Supporting Information

Tolerogenic nanoparticles impacting B and T lymphocyte responses delay autoimmune arthritis in K/BxN mice

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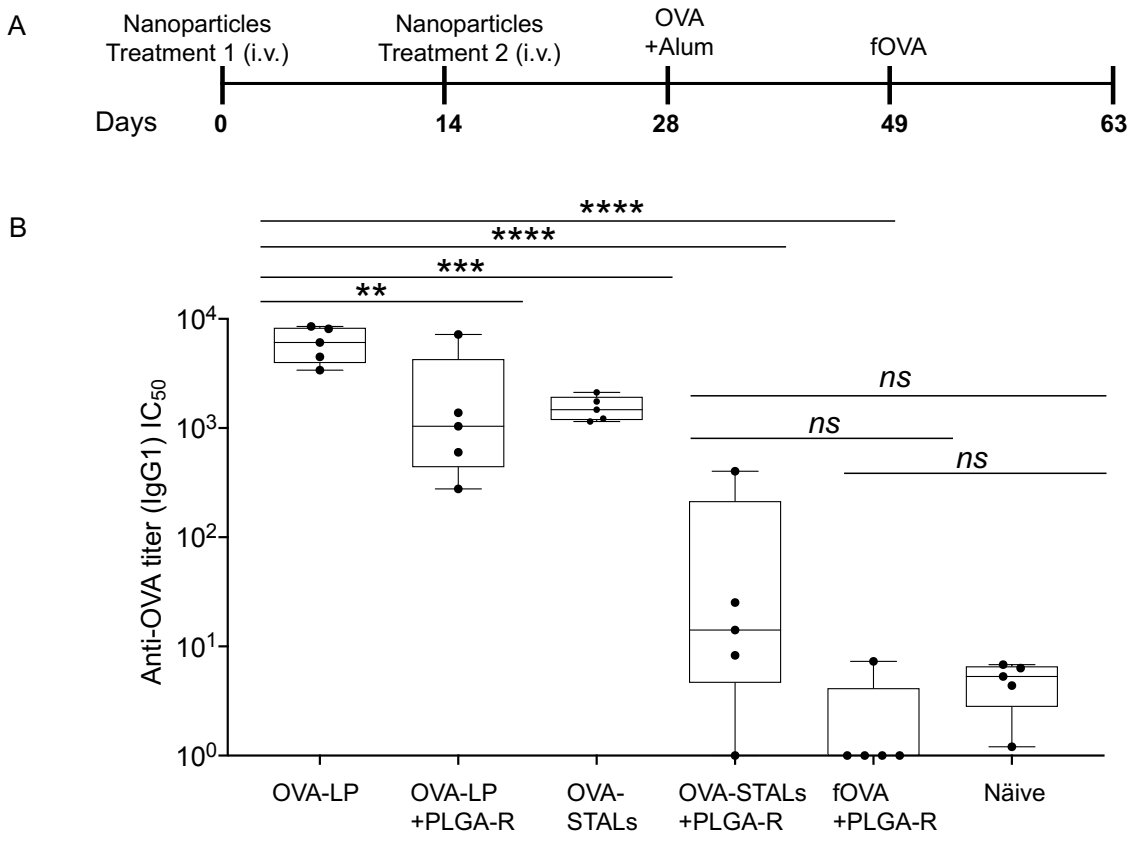

Figure S1. Anti-OVA titers following two treatments with PLGA-R with OVA-LP, OVA-STALs or free OVA. Shown are results summarizing the experiment described in Figure 2B with the addition of a group treated with free OVA (fOVA) and PLGA-R, comparing final anti-OVA titers at day 63. (**A**) C57BL/6J mice ($n = 5$) were immunized on days 0 and 14 with the two doses of indicated nanoparticle treatments i.v. and then challenged i.p. with OVA/Alum on day 28 and fOVA on day 49 and results are representative of two independent experiments. PLGA-R nanoparticles contained 100 μg of rapamycin. Mice were bled weekly after the treatment, and IgG1 titers are shown in figure 2B for complete time course. (**B**) Final IgG1 titers at the end of study (day 63) are shown here in comparison with fOVA + PLGA-R. Data were normalized by dividing the titers by naïve mean and are shown here. All data represent the mean ± SEM. All statistical analyses were performed on raw data using one-way ANOVA with Tukey's post-test (**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$ and *ns* indicates not significant).

