Appendix

Memory IgM protects endogenous insulin from autoimmune destruction

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Appendix Figure S1

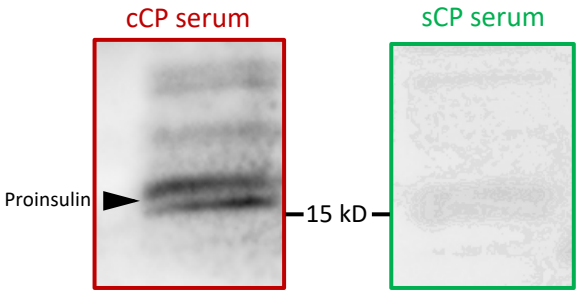

Figure S1. IgG generated by immunization with polyvalent full-length C–peptide recognizes intrapancreatic Proinsulin.
Western blot of pancreas lysates with sera of mice immunized with C-peptide (CP) in complex (cCP) or soluble form (sCP) used as primary antibodies. Anti-mouse-IgG-HRP was used for detection. Proinsulin (15 kD). Images shown are representative for 2 independent experiments with multiple sera.

Appendix Figure S2

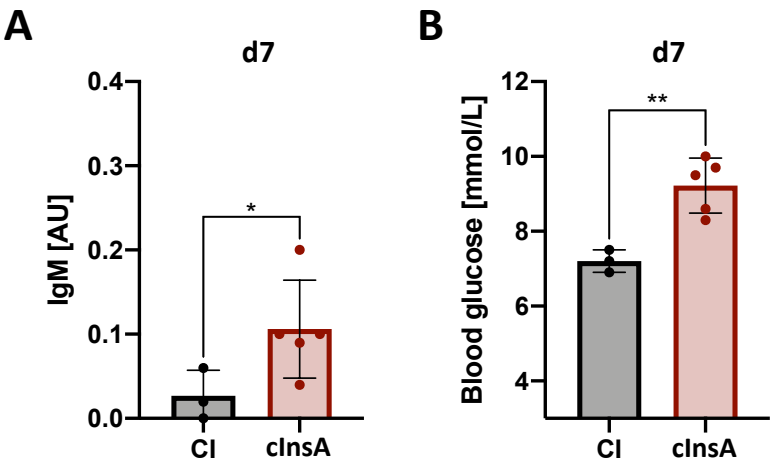

Figure S2. CpG adjuvant is not required for initiation of autoantibody responses against InsA peptides.

A: Serum anti-Insulin-IgM titers of mice injected with complex Insulin-A peptides (cInsA, n=5) or control injection (PBS, n=3) measured by ELISA (coating: Insulin). Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Mann-Whitney-U test, *P < 0.05.

B: Blood glucose levels of mice injected with complex Insulin-A peptides (cInsA, n=5) or control injection (PBS, n=3) were monitored with a commercial blood glucose monitor device. Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Mann-Whitney-U test, **P < 0.01.

Appendix Figure S3

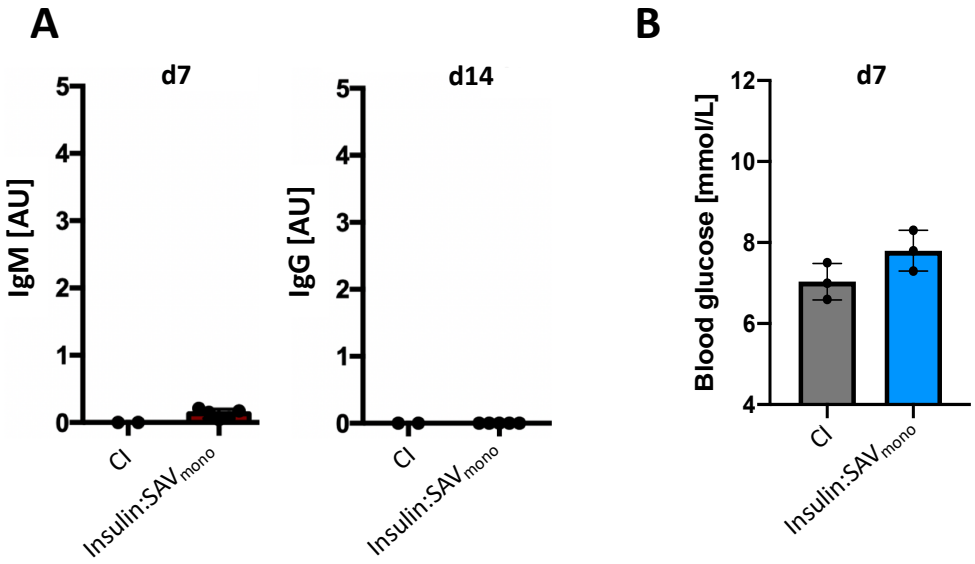

Figure S3. Anti-Insulin antibody responses are independent of the foreign carrier, but depend on antigen valency.

