Investigation of the protective properties of glycosylphosphatidylinositol based vaccine candidates in a *Toxoplasma gondii* mouse challenge model

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Carbohydrate Microarray Analysis of Mouse Serum

Each serum sample was diluted with PBS (pH 7.4) containing 0.1% v/v Tween20 and 3% w/v BSA, thoroughly vortexed for 15 s and incubated at 37 °C for 15 min to dissolve potential lipid aggregates. Insoluble residual sample components were removed by centrifugation for 30 s in a table-top centrifuge at 13,000 rpm. Samples were transferred to the microarray, which was previously blocked with BSA (2.5%, w/v) in PBS for 1 h at room temperature, and gently rocked in a sealed humidified incubator for 2 h at 37 °C. Unbound sample components were removed with a series of washes with 0.1 (1 time) and 0.001% v/v Tween 20 (2 times) in PBS. Afterwards microarrays were incubated for 1 h at r.t. with secondary fluorescently labeled antibodies (ALEXA-Fluor®635 goat anti-mouse IgG antibody (Invitrogen, dilution 1:400); ALEXA-Fluor®594 goat anti-mouse IgG1 antibody (Invitrogen, dilution 1:200); ALEXA-Fluor®635 goat anti-mouse IgG2a antibody (Invitrogen, dilution 1:200); ALEXA-Fluor®488 goat anti-mouse IgG3 antibody (Invitrogen, dilution 1:200) diluted in PBS containing 0.1% v/v Tween20 and 3% w/v BSA. The slides were washed as described above, dipped into double-distilled water for 3 s and subsequently dried by centrifugation. The slides were analyzed using a fluorescence microarray scanner (Genepix® 4300A, Molecular Devices) using a PMT gain that saturated the highest signals to a minimum amount to ensure reproducibility. All analyzed spots were manually adjusted and the "mean fluorescence values - background" value was used for analysis.

Cultivation and Isolation of T. gondii Tachyzoites

HFF cells were grown confluent in DMEM (4.5 g/mL glucose, 3.7 g/mL sodium bicarbonate) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/mL Penicillin and 0.1 mg/mL Streptomycin at 37 °C and 5% CO_2 (all cell culture material was purchased from Pan Biotech). On the day of infection with *T. gondii* (RH strain) the medium of HFF cells was exchanged for fresh medium. Afterwards the bottom of a fully infected cell culture flask was gently scrapped with a cell scrapper and the resulting cell suspension was centrifuged (5 min, 300 rcf). The supernatant was removed and the resulting pellet was suspended in 5 mL cell culture medium. 500 µL of this suspension was added to a fresh confluent flask of HFF cells of the same size. After approximately 72 h all host cells are lysed and the infection cycle can start again. To isolate tachyzoites for challenge experiments two 300 cm² cell culture flasks (TPP) were infected. The bottom of the fully infected cell culture flasks was gently scrapped with a cell scrapper and the resulting cell suspension was centrifuged (5 min, 300 rcf). The supernatant was removed and the resulting pellet was suspended in 10 mL DMEM (without supplements). The suspension was pushed consecutively through needles of different diameters (20, 23, 25, 27 G) using a Luer-Lock

system to destroy remaining HFF cells. The resulting suspension was applied to a glass wool column (12 mL syringe filled with ~ 1.5 g of loose packed glass wool) that was previously equilibrated with ~ 15 mL DMEM. Afterwards the column was eluted with ~ 20 mL PBS and the eluent was centrifuged (5 min, 300 rcf). The supernatant was removed and the pellet was washed three times with PBS (10 mL each).

Immunofluorescence Microscopy

Freshly isolated tachyzoites were fixed for 20 min at room temperature in 4 % paraformaldehyde in PBS (pH 7.4). The cells were washed three times with PBS and blocked for 1 h at room temperature in PBS containing 2% BSA. A 96 polystyrene round bottom microwell plate (Costar) was simultaneously blocked for 1 h using PBS containing 2% BSA. Afterwards tachyzoites were washed with PBS and ~ 10^6 tachyzoites were added per well of the blocked 96 well plate. Tachyzoites were incubated for 2 h at room temperature with the following samples diluted in 2% BSA in PBS:

1. negative controls = pooled pre-bleed serum of groups **A** and **B** (dilution 1:25)
2. samples = pooled serum of groups **A** and **B** (collected at day 35, 5 weeks after prime immunization, dilution 1:25)
3. positive control = NCL-TG antibody (Leica Biosystems, dilution 1:200), which is directed against the major cell surface protein P30 of *T. gondii*.

The plates were centrifuged (5 min, 300 rcf) and washed three times with PBS. The cells were stained with 2% BSA in PBS containing a FITC-labelled anti-mouse IgG (Sigma Aldrich, dilution 1:100) and DAPI (Invitrogen, dilution 1:5000 of a 143 µM aliquot solution) for 45 min. The plates were centrifuged (5 min, 300 rcf) and washed three times with PBS and the samples were transferred to an untreated flat bottom 96 well plate (TPP) to get a better quality for the microscopy images. Images were taken on an Observer Z1 (Zeiss) fluorescence microscope using a LD Plan-Neofluar 40x/0.6 Corr Ph2 M27 (Zeiss) objective and HXP 120 Kübler Codix (Zeiss) illumination laser. Pictures were edited using the AxioVision40 software v. 4.6.3.0 (Zeiss) and are shown in Fig. S14-S16.

