

Supplemental Results

Testing individual TLR KO NOD mice. Genetic approaches to the *in vivo* analysis of the role of TLRs in development of autoimmunity are complicated by the fact that multiple TLRs can be activated by distinct ligands derived from the same microbe. Hence, TLRs can participate in disease induction (or protection from it) in a redundant fashion. Because multiple TLRs signal through the MyD88 adaptor, follow-up studies were conducted in NOD mice lacking individual TLRs (TLRKO). We found that TLR2 and TLR4 (as well as TLR3, data not shown) were dispensable for development of T1D (or protection from it by complete Freund's adjuvant) when deleted *individually*, in contrast to the effect of complete protection from diabetes associated with loss of MyD88 (Fig. 1). Inactivating TLR4 eliminates signaling through TLR4 via both TRIF- and MyD88-mediated pathways, whereas inactivation of the TLR2 gene eliminates signaling through TLR2, 1 and 6. NOD.TLR4KO mice were derived at The Jackson Laboratory (J) and observed there and also at Yale University (J→Y). SPF mice at both sites showed a normal incidence of diabetes, which was slightly although significantly ($p=0.0013$) higher in J→Y mice (Supplemental Fig. 1a). We have observed mouse family-specific differences in the composition of the cecal microbiota (see Fig. 4c) that may explain why J→Y TLR4KO and their control littermates had a slightly higher incidence of diabetes than the same mice at J. These animals originated from two sibling females that were transferred J→Y, while mice observed at J came from multiple breeders.

Mice lacking TLR2 (independently derived at J and Y) developed diabetes with slower kinetics and reduced incidence compared to NOD.TLR4KO mice (Supplemental Fig. 1b). However, the heterozygous control groups (both J and Y) also showed reduced

incidence compared to wild-type NOD mice (Supplemental Fig. 1b). It is possible that the insulin-dependent diabetes locus 17 (*Idd17*), which influences diabetes in NOD mice and is currently inseparable from the *tlr2* gene by genetic means, impaired T1D development in congenic mice expressing the genomic segment from the TLR2 knockout strain. Loss of both TLR2 and TLR4 in double-KO mice (Supplemental Fig. 1b) led to an incidence of diabetes similar to that in mice without TLR2 ($p=0.49$).

Testing mutant mice for protection from T1D by CFA. To test the possibility that loss of TLR4 and TLR2 could have affected T1D *prevention* by microbial stimuli, TLR2 or TLR4 KO mice were treated at 3 wks of age with *M. tuberculosis* containing Complete Freund's Adjuvant (CFA): previous work had shown that CFA acts systemically to protect NOD mice from T1D development^{4, 5, 40, 41}. CFA protected both control and KO animals (Supplemental Fig. 1C versus Supplemental Fig. 1a,b), and did not affect the loss of diabetes in NOD.MyD88KO mice (Supplemental Fig. 1d).

Composition of Altered Schaedler Flora (ASF) in gnotobiotic mice. Bacteria detected in the cecal contents of the ASF-colonized NOD mice belong to divisions that are also represented in the human gut microbiota: (i) the Firmicutes [a strain of *Lactobacillus murinis* (ASF 361) which is a member of the family Lactobacillaceae, as well as ASF 500, a relative of *Eubacterium plautii*, plus two members of Clostridium cluster XIV (ASF 356 related to *Clostridium propionicum* and ASF 502 related to *Ruminococcus gnavus*)]; (ii) the Bacteroidetes (ASF 519, related to *Bacteroides distasonis*); and (iii) the Deferribacteres [*Mucispirillum schaedleri* (ASF 457)]. These same 6 bacterial species were also detected as components of the normal cecal microbiota of NOD/LtJ mice housed under SPF conditions (Supplemental Fig. 5).

Regulatory T cells (Tregs) in GF and MyD88KO NOD mice. The tolerizing effect of MyD88KO is linked to presentation of pancreatic antigens in PLN, as it is not seen in MLN, which also drain the intestine. It is likely due to modifications of antigen presenting cells (APC) by microbial stimuli or due to an indirect effect produced by activation of regulatory T cells. Therefore, the presence of Tregs was measured by staining T cells with antibodies against CD4, CD25 and FoxP3. No statistically significant increase in the presence of Tregs was found in MyD88KO, MLN or PLN compared with Tregs in MyD88-sufficient mice (Supplemental Figure 8a). Interestingly, GF MyD88-sufficient animals did not show any decrease, but rather a small increase (Supplemental Figure 8b) in Tregs in gut-draining lymph nodes, suggesting that the gut microbiota produces stimuli that actually reduce the number of Tregs. Importantly, it has been shown that Tregs exert their function within the pancreatic tissue rather than in the PLN⁴². However, the paucity of infiltrating cells in NOD.MyD88KO pancreata, made it difficult to test this possibility directly. Thus, it is likely that the alterations in gut commensals as a consequence of MyD88 disruption, directly lead to modification of APCs towards a tolerance-inducing state in PLNs, where both microbial products (or mediators induced by them) and pancreatic antigens are available¹⁷.