A: Serum anti-Insulin-IgM and IgG titers of mice immunized with Insulin coupled to monomeric streptavidin (Insulin:SAV_{mono}, n=5) or control immunization (CpG only, n=2) measured by ELISA (coating: Insulin). Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Mann-Whitney-U test, all comparisons were not significant.

B: Blood glucose levels of mice immunized with Insulin coupled to monomeric streptavidin (Insulin:SAV_{mono}, n=3) or control immunization (n=3) were monitored with a commercial blood glucose monitor device. Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Mann-Whitney-U test.

Appendix Figure S4

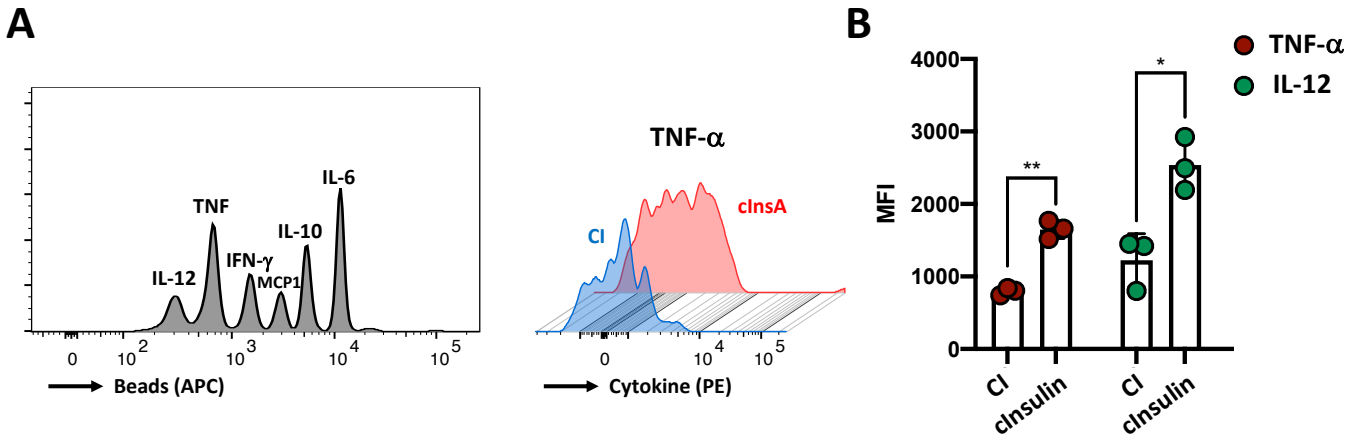

Figure S4. Immunization of mice with cInsulin induces acute inflammatory pancreatitis.
A, B : Flow cytometry-based bead array of pancreas supernatant of mice immunized with cInsulin (n=3) or control immunization (n=3). **(A)** representative histograms of cytokine beads (left) and cytokine detection (right), **(B)** quantification of FACS bead array. Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Mann-Whitney-U test.