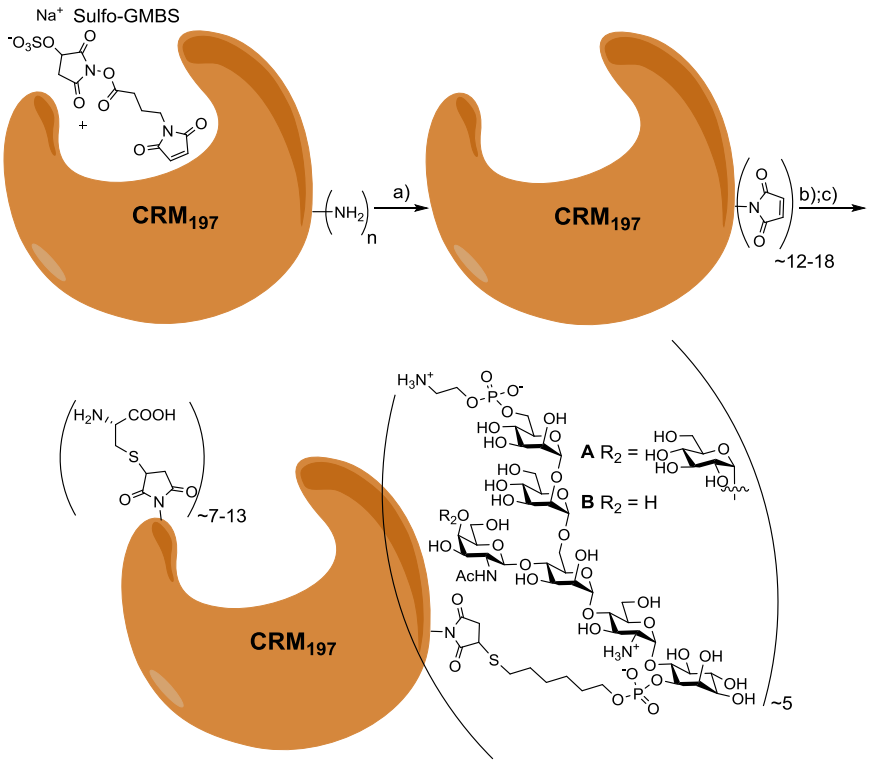


Fig. S1. Reaction scheme for the preparation of GPI glycoconjugates **A** and **B** using maleimide chemistry. Reagents and conditions: a) H_2O, $NaHCO_3$; b) **1** or **2**, PBS @ pH7.4; c) H_2O, L-cysteine, 4 °C.

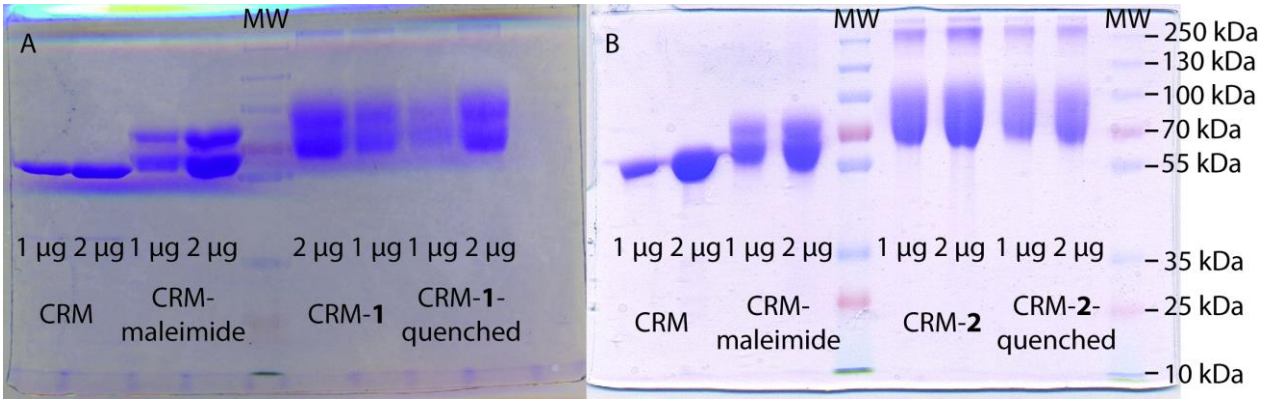


Fig. S2. SDS-PAGE analysis of CRM_{197} and the corresponding conjugates for A) **A** and B) **B**. The bands were visualized using Coomassie staining and the molecular weight marker lane is labeled with MW.

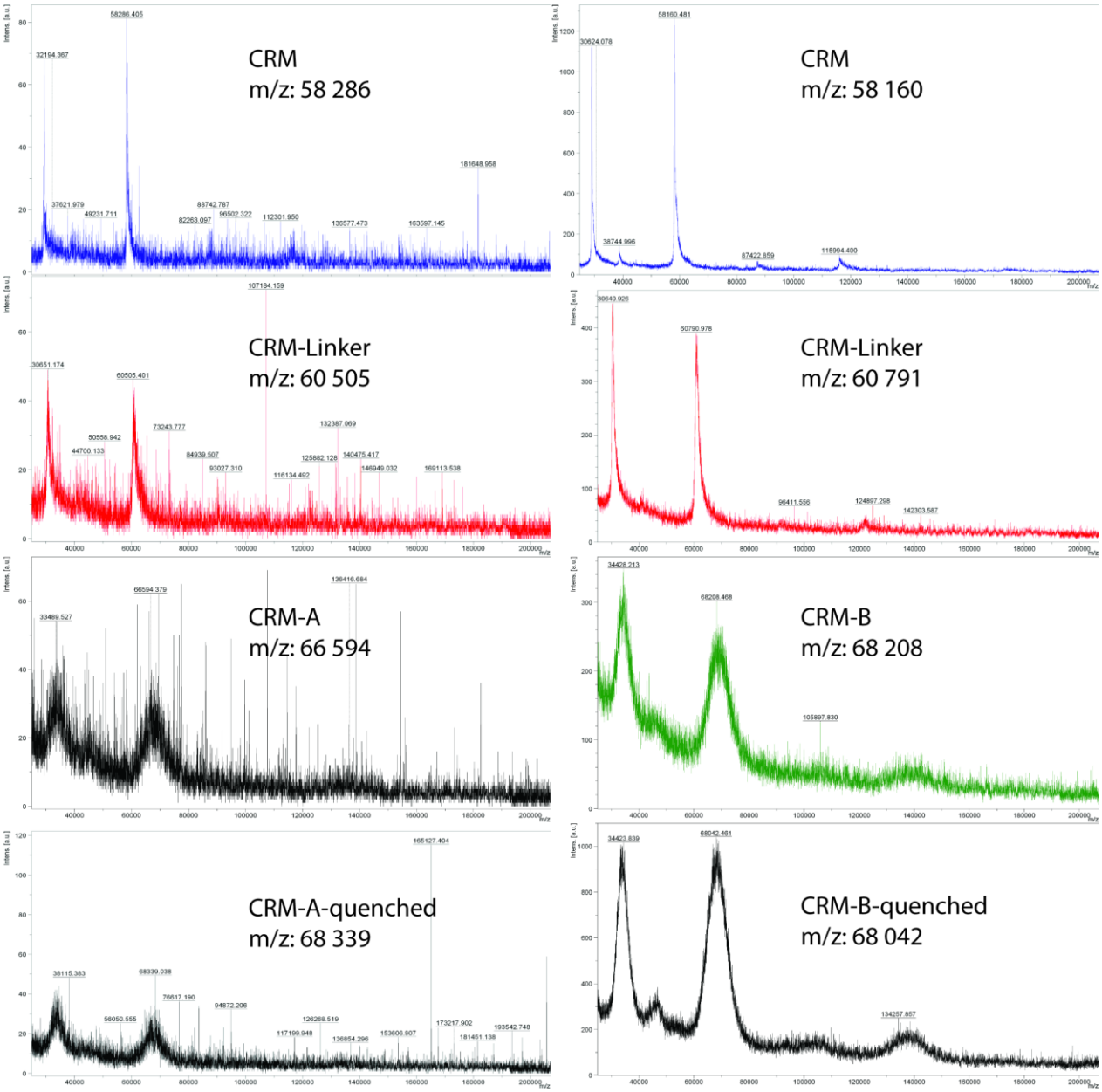


Fig. S3. MALDI-TOF analysis of the different stages during glycoconjugate preparation (**A** and **B**).

