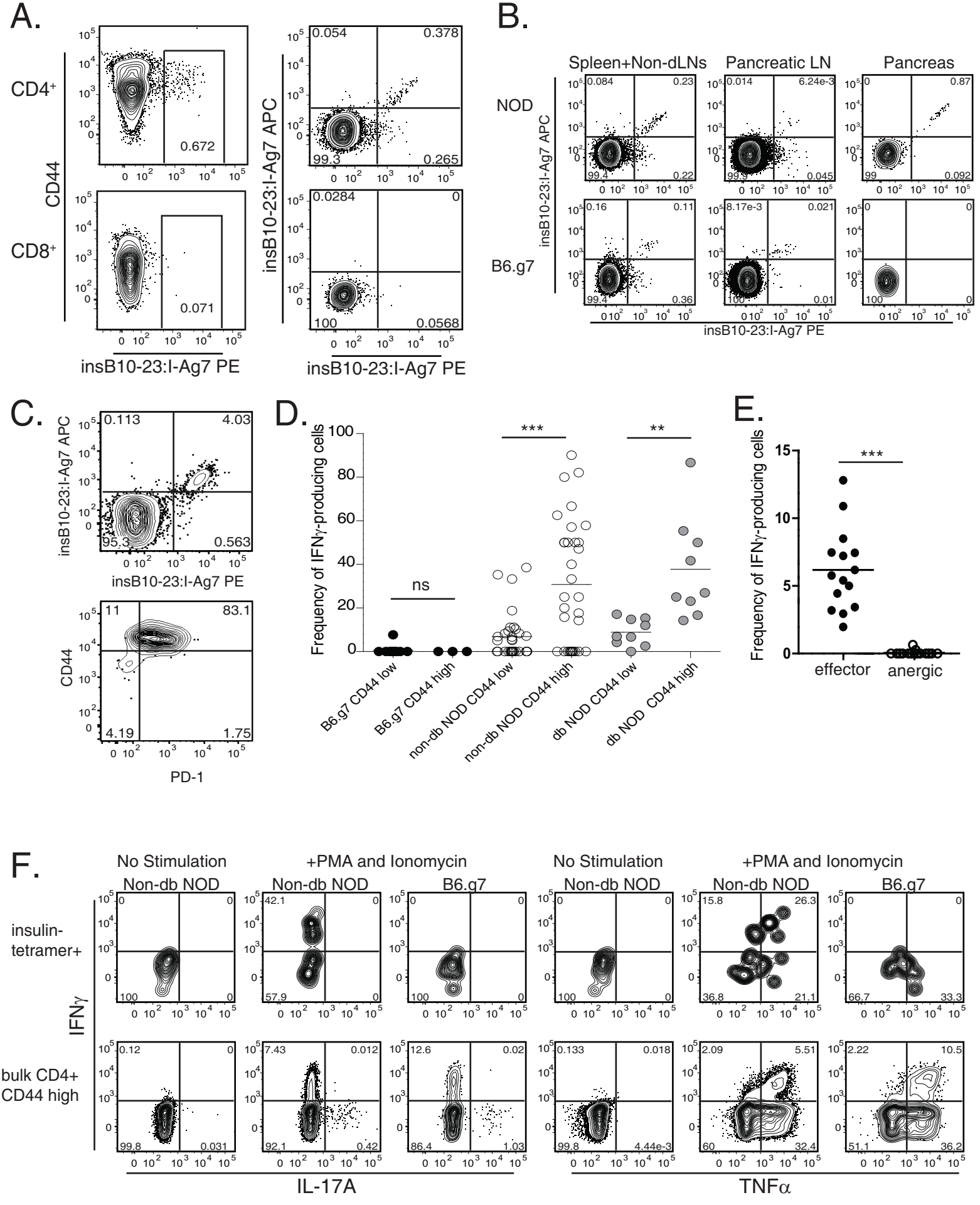
Supplemental Figure 1



Supplemental Data Figure 1

Flow cytometric staining of tetramer positive $CD4^+$ T cells and intracellular cytokines.

(A) (Left) CD44 and $InsB_{10\text{-}23r3}$:I-A^{g7} staining from $CD4^+$ (top) and $CD8^+$ T cells (bottom) from the spleen and non-draining LNs of a non-diabetic NOD. (Right) Double tetramer staining with the $InsB_{10\text{-}23r3}$:I-A^{g7} tetramers in PE and allophycocyanin (APC). Data is representative of fifty-two mice from fifteen independent experiments. Cells were gated on singlet$^+$, $CD3^+$,$B220^-$, $CD11b^-$, $CD11c^-$, and $CD4^+$ or $CD8^+$ cells.