Appendix Figure S5

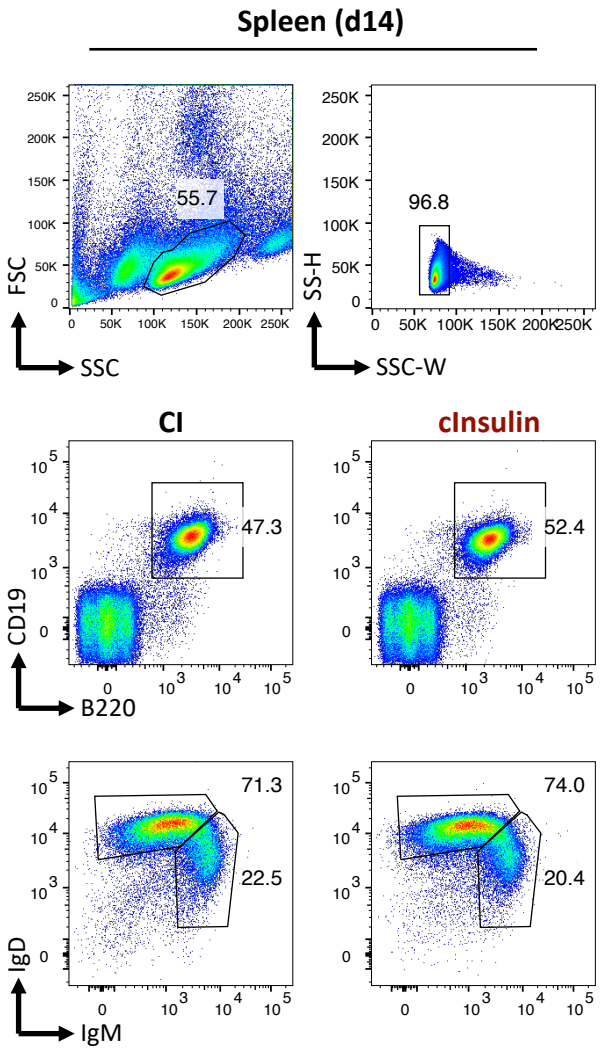

Figure S5. No alteration of splenic B cell compartment after primary immunization with self-antigen.
Flow cytometric analysis of splenocytes derived from cInsulin (n=5) immunized and control immunization (CI, n=5) mice. Top panel showing gating strategy for lymphocytes and single cells. Middle panel showing B cells ($CD19^+$ $B220^+$) pre-gated on lymphocytes. Lower panel showing IgM and IgD expression on B cells. Data are representative for three independent experiments.

Appendix Figure S6

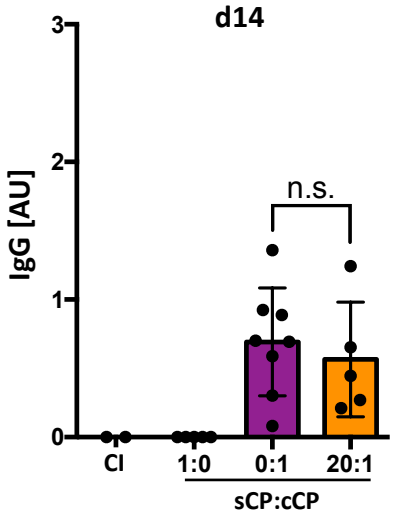

Figure S6. Antibody responses to the protein-carrier are not affected by the presence of soluble C-peptide.
Serum anti-streptavidin IgG titers of mice immunized with C-peptide (CP) measured via ELISA (coating: streptavidin). Ratios on the x-axis refer to molar ratios of soluble (sCP) to complex CP (cCP). cCP was generated by coupling of sCP to streptavidin (carrier). Dots represent individual mice (CP: n=7/group, CI: n=2). Mean ± SD, statistical significance was calculated using Kruskal-Wallis test, n.s. = not significant.