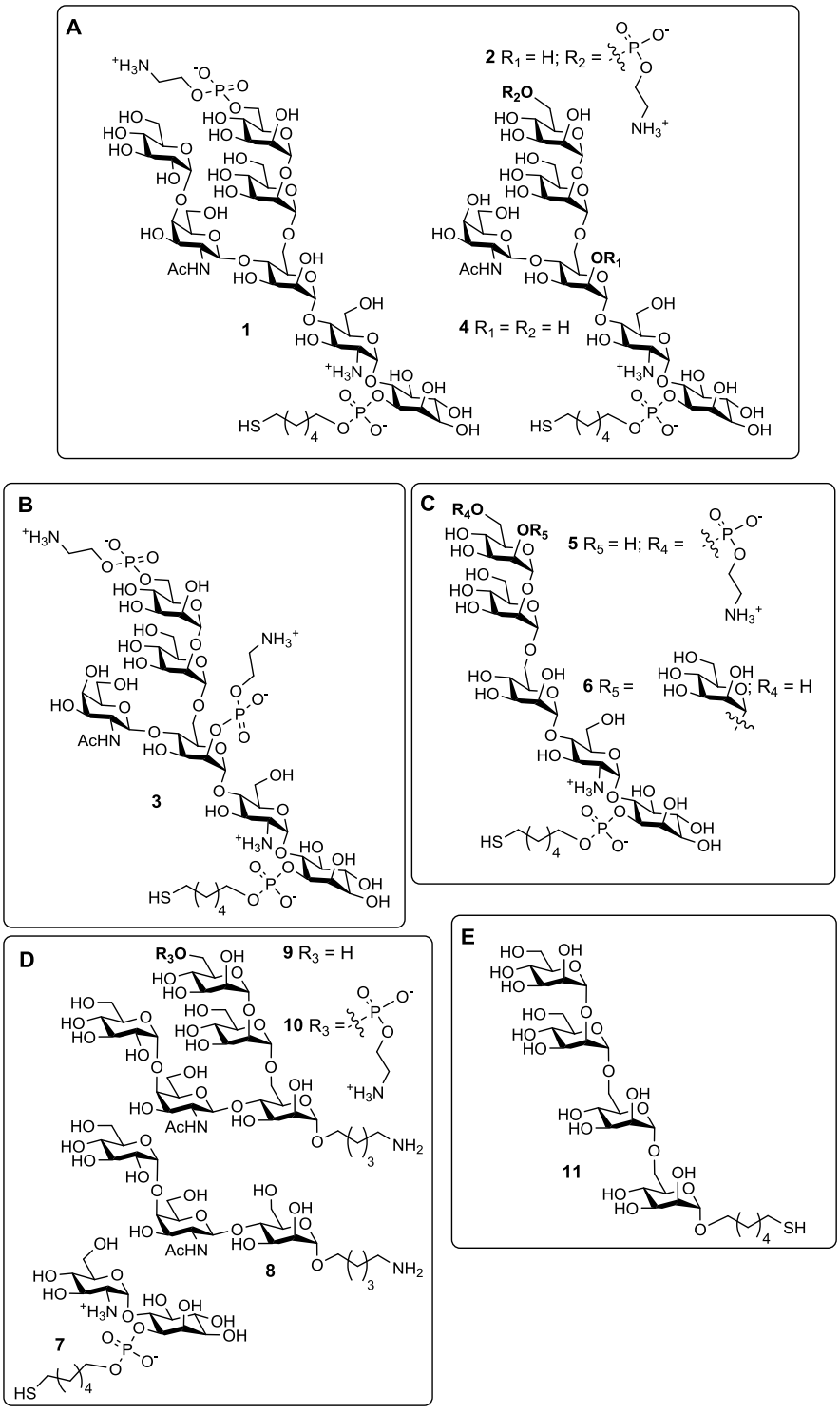


Fig. S4. Chemical structures of all compounds used for the fabrication of the synthetic carbohydrate microarray. (**A**) GPIs found specifically in *T. gondii*. (**B**) A mammalian GPI structure. (**C**) GPIs of *C. parvum* (**5**) and *P. falciparum* (**6**). (**D**) Substructures of GPI **1**. (**E**) *M. tuberculosis* related mannan-oligosaccharide.

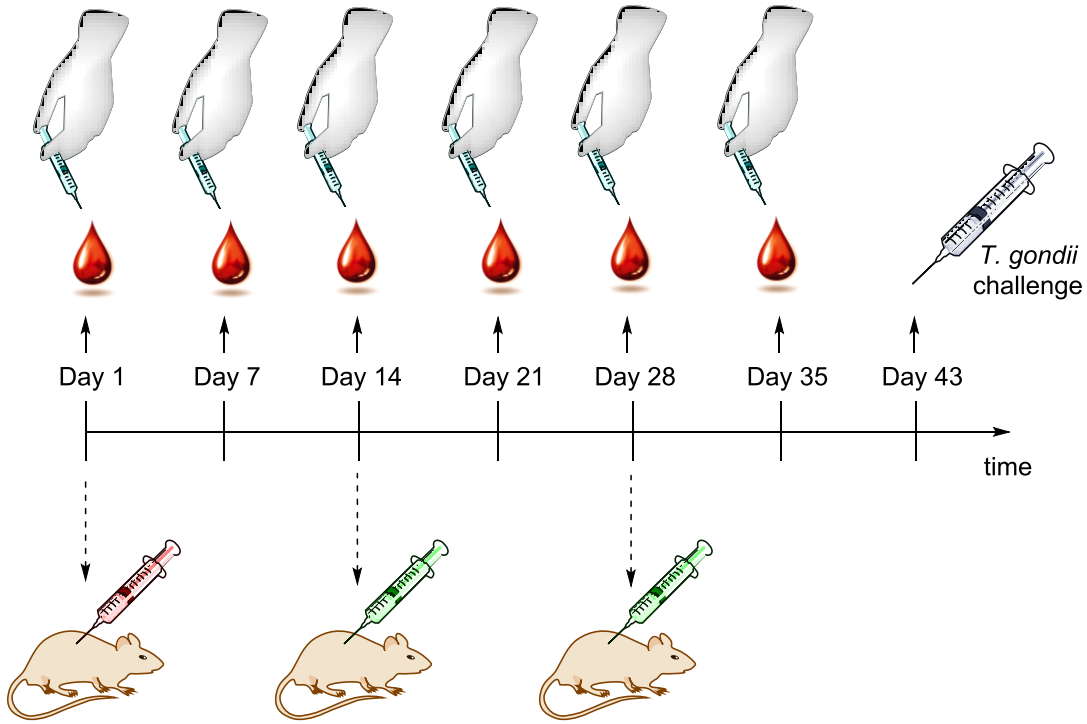


Fig. S5. Time schedule for the prime-boost immunization studies of glycoconjugates **A** and **B**. Employed adjuvants are reflected by the color of the syringe (red = complete Freund's Adjuvant; green = incomplete Freund's Adjuvant). Blood was drawn at the days indicated.