Additional references for Supplemental results

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41. Qin, H. Y., Suarez, W. L., Parfrey, N., Power, R. F. & Rabinovitch, A. Mechanisms of complete Freund's adjuvant protection against diabetes in BB rats: induction of non-specific suppressor cells. *Autoimmunity* **12**, 193-9 (1992).
42. Chen, Z., Herman, A. E., Matos, M., Mathis, D. & Benoist, C. Where CD4+CD25+ T reg cells impinge on autoimmune diabetes. *J Exp Med* **202**, 1387-97 (2005).
43. Sarma-Rupavtarm, R. B., Ge, Z., Schauer, D. B., Fox, J. G. & Polz, M. F. Spatial distribution and stability of the eight microbial species of the altered schaedler flora in the mouse gastrointestinal tract. *Appl Environ Microbiol* **70**, 2791-800 (2004).

Supplemental Figure Legends

Supplemental Figure 1 Diabetes development and sensitivity to CFA in TLR2KO and TLR4KO NOD mice.

- a. Diabetes incidence in NOD.TLR4KO mice (10 back-crosses to NOD/LtJ) derived at The Jackson Laboratory (J) and observed there, and at the Yale University animal facility (J→Y). Heterozygous NOD.TLR4KO/+ littermates served as controls.

The difference between incidence curves for NOD.TLR4KO(J) and NOD.TLR4KO (J→Y) was significant ($p=0.0013$), as it was for the comparison with their heterozygous littermates ($p=0.033$).
- b. Diabetes incidence in two independently derived (J and Y) NOD.TLR2KO mouse stocks (12 back-crosses to NOD/LtJ), heterozygous control animals, and in double TLR2/TLR4KO (J).
- c. Diabetes incidence in NOD mice lacking TLR4 or TLR2 upon administration of Complete Freund's Adjuvant (CFA).
- d. Diabetes incidence in NOD.MyD88KO/+ and NOD.MyD88KO (J) mice injected with CFA into their footpads at 3 wks of age.

Supplemental Figure 2 Primary results of Elispot analysis of T cell reactivity to peptides recognized by diabetogenic T cells.

- a. IFN- γ producing CD8⁺ or CD4⁺ T cells were enumerated in the spleens of NOD MyD88-sufficient (solid bars) and NOD.MyD88KO (open bars) mice by Elispot

analysis using peptides derived from islet-specific antigens (Insulin B¹⁵⁻²³) or peptide mimics recognized by known diabetogenic CD8 T⁺ cells (AI4, 8.3) or CD4⁺ T cells (BDC2.5). Data (IFN- γ producing cells per 10⁶ CD8⁺ or CD4⁺ T cells) obtained from individual animals were combined (mean \pm SE). n, number of animals per group.

- b. The frequency of IFN- γ producing cells was determined in mesenteric (MLN) and pancreatic (PLN) lymph nodes of individual control (black dots) and NOD.MyD88KO (open dots) mice.
- c. Primary data used in Figure 2A: % of mice reacting to listed peptides. Mean (% \pm SE) refers to overall reactivity to diabetogenic peptides in a group.
Abbreviations: +/-, heterozygous control; KO, MyD88-negative NOD mice. 100% reactivity reflects the level that would have been achieved if T cells from all mice of a given genotype responded to all four peptides.

Supplemental Figure 3 Development of diabetes in GF NOD mice.

- a. GF MyD88-sufficient NOD males and females were monitored for hyperglycemia weekly for a total of 30 weeks. The kinetics of disease occurrence was similar to what was normally observed in our high health status, conventional SPF facilities, although males showed a higher incidence of diabetes (80%) at 30 weeks of age. Thus, NOD mice can develop diabetes without their microbiota, and disease development is not synchronized in all GF animals, indicating that onset of autoimmunity is prone to intrinsic variability, likely due to the stochastic nature of production of a relevant T cell receptor repertoire.

- b.** GF MyD88KO NOD females develop diabetes with 100% incidence at 30 wks of age.

