CXCR5+
(% of tet+ CD4 T cells)



Supplemental Figure 3. DNase that is not contaminated with proteases does not reduce the numbers of T follicular helper cells generated by immunisation with alum.

Mice were immunized with NP-OVA without adjuvant (control) or adjuvanted with alum with the indicated additional proteins. Seven days later, popliteal lymph nodes were harvested and assayed for the presence of T follicular helper cells. Data are combined from two independent experiments with 3 mice per group. Statistical differences were determined using unpaired Student's t test; * $p < 0.05$.