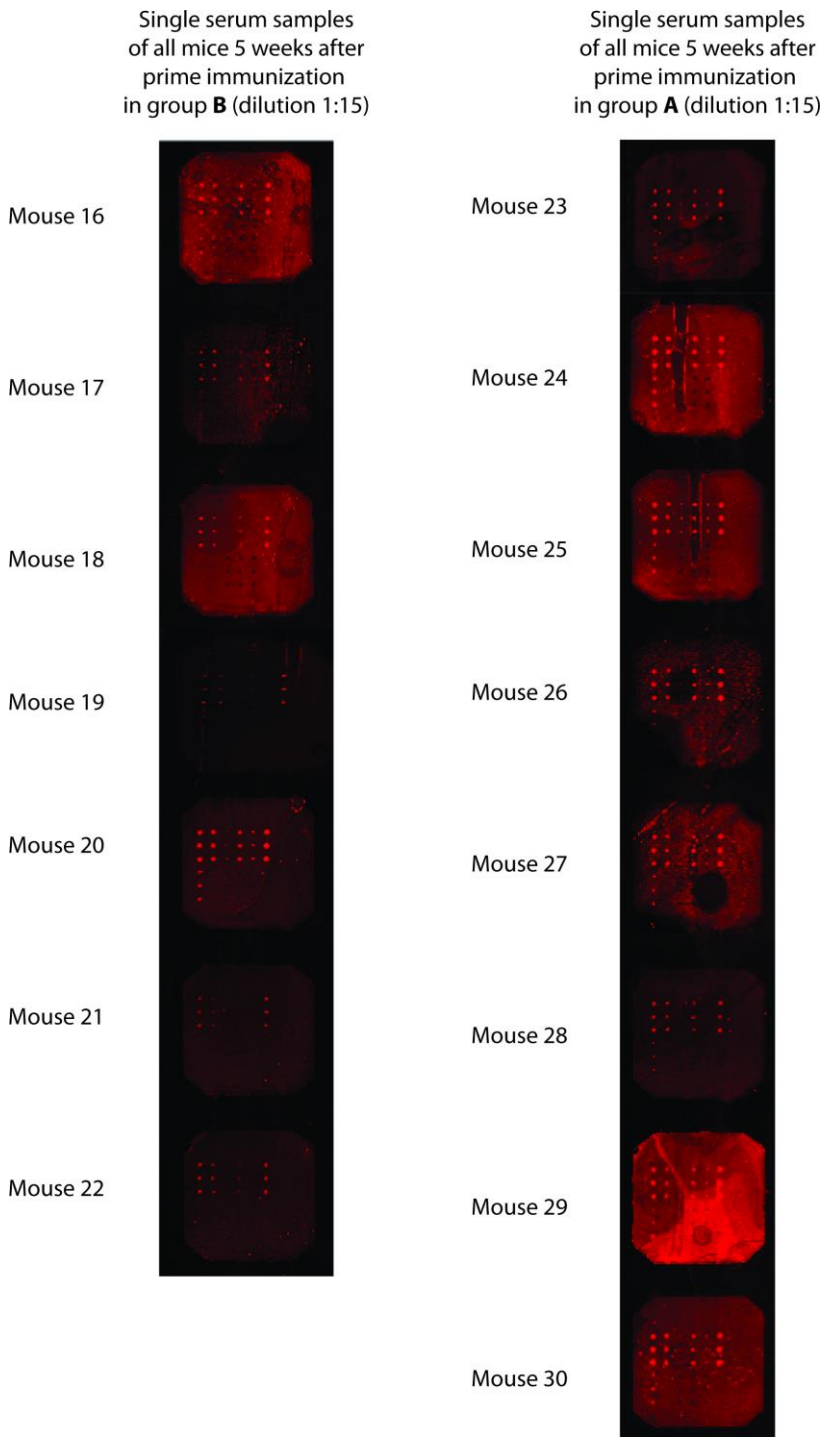


Fig. S6. Scans of carbohydrate microarrays incubated with single mouse sera (dilution 1:15) collected at day 35 (5 weeks after prime immunization). Total IgG analysis is shown. This experiment was performed to ensure that all mice had developed IgG antibodies against GPIs **1** or **2**, respectively.

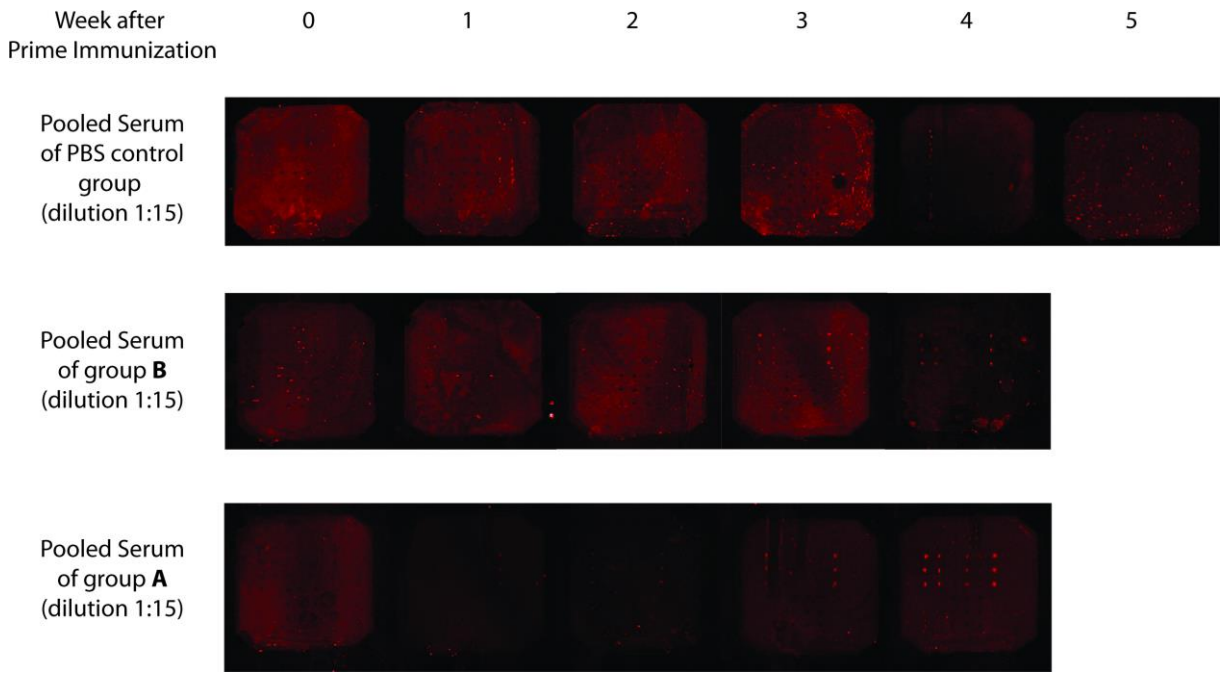


Fig. S7. Scans of carbohydrate microarrays incubated with pooled mouse sera at different time points after prime immunization. Total IgG analysis is shown.