Supplemental Figure 4 Evidence that despite the fact that GF mice are moderately lymphopenic, the absolute numbers of the anti-islet T cells responding to islet peptides are not significantly higher than in SPF NOD animals.

- a.** IFN- γ producing CD8⁺ or CD4⁺ T cells were enumerated in the spleens of SPF NOD mice (solid bars) and GF NOD mice by Elispot analysis using peptides derived from islet-specific antigens (Insulin B¹⁵⁻²³) or peptide mimics recognized by known diabetogenic CD8 T⁺ cells (AI4, 8.3) or CD4⁺ T cells (BDC2.5). Data (IFN- γ producing cells per 10⁶ of CD8⁺ or CD4⁺ T cells) obtained from individual animals were combined (mean \pm SE). n, number of animals per group.
- b.** No significant differences were found in proliferation of BDC2.5 cells injected into GF NOD or NOD.MyD88 KO recipients (performed and analyzed as in Fig. 2c).

Supplemental Figure 5 PCR analysis of cecal contents from gnotobiotic and SPF NOD mice.

- a.** Six out of eight bacterial components of the ASF are present in the cecal contents of all mice tested (n=25) by PCR. 16S rRNA genes were amplified using a protocol from Sarma-Rupavtarm et al.⁴³. 1-8, correspond to ASF strain numbers as defined by Sarma-Rupavtarm et al.⁴³ (Firmicutes unless noted otherwise): ASF 356, related to *Clostridium propionicum*; ASF 502, related to *Ruminococcus*

- gnavus*; ASF 360, related to *L. acidophilus*; ASF 361, a strain of *Lactobacillus murinis*; ASF 457, *Mucispirillum schaedleri* (phylum Deferribacteres); ASF 492, *Eubacterium plexicaudatum*; ASF 500, a relative of *Eubacterium plautii*; ASF 519, related to *Bacteroides distasonis* (phylum Bacteroidetes). *- positive control using DNA from monocultures of ASF 360 and ASF 457, designed to show that the corresponding primers work, and that these two bacterial lineages were not present in experimental mice.
- b. All eight 16S rRNA genes representing ASF consortium members are found in a randomly chosen NOD/LtJ mouse. Note that primers amplifying ASF 360 and ASF 361 may amplify other *Lactobacilli*⁴³ in the SPF mice.

Supplemental Figure 6 Histological grading of infiltration in pancreatic islets during development of T1D.

Hematoxylin and eosin (HE) staining of 6µm thick paraffin sections cut at 40µm intervals. All images were taken with the same magnification. Bar shown in the far right lower panel, 100µm.