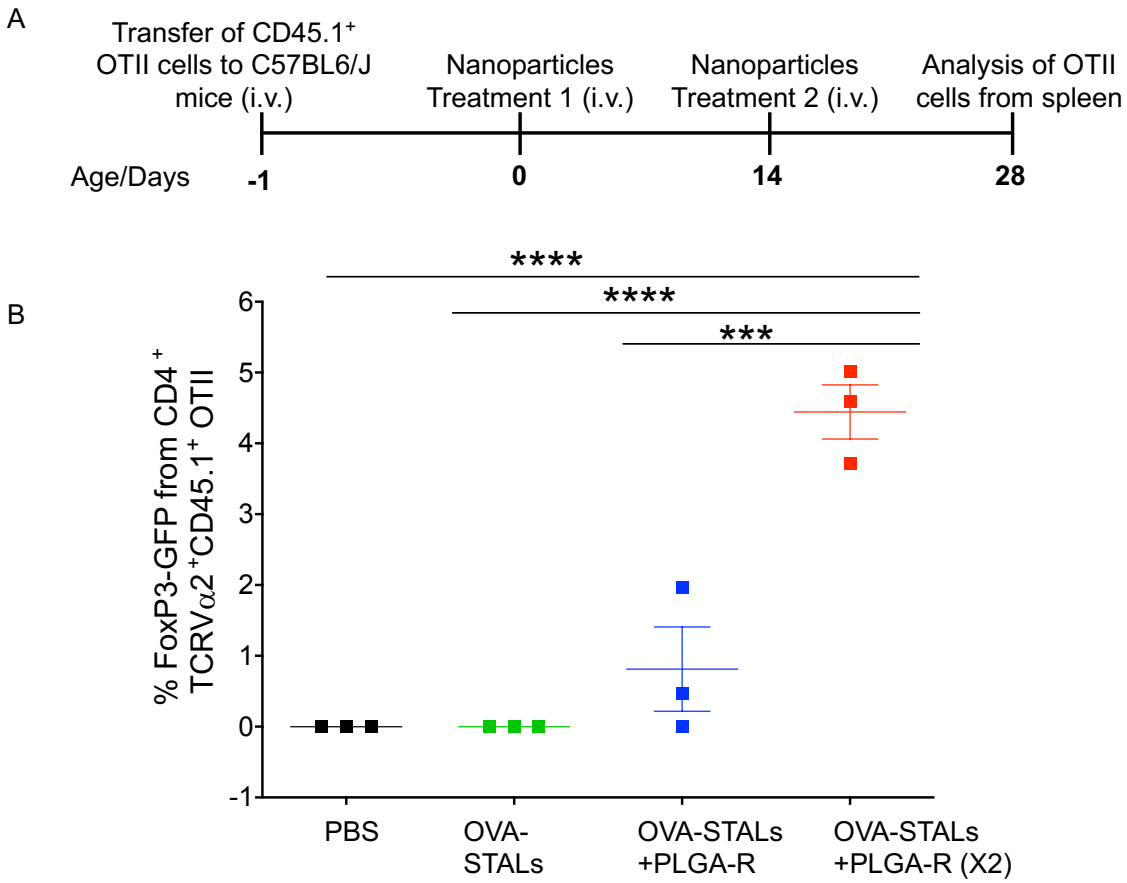

Figure S2. Co-delivery of OVA-STALs (OVA-LP-CD22L) and PLGA-R nanoparticles induces regulatory T cells (Tregs). (**A**) Sorted $CD4^+FoxP3GFP^-CD44^-CD62L^+$ OTII T cells (0.2 x 10^6) were transferred into $CD45.2^+$ C57BL/6J mice on day –1. At day 0, mice (n = 3) received i.v. immunizations with PBS, OVA-STALs alone and OVA-STALs + PLGA-R. Few mice (n = 3) received a second dose of OVA-STALs + PLGA-R on day 14. PLGA-R nanoparticles containing 100 µg of rapamycin. At day 28, spleens were harvested and analyzed by flow cytometry. (**B**) Total percentage of $Foxp3^+CD25^+$ OTII T cells ($PI^-CD19^-CD4^+TCRV\alpha2^+CD45.1^+FoxP3GFP^+CD25^+$) in spleen. All data represent the mean ± SEM. All statistical analyses were performed using one-way ANOVA with Tukey's post-test (**** $p \leq 0.0001$; *** $p \leq 0.001$).