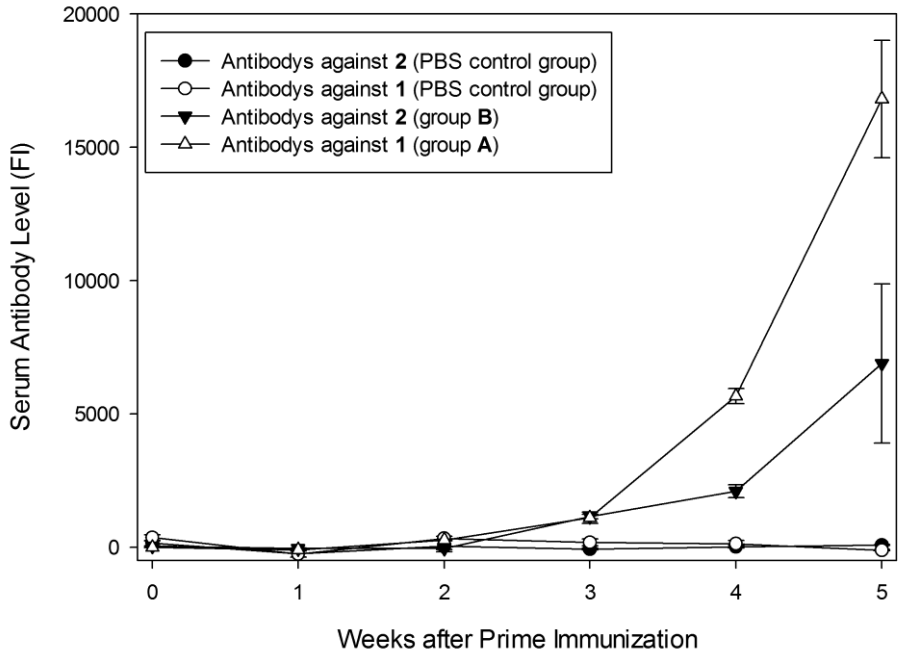


Fig. S8. Numerical read-out of microarrays shown in Fig. S6 and S7.

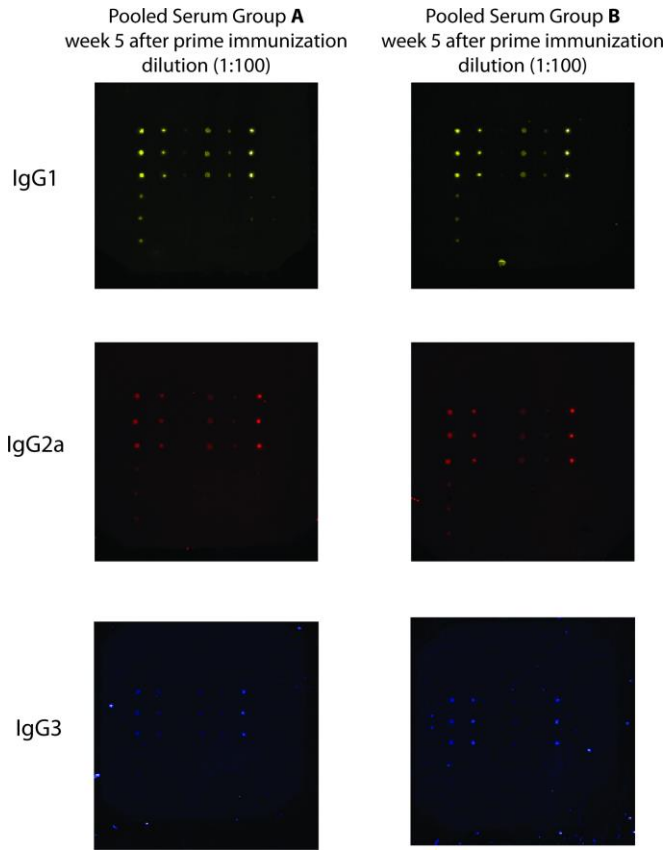


Fig. S9. Scans of carbohydrate microarrays incubated with pooled mouse sera (dilution 1:100) collected at day 35 (5 weeks after prime immunization) and analyzed for isotype distribution. All isotpyes were scanned at the same PMT gain.

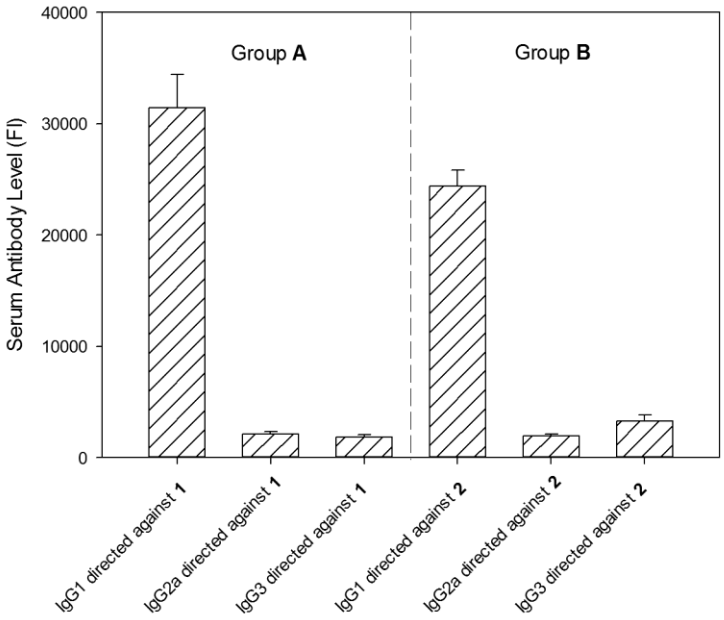


Fig. S10. Numerical read-out of microarrays shown in Fig. S9.