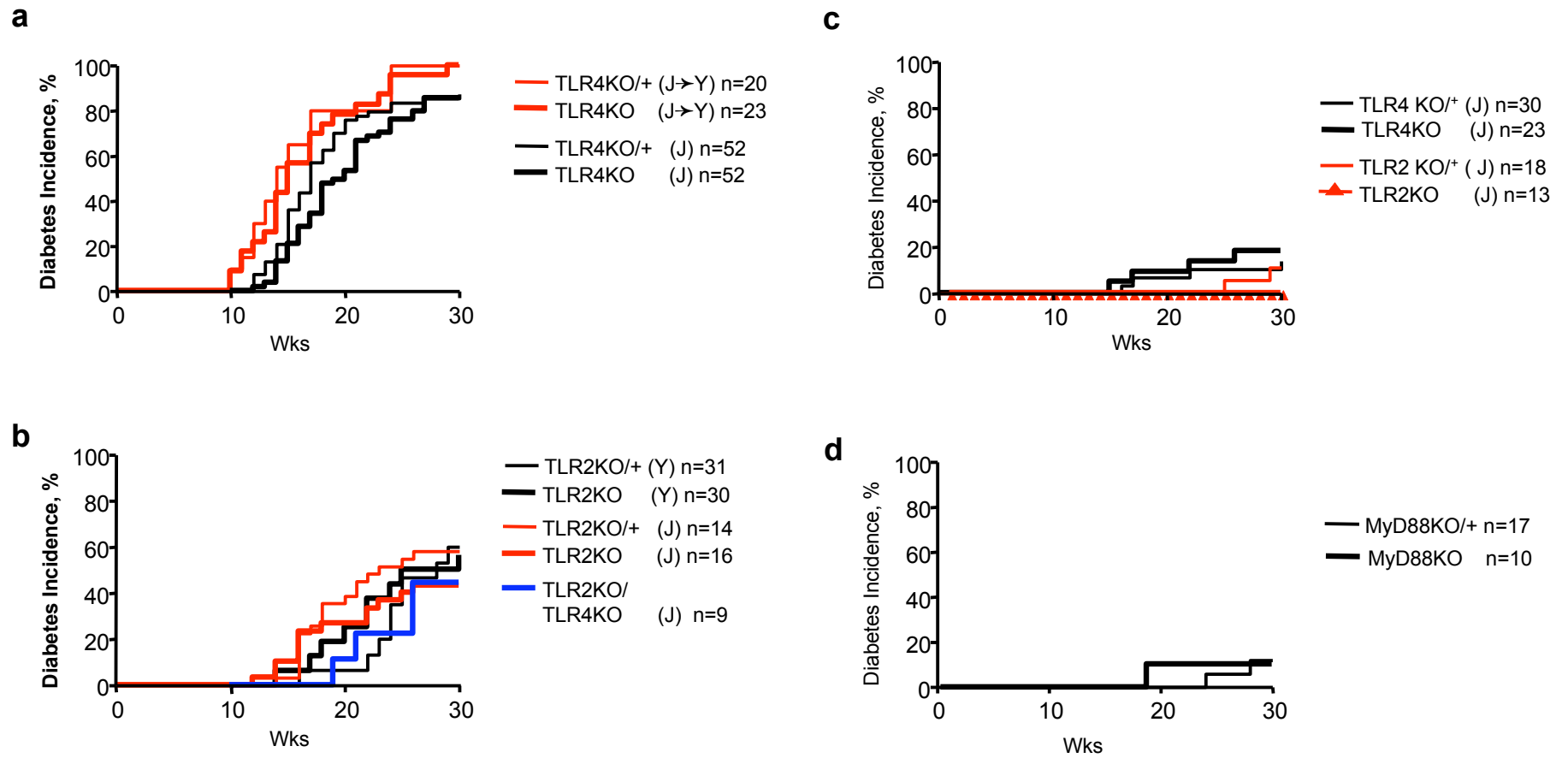
Supplemental Figure 7 Pancreatic islet infiltration in 8wk-old female mice used for sequence analysis of gut microbiota 16S rRNA genes.

- a. Insulinitis score.
- b, c. Staining for insulin or with HE, respectively.

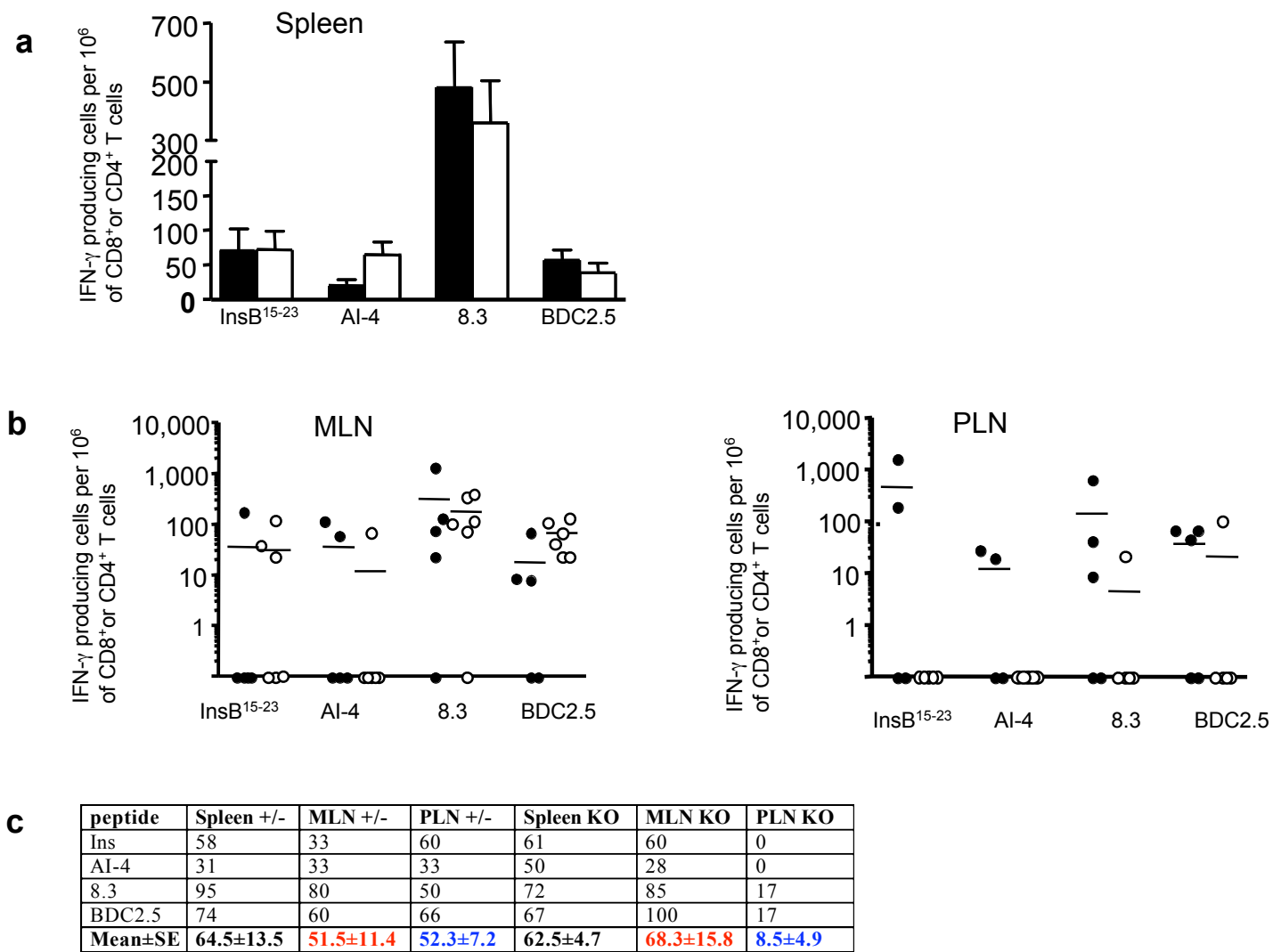
Islets of Langerhans were only moderately infiltrated with leukocytes in NOD.MyD88KO animals. Infiltration was more pronounced in NOD.MyD88KO/+ mice. Sulfatrim treatment restored infiltration in NOD.MyD88KO mice.