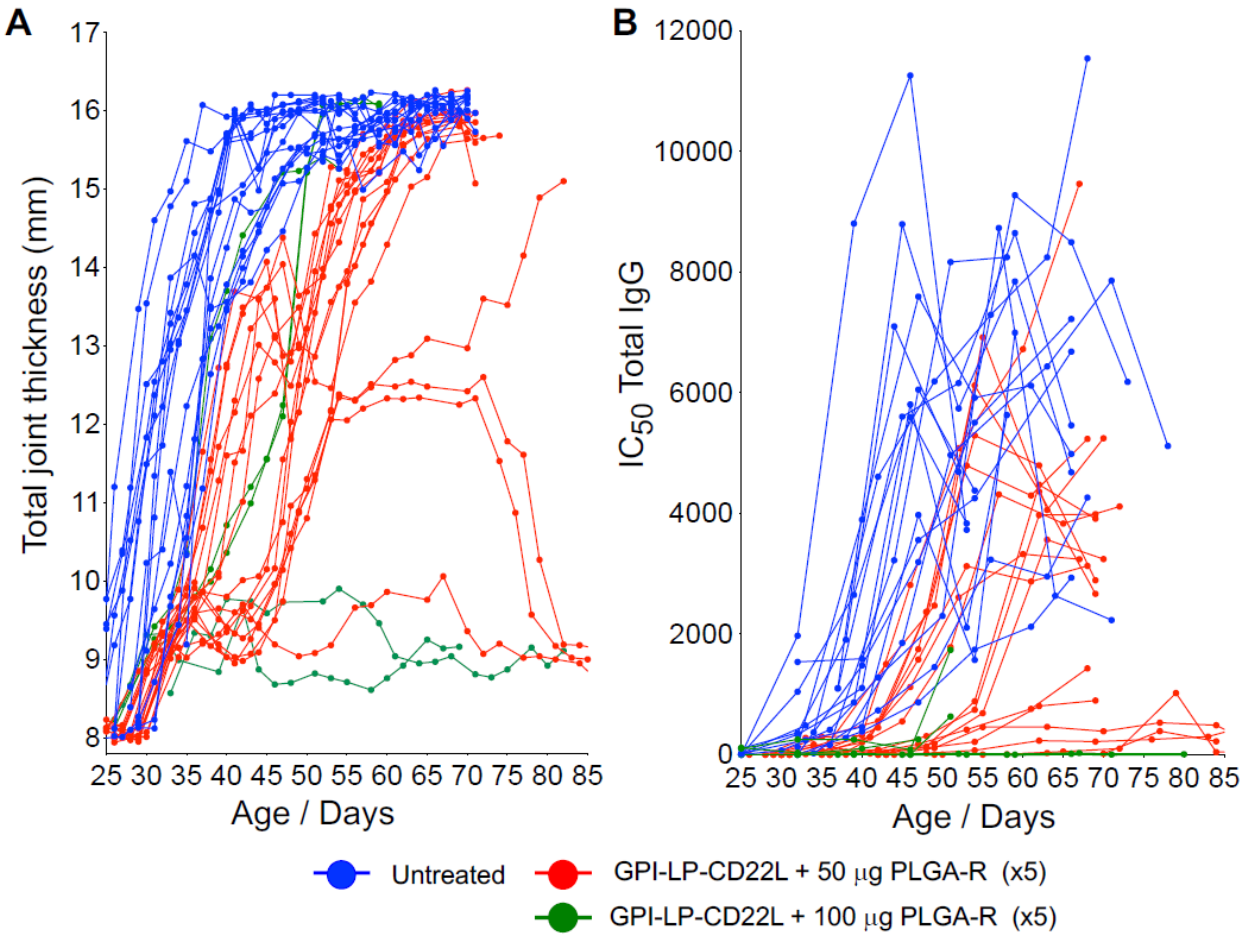

Figure S3. Comparison of disease severity and anti-GPI titers. K/BxN mice were treated after disease onset, measured as > 3 mm in one or more joints. Mice were either left untreated (blue) or treated with 5 weekly injections of GPI-STALs (GPI-LP-CD22L) co-administered with PLGA-R nanoparticles containing either 50 μg (red) or 100 μg (green) of rapamycin. (**A**) Progression of disease severity measured by total joint thickness. (**B**) Anti-GPI antibody titers from untreated and GPI-STALs + PLGA-R treated K/BxN mice.