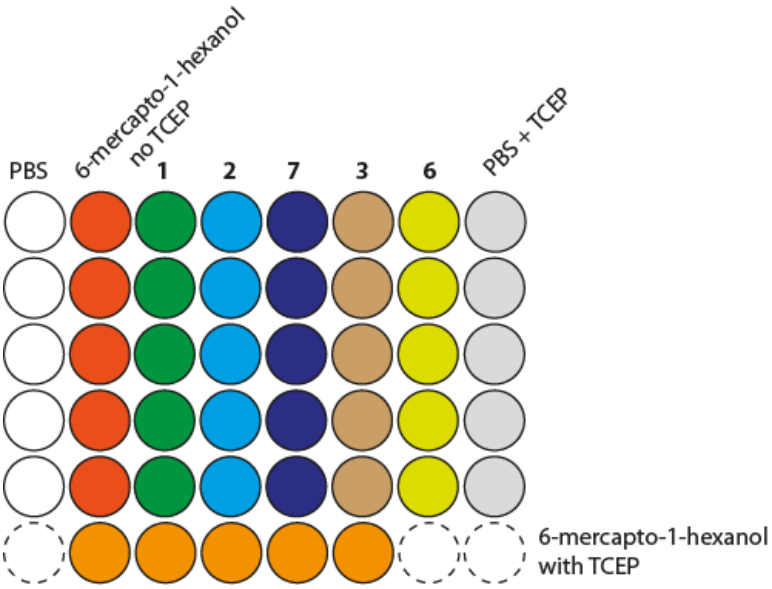
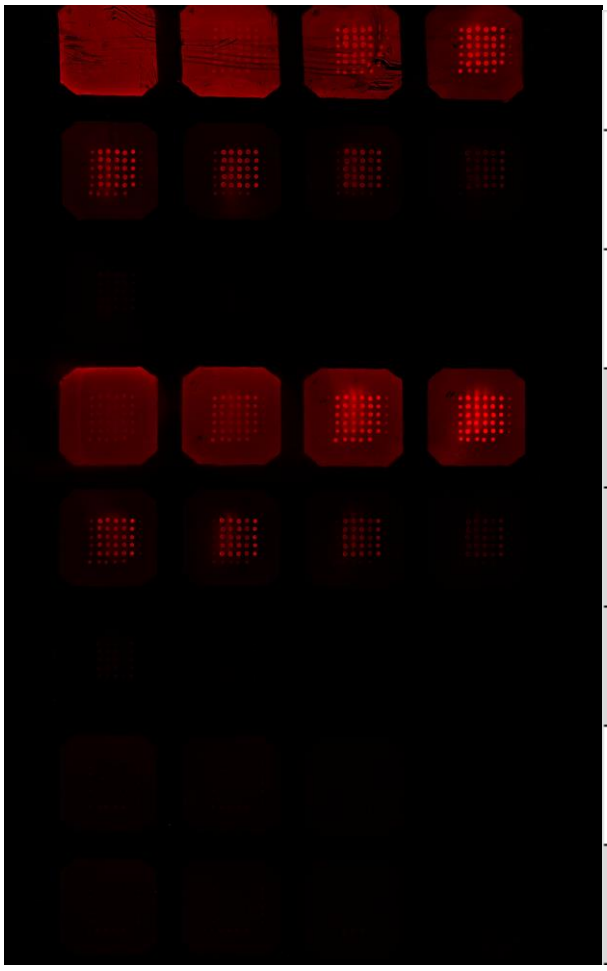
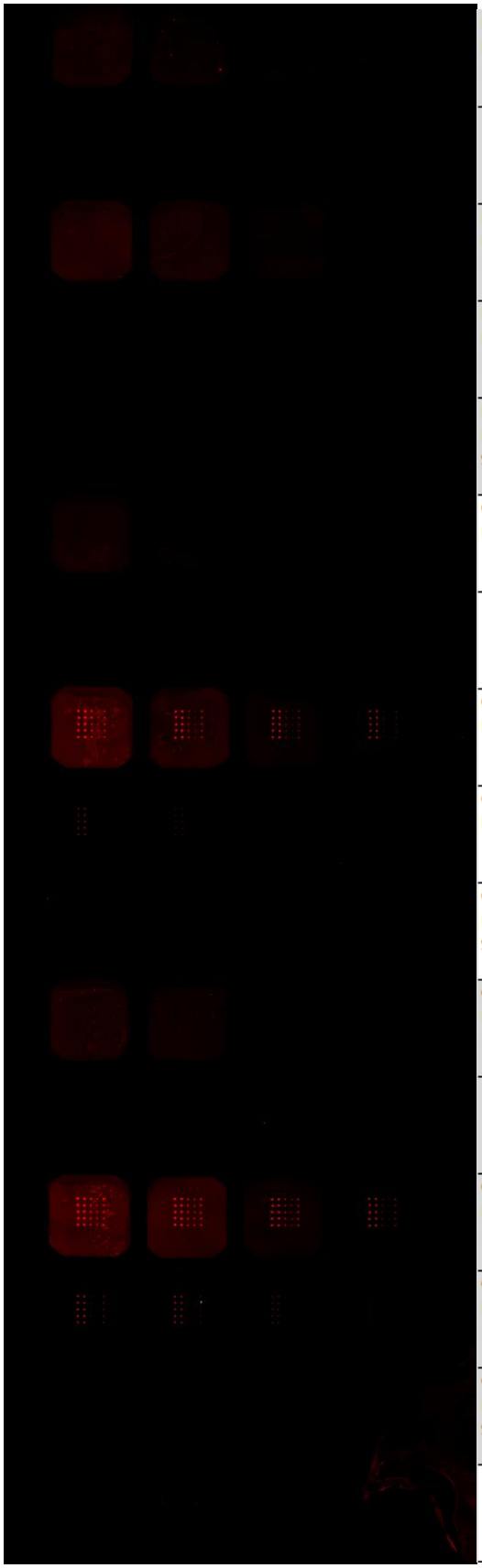


Fig. S11. Printing pattern of microarray experiments shown in Figure S12 and S13. 6-Mercapto-1-hexanol was purchased from Sigma-Aldrich.



Group B Post 2nd 15	Group B Post 2nd 45	Group B Post 2nd 135	Group B Post 2nd 405
Group B Post 2nd 1215	Group B Post 2nd 3645	Group B Post 2nd 10935	Group B Post 2nd 32805
Group B Post 2nd 98415	Group B Post 2nd 295245	Group B Post 2nd 885735	Group B Post 2nd 2657205
Group A Post 2nd 15	Group A Post 2nd 45	Group A Post 2nd 135	Group A Post 2nd 405
Group A Post 2nd 1215	Group A Post 2nd 3645	Group A Post 2nd 10935	Group A Post 2nd 32805
Group A Post 2nd 98415	Group A Post 2nd 295245	Group A Post 2nd 885735	Group A Post 2nd 2657205
Group B Preimmune 15	Group B Preimmune 45	Group B Preimmune 135	Group B Preimmune 405
Group A Preimmune 15	Group A Preimmune 45	Group A Preimmune 135	Group A Preimmune 405

Fig. S12. Scan of a carbohydrate 'maleimide' microarray incubated with a serial dilution of pooled preimmune and pooled sera (day 35 after prime immunization) of groups **A** and **B**.