Supplemental Figure 8 Enumeration of FoxP3⁺ CD4⁺ T cells in secondary lymphoid organs of manipulated NOD mice.

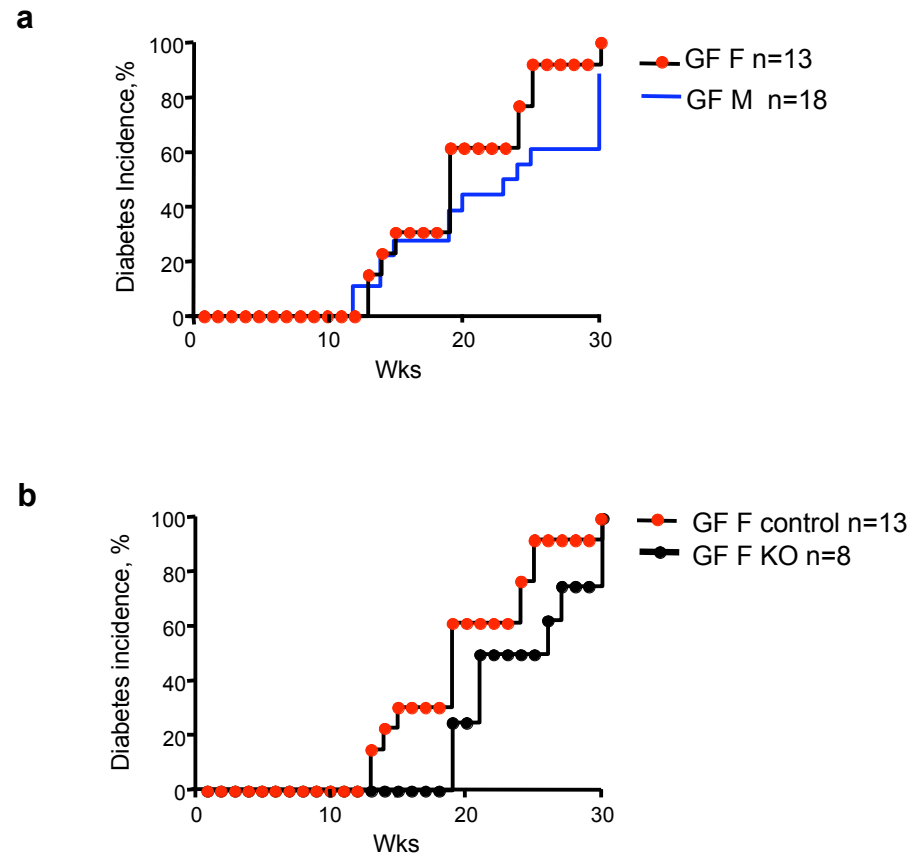
- a. The number of FoxP3⁺ Treg cells is not significantly increased in the gut-draining lymph nodes in NOD.MyD88KO mice. Percentage of CD4⁺ cells positive for FoxP3 is shown for individual mice. *p* values were determined using unpaired Student's *t* test.
- b. The numbers of FoxP3⁺ T reg cells are slightly elevated in gut-draining lymph nodes of GF NOD.MyD88KO/+ mice compared to SPF NOD.MyD88KO/+ mice. For MLN the *p* value was 0.04 and for PLN it was more of a trend (*p*=0.12).