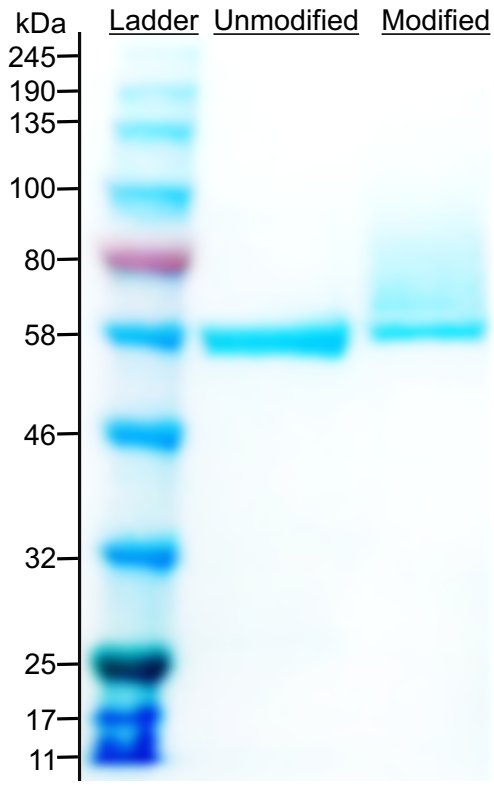

Figure S4. SDS-PAGE analysis of GPI protein linked to pegylated lipid. Gel is loaded as follows; lane 1: protein ladder, lane 2: unmodified protein, lane 3: modified protein. Modified protein had between 1-3 lipids attached per protein.

MATERIALS AND METHODS

Sugar-lipid conjugation. Reaction of murine CD22 ligand (9-biphenylacetyl-*N*-glycolylneuraminic acid-α-2-6-galactose-β-1-4-*N*-acetylglucosamine-β-ethylamine (6'-^{BPA}NeuGc)) with NHS-PEG2000-DSPE (NOF America) produced the sugar-lipid conjugate (Figure 1A) as reported previously.[1] 1.5 Mol % of mCD22 ligand conjugated lipid was used for the Ag-LP-CD22L formulation.

Protein-lipid conjugation. Ovalbumin (OVA; Worthington Biochemical LS003062) or purified recombinant mouse glucose-6-phosphate isomerase (GPI) expressed in *E. coli* (pGEX-4T-3-gpi expression vector kindly gifted by Diane Mathis and Christophe Benoist (Harvard Medical School)) was conjugated to PEGylated distearoylphosphoethanolamine (PEG2000-DSPE) using maleimide chemistry.[2] Proteins were first reacted to approximately 2.5 equivalents of sulfosuccinimidyl 6-[3'-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP, Pierce), a heterobifunctional crosslinker, to introduce a thiol group. The reaction mixture was shaken at RT for 1 h, purified on Sephadex G-50, and treated with 1,4-dithiothreitol (25 mM) at RT for 10 min. Extent of protein modification was calculated by quantification of released thiol 2-pyridyl by absorbance at 343 nm. After reaction completion, the thiol-derivatized protein obtained was purified on Sephadex G-50 column and reacted with maleimide-PEG2000-DSPE (10 eq, NOF America) under nitrogen overnight at RT. Lipid-modified protein was purified by passage over a Sephadex G-100 column and stored at 4 °C. Under these reaction conditions, proteins were modified with 1–3 lipids as determined by increased MW by SDS-PAGE (Supplementary figure S4). The final concentration of protein-lipid conjugate was calculated based on the absorbance at 280 nm.