Appendix Figure S7

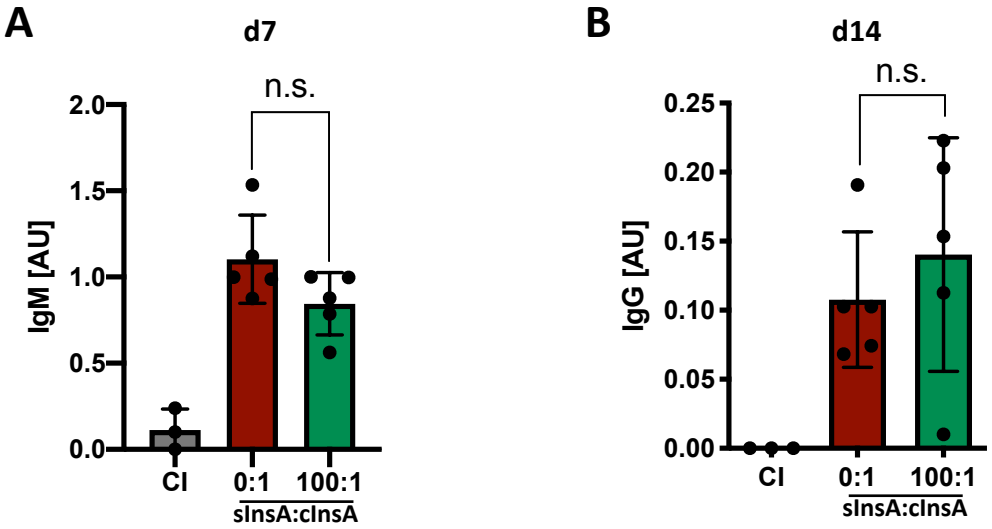

Figure S7. Antibody responses to the protein-carrier are not affected by ratios of soluble InsA.
A: Serum anti-KLH-IgM titers of Insulin-A-peptide (InsA) immunized mice were measured via ELISA (coating: KLH). Ratios on the x-axis refer to molar ratios of soluble to complex InsA used (sInsA: soluble, cInsA: complex). Dots represent individual mice. Mean ± SD, statistical significance was calculated using Kruskal-Wallis test, n.s. = not significant.
B: Serum anti-KLH-IgG titers of Insulin-A-peptide (InsA) immunized mice were measured via ELISA (coating: KLH). Ratios on the x-axis refer to molar ratios of soluble to complex InsA used (sInsA: soluble, cInsA: complex). Dots represent individual mice. Mean ± SD, statistical significance was calculated using Kruskal-Wallis test, n.s. = not significant.