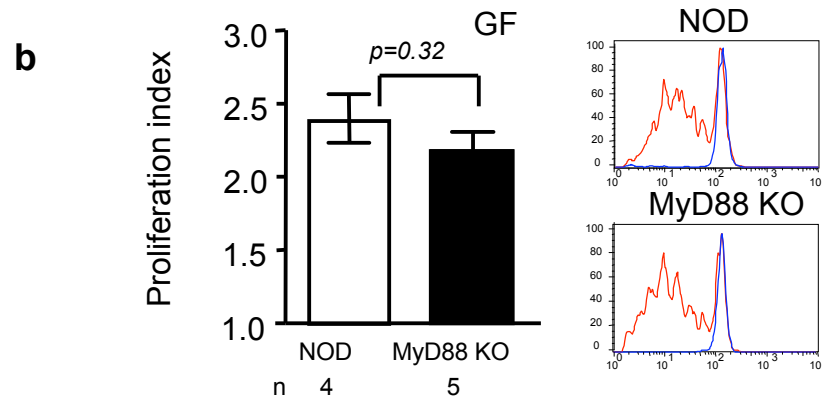
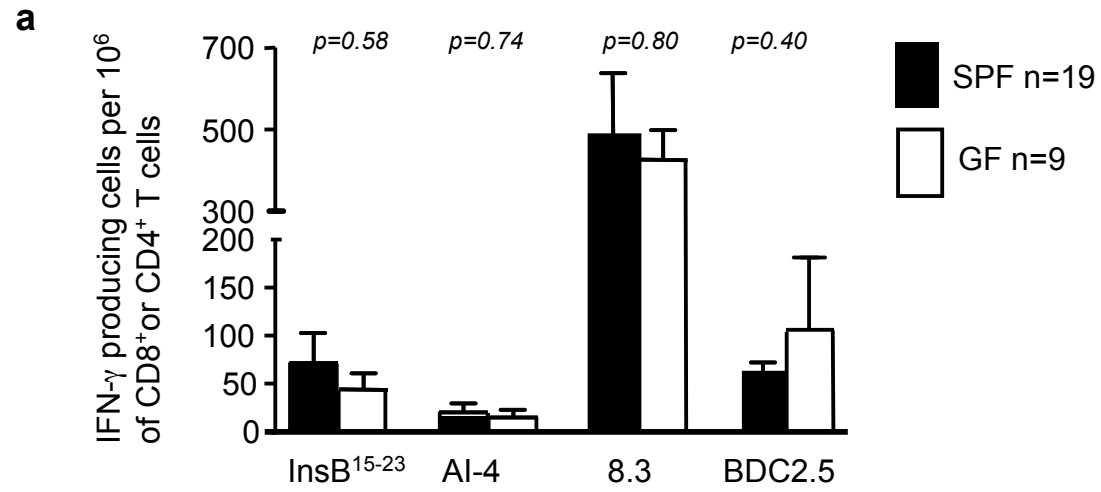