PBS Preimmune 15	PBS Preimmune 45	PBS Preimmune 135	PBS Preimmune 405
PBS Post 2nd 15	PBS Post 2nd 45	PBS Post 2nd 135	PBS Post 2nd 405
PBS Post 2nd 1215	PBS Post 2nd 3645	PBS Post 2nd 10935	PBS Post 2nd 32805
PBS Post 2nd 98415	PBS Post 2nd 295245	PBS Post 2nd 885735	PBS Post 2nd 2657205
Group B Preimmune 15	Group B Preimmune 45	Group B Preimmune 135	Group B Preimmune 405
Group B Post 2nd 15	Group B Post 2nd 45	Group B Post 2nd 135	Group B Post 2nd 405
Group B Post 2nd 1215	Group B Post 2nd 3645	Group B Post 2nd 10935	Group B Post 2nd 32805
Group B Post 2nd 98415	Group B Post 2nd 295245	Group B Post 2nd 885735	Group B Post 2nd 2657205
Group A Preimmune 15	Group A Preimmune 45	Group A Preimmune 135	Group A Preimmune 405
Group A Post 2nd 15	Group A Post 2nd 45	Group A Post 2nd 135	Group A Post 2nd 405
Group A Post 2nd 1215	Group A Post 2nd 3645	Group A Post 2nd 10935	Group A Post 2nd 32805
Group A Post 2nd 98415	Group A Post 2nd 295245	Group A Post 2nd 885735	Group A Post 2nd 2657205

Fig. S13. Scan of a carbohydrate 'epoxy' microarray incubated with a serial dilution of pooled preimmune and pooled sera (day 35 after prime immunization) of groups **A** and **B**.

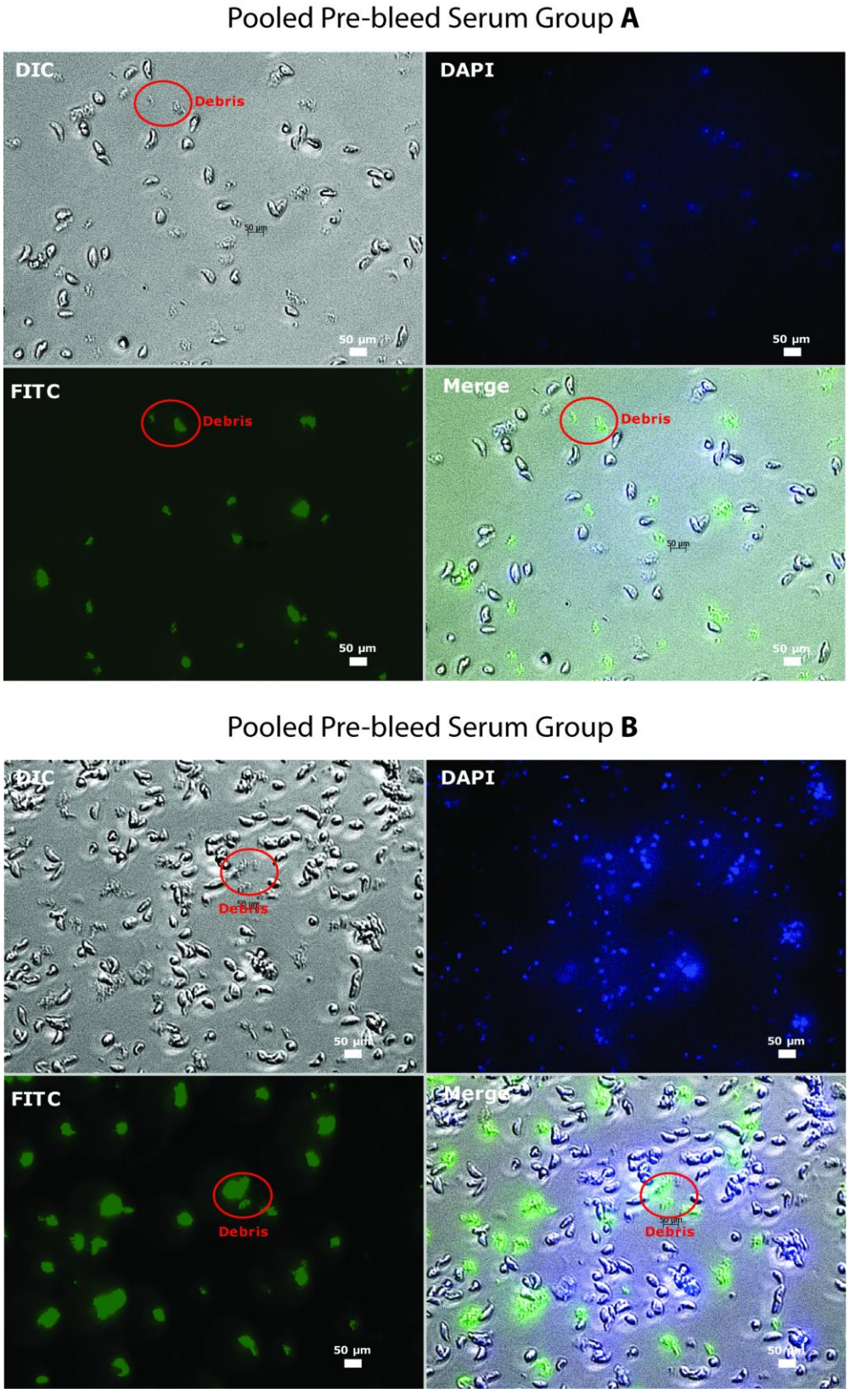


Fig. S14. Immunofluorescence (IF) pictures of tachyzoites incubated with pooled pre-bleed serum of the mouse groups **A** and **B**. The serum does not contain IgG antibodies recognizing *T. gondii* tachyzoites. Cell debris is partially indicated.