Appendix Figure S8

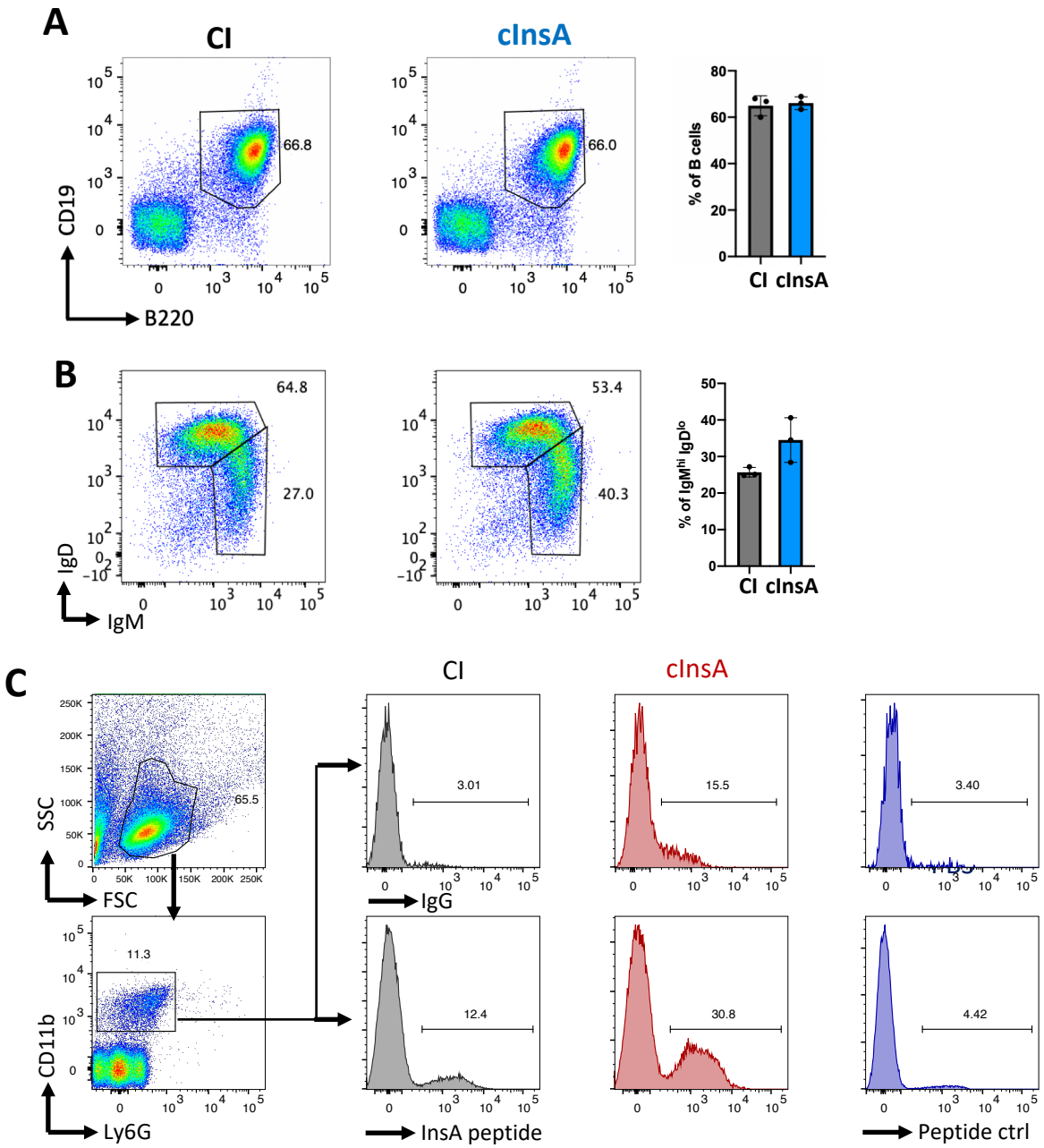

Figure S8. Increased IgM^{high}/IgD^{low} positive splenic compartment and pancreatic macrophages positive for InsA peptide and IgG after secondary immunization with self-antigen.

A, B: Flow cytometric analysis of splenocytes derived from Insulin-A peptide (InsA) or control immunized (CI) mice. **(A)** Panel showing B cells (CD19^{+} B220^{+}) pre-gated on lymphocytes. **(B)** Panel showing B cell subsets: mature B cells (IgD^{hi} IgM^{lo}), transitional/marginal zone B cells (IgD^{lo} IgM^{hi}). Cells were pre-gated on B cells. Left: CI (grey), right: cInsA (teal). Data shown are representative for 3 independent experiments. Mean ± SD, statistical significance was calculated using Mann-Whitney-U test.

C: Flow cytometric analysis of pancreatic cells. Left panel showing gating strategy for cells (top) and Macrophages (bottom). Right panel showing histograms for InsA-peptide and peptide control binding as indicated. Data shown are representative for 3 independent experiments.

Appendix Figure S9

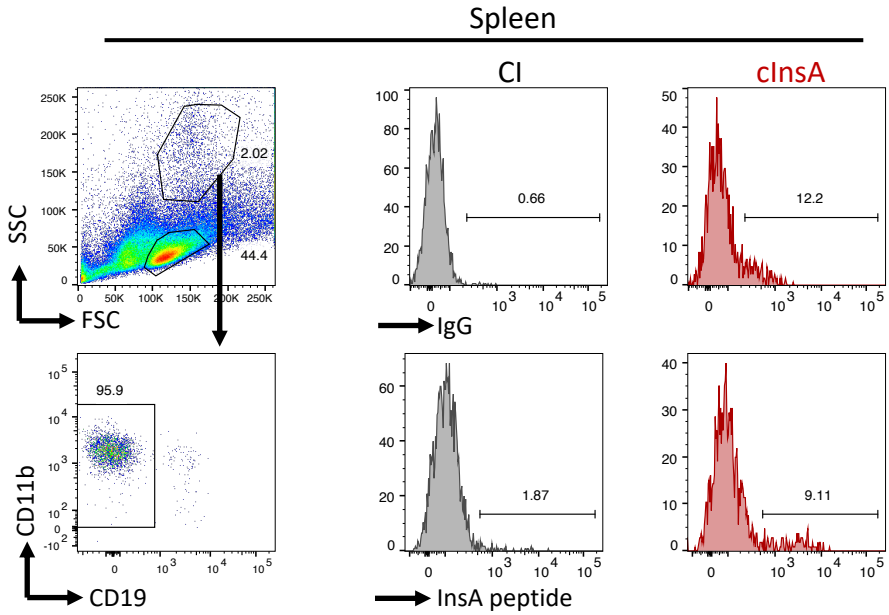

Figure S9. Splenic macrophages bind insulin-specific IgG in complex InsA-peptide immunized mice.

Flow cytometric analysis (FACS) of splenocytes of complex Insulin-A peptide (cInsA) immunized mice. Left panel showing gating strategy for macrophages ($CD11b^+$ $CD19^-$). Histograms showing IgG and InsA peptide binding of macrophages of control immunization (CI) (middle panel) and cInsA-immunized mice (right panel). Representative data for three independent experiments with n=5/group.