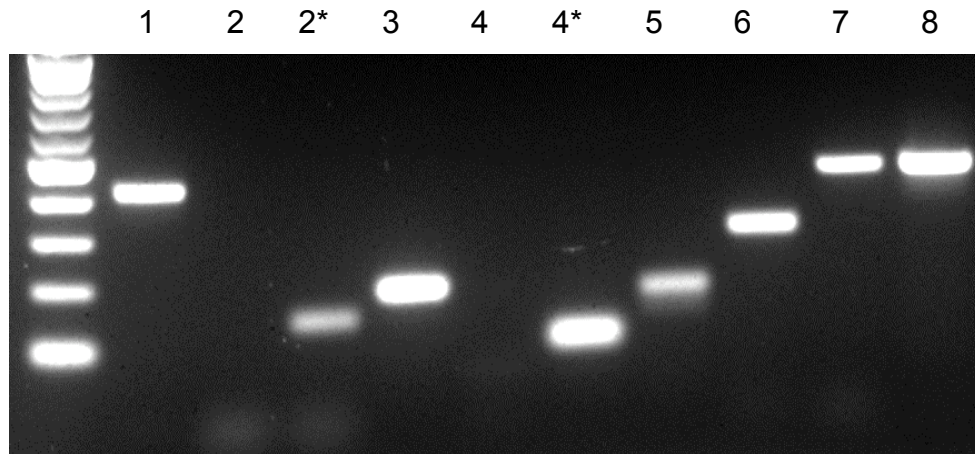
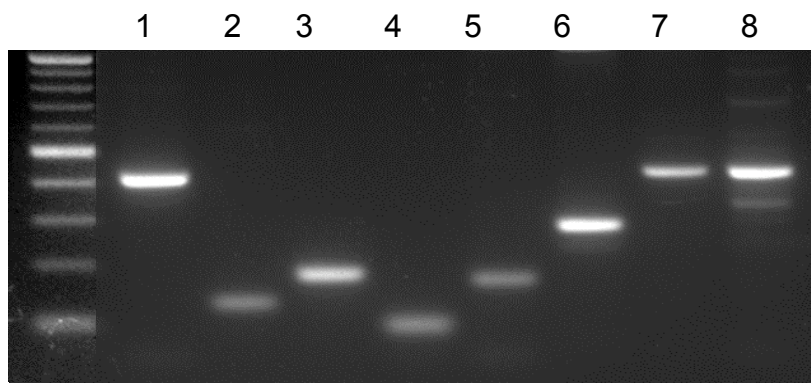
Supplemental Figure 1



Supplemental Figure 2





a**b**

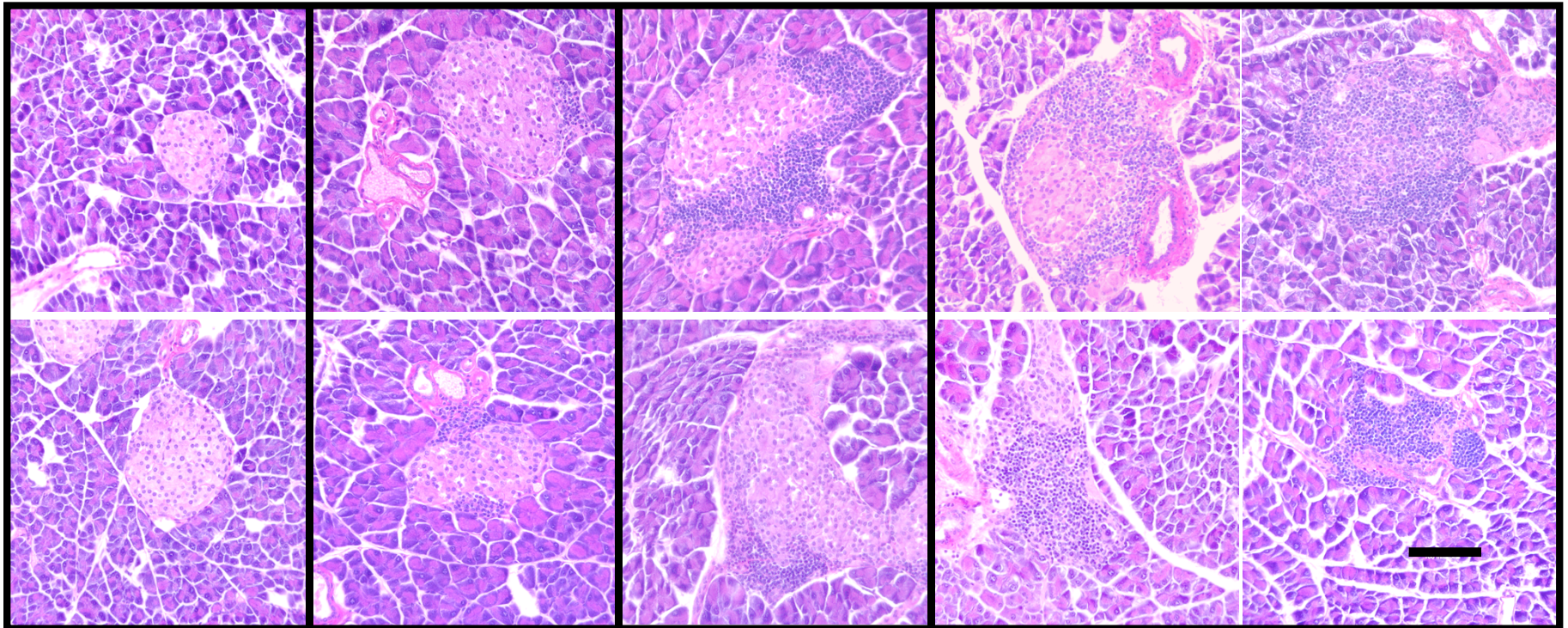
- 1 ASF 356
- 2 ASF 360
- 2* ASF 360 culture
- 3 ASF 361
- 4 ASF 457
- 4* ASF 457 culture
- 5 ASF 492
- 6 ASF 500
- 7 ASF 502
- 8 ASF 519