Liposome formulation. Liposomes were prepared by the thin film hydration method as reported previously[3] using distearoyl phosphocholine (DSPC) (Avanti Polar Lipids), cholesterol (Sigma-Aldrich), and PEGylated lipids in a molar ratio of 57:38:5. The total molar fraction of PEGylated lipids, consisting of PEG2000-DSPE (Avanti Polar Lipids), ^{BPA}NeuGc-PEG2000-DSPE, and protein-PEG2000-DSPE, was kept constant at 5%. DSPC, cholesterol and PEG2000-DSPE were dissolved in chloroform. After mixing appropriate amounts of lipids, the chloroform was removed under a stream of nitrogen gas. To the dried lipid thin film, 1.5 mol % of ^{BPA}NeuGc-PEG2000-DSPE (DMSO stock) was added, and this mixture was lyophilized overnight. The dried lipids were then hydrated with 0.1 mol % of protein(OVA/GPI)-PEG2000-DSPE[4] in PBS to yield a lipid concentration of 5 mM. This

hydrated mixture was sonicated for at least 5×30 sec with 10 min intervals. Liposomes were passed a minimum of 20 times through 800-nm, 200-nm, and 100-nm polycarbonate membranes (Avanti Polar Lipids) using a mini-extrusion device (Avanti Polar Lipids). The liposomes were purified over a CL–4B column and detected using a Nanodrop 2000 (Thermo Scientific) at 280 nm. The prepared liposomes were stored as a 2.5 mM stock based on molar content of total lipids, in PBS at 4 °C. Hydrodynamic diameter of the liposomes was found to be 156±32 nm as determined by dynamic light scattering (DynaPro NanoStar). Concentrations of liposomes are based on lipid content, and the amounts of CD22L and protein antigen as mol % based on total lipids.

PLGA nanoparticle formulation. PLGA nanoparticles were prepared using the oil-in-water single emulsion method as described previously with slight modification.[5] PLGA, a 75:25 D,L-lactide/glycolide ratio and mPEG-PLGA with 50:50 D,L-lactide/glycolide ratio were dissolved in 1mL of dichloromethane in 3:1 ratio. To this solution, 13% w/w rapamycin was added. PLGA drug solution was added dropwise to an aqueous PVA solution while vortexing, followed by sonication under ice. The single emulsion so obtained was added to 30mL of phosphate buffer, stirred for 2.5 h at RT to evaporate dichloromethane. The subsequent NP suspension was washed twice by centrifuging at 27000 rpm at 4 ºC and then resuspended in 1X DPBS. The hydrodynamic diameter was found to be around 182 ±31 nm based on dynamic light scattering. PLGA NPs injections contain 50 or 100 μg of rapamycin content.

Drug loading and encapsulation efficiency. To measure drug loading of rapamycin, PLGA-R stock (20 μL) was mixed with water (130 μL). An equal amount of acetonitrile was added to this solution to make 50/50 acetonitrile/water medium and then the mixture was sonicated for 30 min and vortexed time to time. Prior to HPLC analysis, the mixture was filtered using a 0.45 μm PVDF syringe filter. The resulting free drug was assayed using an Agilent 1100 HPLC series with a quaternary pump equipped with an Alltech C18, 5 μm, 4.6 × 150 mm column. Rapamycin absorbance was measured by a UV-vis detector at 278 nm and a retention time of 13 min in 1 mL/min using 50/50 acetonitrile/water mobile phase. Peak area of each sample was calculated using ChemStation software (Agilent). Standard solutions of known rapamycin concentrations from 30–210 μg/mL were used to make a calibration curve. Rapamycin drug loading was 10–11% by weight. Rapamycin encapsulation efficiency (EE) was found to be 83% as calculated using the following equation.

$$EE\ (\%) = \frac{Amount\ of\ drug\ encapsulated\ in\ PLGA - R\ NP}{Amount\ of\ initial\ drug\ input} \times 100$$

Animals. Wild-type C57BL/6J mice were obtained from the rodent breeding colony of The Scripps Research Institute (TSRI). All experimental procedures involving mice in this work were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (La Jolla, CA).

Immunization and blood collection. Liposomal and PLGA-R nanoparticles were delivered via the lateral tail vein in a volume of 200 μL comprising 150 μM of liposome based on total lipid concentration containing 0.1 mol % of protein (OVA/GPI) and 1.5 mol% of CD22 ligand and 50 or 100 μg of rapamycin containing PLGA-R.[6, 7] For OVA challenges, mice were injected intraperitoneally with 100 μg of OVA (Worthington Biochemical LS003062) plus Alum (Thermo Scientific, 77161) or 100 μg of free OVA. Serum for ELISA was obtained by centrifuging (15,000 *g*, 1 min) whole blood (50 μL, retro-orbital bleed).