Appendix Figure S10

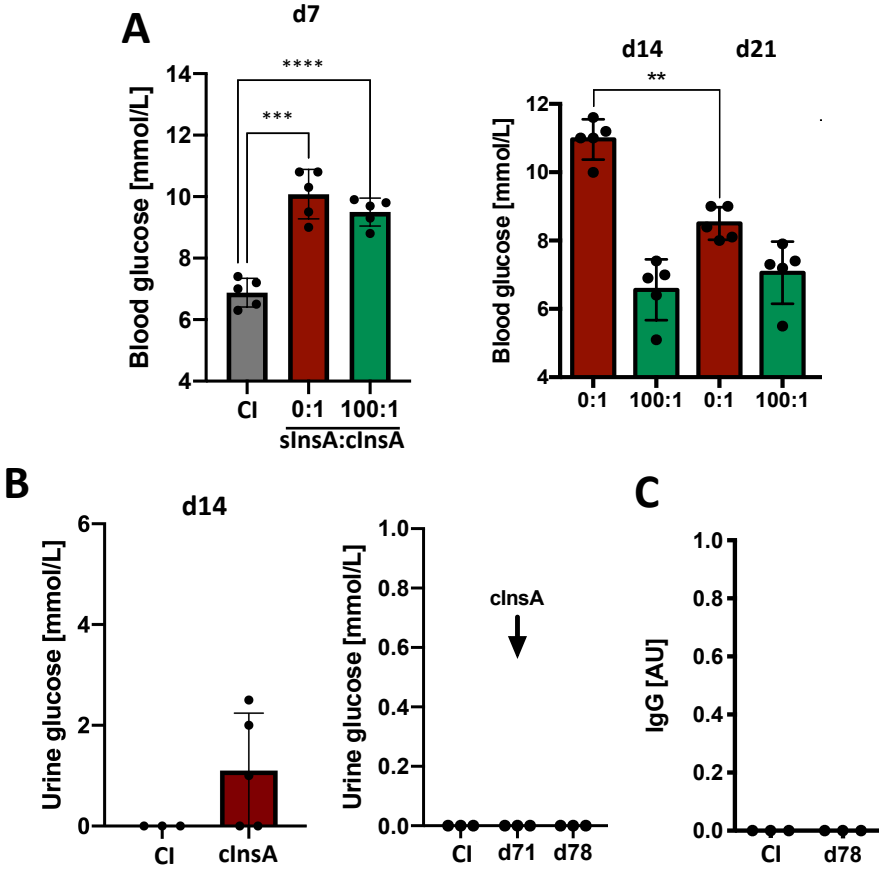

Figure S10. Primary IgM induced by InsA-peptide immunization leads to diabetes symptoms depending on the antigen valence and day.

A: Blood glucose levels of mice immunized with complex Insulin-A peptide (cInsA), soluble InsA (sInsA), or control immunization (CpG only, Cl) were monitored by a commercial glucose monitoring device. Ratios on the x-axis refer to molar ratios of sInsA to cInsA. Dots represent individual mice (n=5/group). Mean ± SD, statistical significance was calculated using Kruskal-Wallis test, ***P < 0.001.

B: Urine glucose levels were monitored by commercial test stripes. Dots represent individual mice (n=3/group). Mean ± SD, statistical significance was calculated using Mann-Whitney-U test, **P < 0.01.

C: Serum anti-Insulin IgG titers of complex Insulin-A-peptide (cInsA) immunized mice measured via ELISA (coating: Insulin). Dots represent individual mice (n=3/group). Mean ± SD, statistical significance was calculated using Mann-Whitney-U test, all comparisons were not significant.

Appendix Figure S11

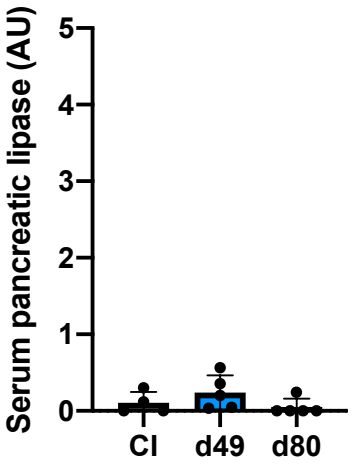

Figure S11. Elevated PR-IgM titers protect complex Insulin immunized mice from acute pancreatitis-mediated pancreas damage.
Serum pancreatic lipase levels of control (CI, n=4) and complex Insulin (cInsulin, n=5/group) immunized mice at indicated days measured by ELISA. Dots represent individual mice. Mean ± SD, statistical significance was calculated by using Kruskal-Wallis test.