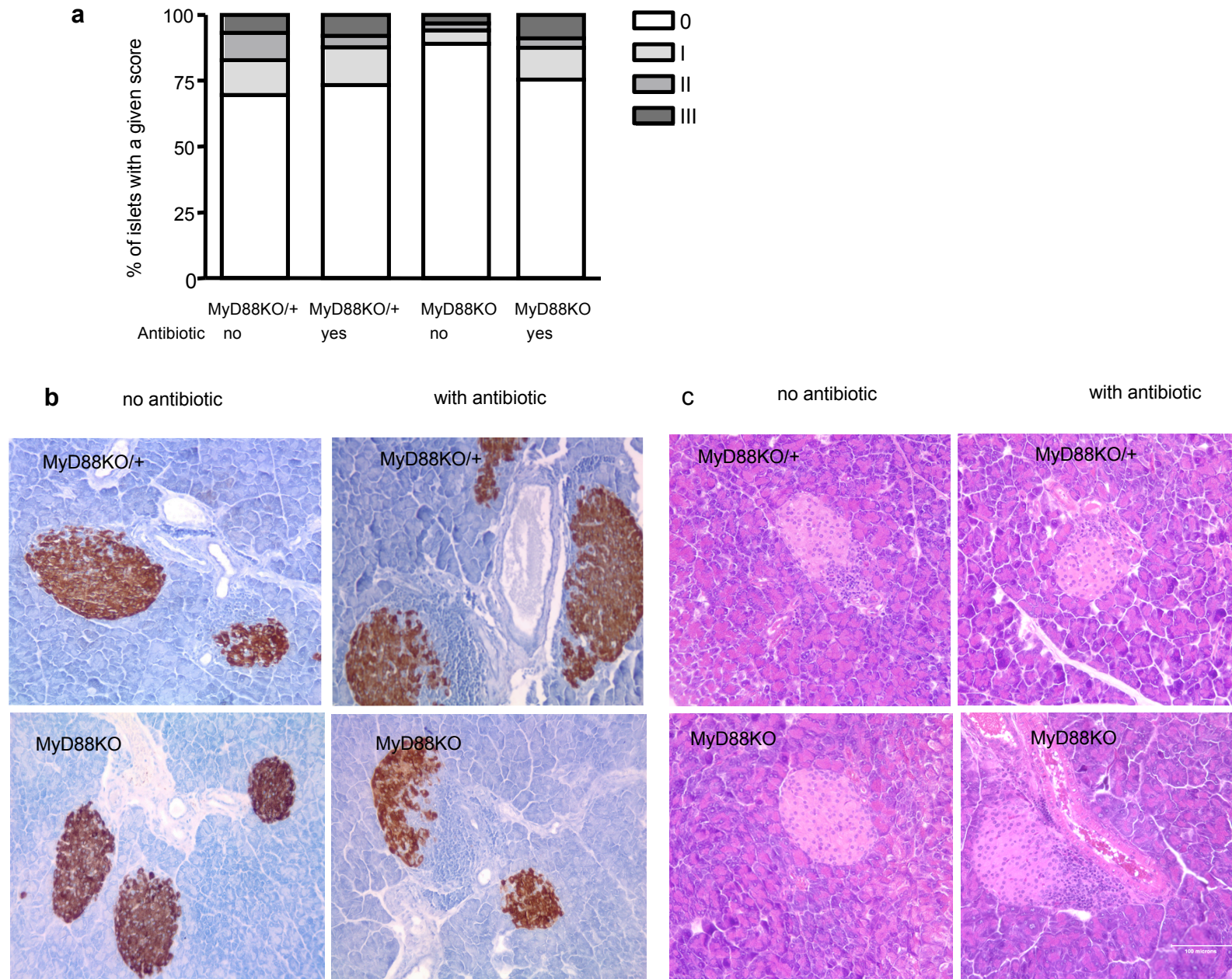
0 - Intact islets

I - peri-insulinitis

II - <50% infiltration

III > 50% infiltration to full destruction





Supplemental Figure 7