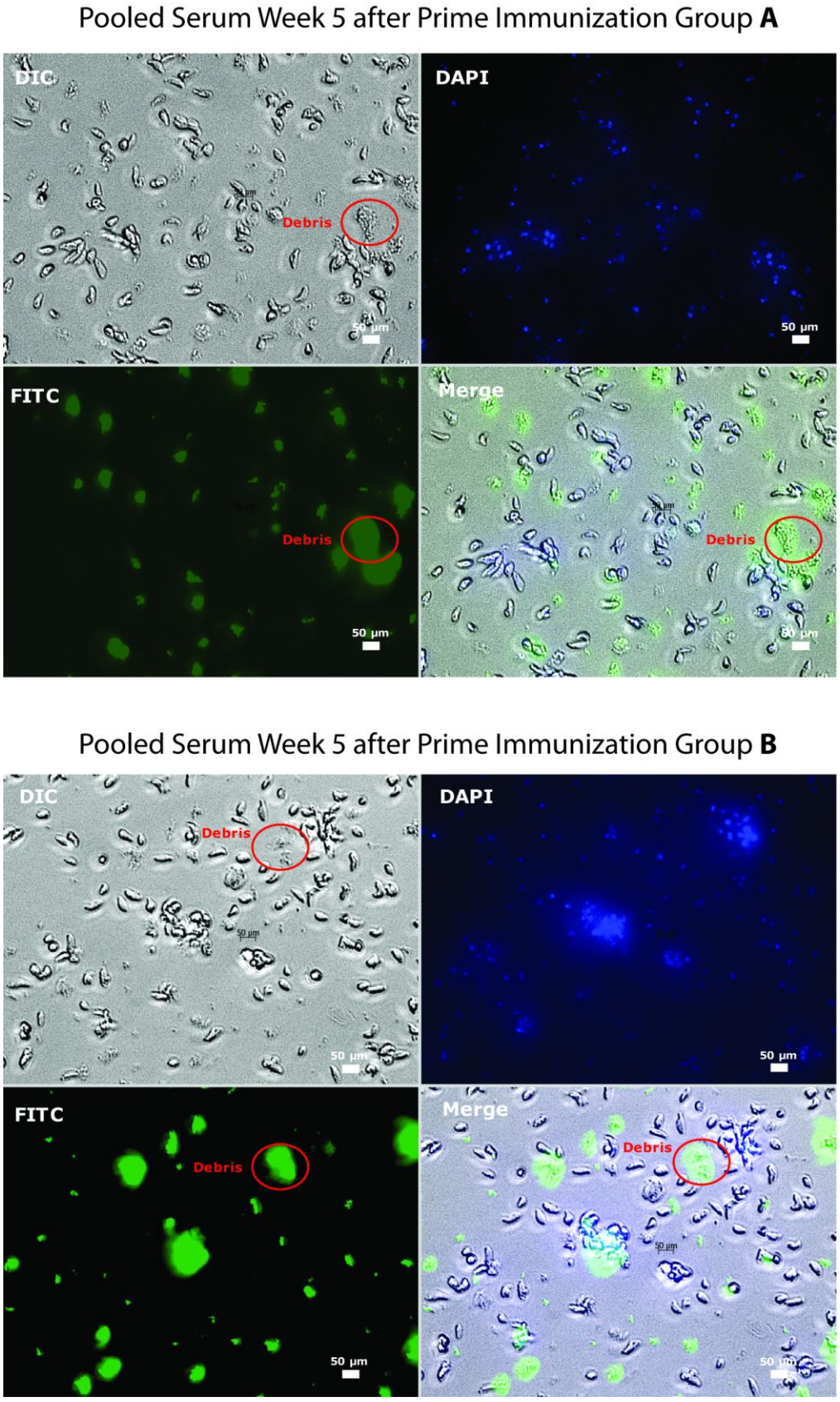


Fig. S15. IF pictures of tachyzoites incubated with pooled serum of the mouse groups **A** and **B** collected at day 35 (5 weeks after prime immunization). The serum does not contain IgG antibodies recognizing *T. gondii* tachyzoites. Cell debris is partially indicated.

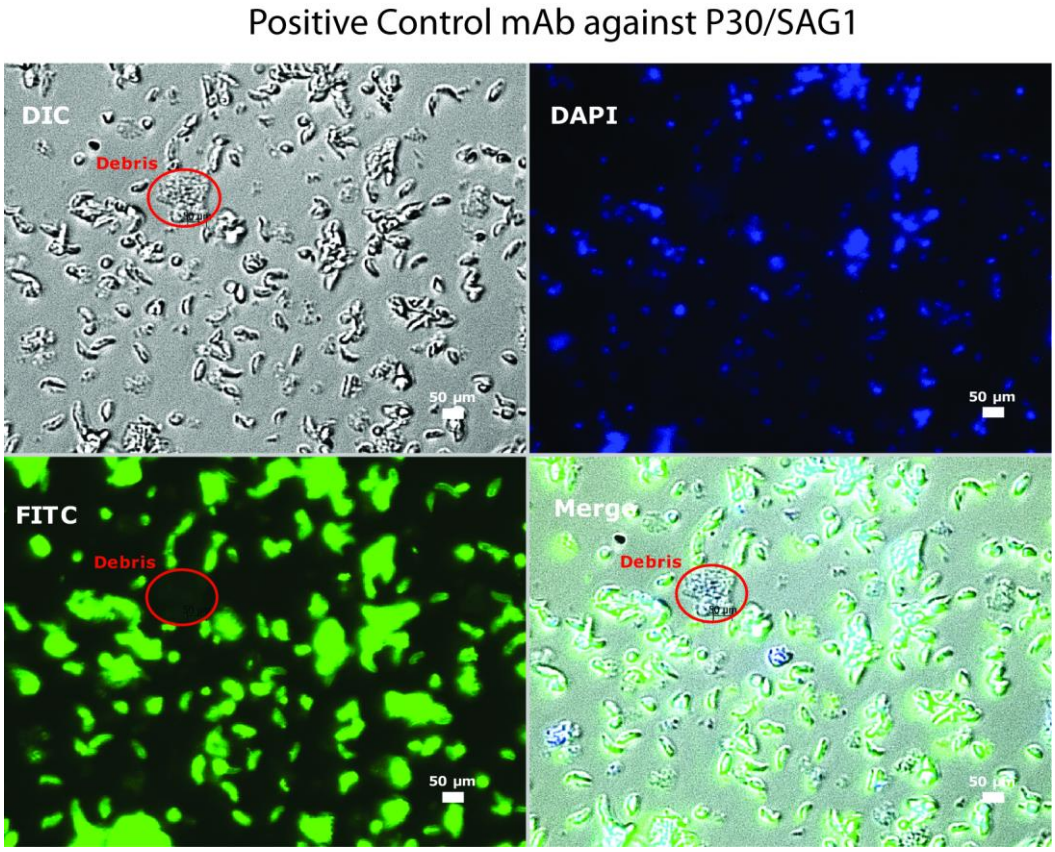


Fig. S16. IF pictures of tachyzoites incubated with the monoclonal antibody NCL-TG (Leica Biosystems). FITC and DAPI staining correlate nicely as can be seen in the merged picture. Cell debris is partially indicated.