Appendix Figure S12

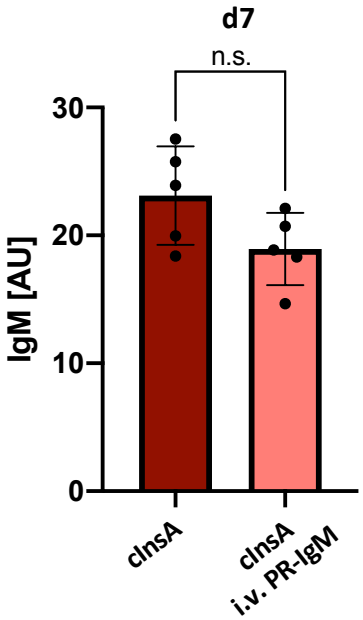

Figure S12. Intravenous injections of PR-IgM do not affect the robustness of induced immune reaction by cInsA to the carrier.
Serum anti-KLH-IgM titers of complex Insulin-A-peptide (cInsA) immunized mice measured via ELISA (coating: KLH). Dots represent individual mice (n=5/group). Mean ± SD, statistical significance was calculated using Mann-Whitney-U test, n.s. = not significant.

Appendix Figure S13

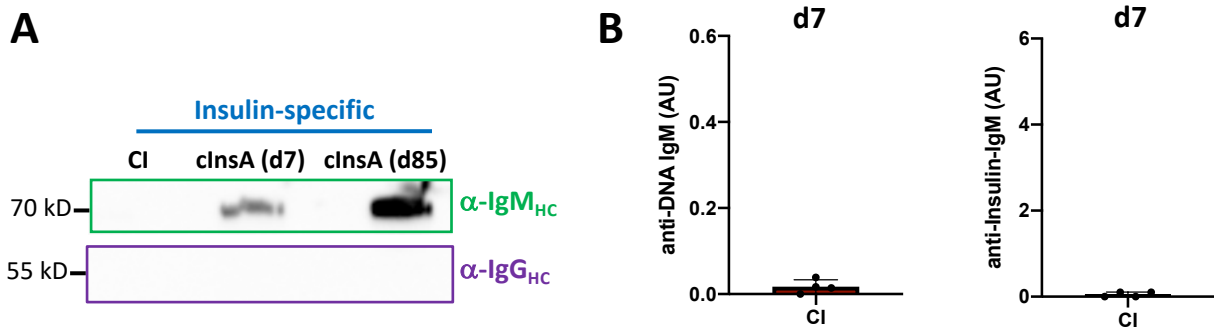

Figure S13. Insulin-specific pulldown of sera of cInsA immunized mice contains only Insulin-reactive IgM.

A: Western blot analysis of Insulin-specific IgM pulldown. Gels were loaded with insulin-specific IgM pulldowns of mice immunized with CpG only (CI), cInsA on day 7, or day 85. Top panel showing IgM heavy chain (IgM HC, 69 kD) and bottom panel showing IgG heavy chain (IgG HC, 55 kD) detected by anti-IgM-HC or anti-IgG-HC HRP-labelled antibodies. Data shown are representative of three independent experiments.

B: Serum anti-dsDNA-IgM (left) and anti-Insulin-IgM (right) of control immunized mice (CpG only, CI) measured via ELISA. Dots represent individual mice, mean ± SD.

Appendix Figure S14

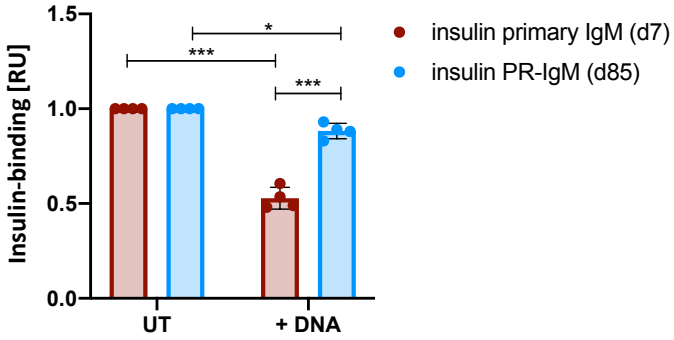

Figure S14. Soluble antigen competition assay reveals poly-specificity for primary IgM but mono-specificity for PR-IgM.
Direct IgM insulin-binding competition assay. Serum of wild-type mice immunized with cInsA at indicated days (n=4/group) was either pre-incubated with BSA (untreated control, UT), or 50 µg/mL calf-thymus dsDNA (+ DNA). Data show insulin-binding of IgM relative to insulin-binding of the UT IgM samples. Mean ± SD, statistical significance was calculated by using Kruskal-Wallis test., *P < 0.05, ***P < 0.001.