ELISAs. The levels of Anti-OVA and anti-GPI IgG titers were measured by ELISA. Assay microplates were coated with protein (50 μL/well, 100 μg/ml in PBS) and incubated overnight at 4 °C. Plates were washed with TBS-T (4 times, 0.1 % Tween 20) and blocked with 1% BSA at RT for 1 h. Serial dilutions of serum sample were added and plates were incubated (50 μL/well) at RT for 1 h. Plates were then washed 4 times and incubated with anti-IgG1 mouse-horseradish peroxidase (HRP) conjugate (1:2000 dilution; Santa Cruz Biotechnology Inc.) at RT for 1 h. After 1 h, plates were washed and developed in 75 μL/well HRP at RT for 15 min and quenched with 75 μL/well 2N H_2SO4. Absorbance at 450 nm was measured using a Synergy H1 microplate reader (BioTek). Anti-IgG1 titers were calculated with Prism (GraphPad Software) by applying a standard four-parameter IC_{50} function.

Statistics. Statistical significance was determined using repeated measures one- or two-way analysis of variance (ANOVA) with Tukey post-test for multiple comparisons, mixed-effect analysis followed by Sidak test, Mann-Whitney test, or the log-rank Mantel-Cox test using the Graphpad Prism 8.0. $P < 0.05$ was considered significant.

K/BxN arthritis model. K/BxN mice on a C57BL/6J background, which express both the T cell receptor (TCR) transgene KRN and the MHC class II molecule A^{g7}, spontaneously produce autoantibodies recognizing glucose-6-phosphate isomerase (GPI) and develop inflammatory arthritis. For prophylactic treatment, K/BxN mice were injected i.v. with 200 μL of nanoparticles every 6–7 days

for 1, 2 or 5 weeks, starting at 22–25 days of age. Nanoparticles injected include PLGA-R containing 100 μg of rapamycin mixed with 1) liposomes decorated with 0.1 mol % recombinant glucose-6-phosphate isomerase (GPI) protein and 1.5 mol % BPA CD22 ligand (GPI-LP-CD22L), 2) liposomes decorated with 0.1 mol % GPI (GPI-LP), or 3) 25 μg of free GPI (fGPI). For therapeutic treatment, K/BxN mice were left untreated or injected i.v. with 200 μL of GPI-LP-CD22L and PLGA-R containing 50 or 100 μg of rapamycin every 6–7 days, starting when the thickness of at least one paw measured 2.8–3.05 mm. Disease progression was monitored by paw thickness (front and hind) measurements collected 3 times per week using digital Vernier calipers and approximation of GPI-specific IgG titers by weekly serum ELISAs. K/BxN mice were produced through breeding of KRN and A^{g7} mice provided by the late Dr. Kerri Mowen (The Scripps Research Institute) with permission from Dr. Diane Mathis and Dr. Christophe Benoist (Harvard Medical School).

Histology. Mouse ankles were fixed for 24 hours in buffered zinc formalin (Anatech Ltd), decalcified for 9 days in Shandon TBD-2, dehydrated by passage through increasing concentrations of EtOH, transferred to xylene, and paraffin-embedded. Tissue sections (3 μm) were mounted on glass slides, deparaffinized using Pro-Par Clearant (Anatech Ltd), and rehydrated in successive baths of absolute EtOH, decreasing concentrations of EtOH, and finally distilled water. Slides were stained in filtered hematoxylin (Ricca Chemical Company 353532) for 4 min and then washed in distilled water, acid alcohol (0.5% HCl in 70% EtOH), distilled water, Scott's Tap Water (0.2% w/v sodium bicarbonate, 2% w/v magnesium sulfate), and finally distilled water. Slides were then immersed in 0.2% Fast Green FCF (MP Biomedicals 211922) for 6 min, washed in 1% glacial acetic acid, and stained with 0.003% Safranine O (Acros Organics 146640250) for 4 min. After staining, tissues were subsequently dehydrated as described above and cover slipped using Refrax mounting medium (Anatech Ltd). Slides were scanned with a Leica SCN400 slide scanner, and images were captured using SlidePath's Digital Image Hub. Specimens were analyzed primarily for abnormalities in cellularity, Safranine O stain distribution, and surface fibrillation.

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