(B) Flow cytometry plots from the spleen combined with non-draining LNs, pancreatic LN (pLN), or pancreas of non-diabetic NOD (20 week old non-diabetic mouse representative of 8 mice from five experiments) and B6.g7 (21 week old mouse representative of 8 mice from three experiments). Double tetramer staining with the $InsB_{10\text{-}23r3}$:I-A^{g7} tetramers in PE and APC. Cells were gated on singlet$^+$, $CD3^+$, $B220^-$, $CD11b^-$, $CD11c^-$, and $CD4^+$.

(C) Flow cytometry plots from the spleen combined with non-draining LNs and the pLN of a NOD mouse primed with whole insulin protein emulsified in CFA (25μg/mouse). (Top) Double tetramer staining with the $InsB_{10\text{-}23r3}$:I-A^{g7} tetramers in PE and APC. Cells were gated on singlet$^+$, $CD3^+$ $B220^-$, $CD11b^-$, $CD11c^-$, and $CD4^+$. (Bottom) CD44 and PD-1 expression on insulin-specific CD4+ T cells from the upper right quadrant of the top flow plot. Cells are gated on singlet$^+$, $CD3^+$, $B220^-$, $CD11b^-$, $CD11c^-$, and $CD4^+$ $InsB_{10\text{-}23r3}$:I-A^{g7} PE^+ and APC^+. Data are representative of 14 NOD and 14 B6.g7 mice (9-10 weeks old) from five independent experiments.

(D) Frequency of $CD44^{high}$ or $CD44^{low}$ insulin-specific $CD4^+$ T cells from combined SLO (spleen+non-draining LN+pLN) producing IFNγ. Data are compiled from nine experiments with B6.g7 (n=12), non-diabetic NOD (n=28), and diabetic NOD (n=9). Cells were gated on singlet$^+$, $B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$, $InsB_{10\text{-}23r3}$:I-A^{g7}-PE^+ and $InsB_{10\text{-}23r3}$:I-A^{g7}-APC^+ and either $CD44^{high}$ or $CD44^{low}$.

(E) Frequency of effector phenotype ($FR4^-CD73^-CD44^{high}Foxp3^-$) and anergic phenotype ($FR4^+CD73^+$ $CD44^{high}Foxp3^-$) IFNγ-producing $CD4^+$ T cells from the spleen of pre-diabetic NOD mice following 2-4 hours in vivo stimulation with 100 μg anti-CD3 intravenously (clone 145-2C11, Bio X Cell). Data are compiled from three independent experiments with a total n=15 mice.

(F) Flow cytometric plots of cytokine staining from insulin-specific $CD4^+$ T cells (top) compared to polyclonal $CD4^+$ $CD44^{high}$ cells (bottom). Cells were pooled from pLN, spleen, and non-draining LNs of non-diabetic NOD or B6.g7 mice. Data are representative of nine experiments with B6.g7 (n=12), non-diabetic NOD (n=28), and diabetic NOD (n=9). Cells were gated on singlet$^+$, $B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$, $CD44^{high}$, $InsB_{10\text{-}23r3}$:I-A^{g7} PE^+ and APC^+ cells (Top row), or singlet$^+$, $B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$. tetramer$^-$, $CD44^{high}$ (bottom row).

Supplemental Table I. Quantification of insulin-specific $CD4^+$ T cells in NOD and B6.g7 mice.

A. Total Tetramer positive cells

NOD	Spleen+non-draining LNs	Pancreatic LN	Pancreas
3 wks	200 ± 26	185 ± 35	2.5 ± 1
5 wks	355 ± 36	211 ± 19	1.8 ± 0.6
14 wks	1416 ± 739	377 ± 71	130 ± 79
20 wks	618 ± 72	183 ± 33	24 ± 10
Diabetic	1156 ± 486	356 ± 64	182 ± 83
B6.g7	**Spleen+non-draining LNs**	**Pancreatic LN**	**Pancreas**
3 wks	159 ± 36	28 ± 6	0 ± 0
5 wks	166 ± 19	102 ± 18	0 ± 0
20 wks	184 ± 22	86 ± 27	0 ± 0

B. $CD44^{high}$ Tetramer positive cells

NOD	Spleen+non-draining LNs	Pancreatic LN
3 wks	69 ± 16	48 ± 18
5 wks	122 ± 16	132 ± 19
14 wks	878 ± 588	201 ± 49
20 wks	218 ± 47	53 ± 10
Diabetic	605 ± 336	139 ± 43
B6.g7	**Spleen+non-draining LNs**	**Pancreatic LN**
3 wks	8 ± 2.5	3 ± 1
5 wks	11 ± 3.5	6 ± 3
20 wks	13 ± 4	5 ± 1.5

(**A**) $InsB_{10\text{-}23r3}$:$I\text{-}A^{g7}$ cells that bind both the PE and allophycocyanin (APC) tetramer (double positive) in the spleen and non-dLNs, pLN, and pancreas of NOD and B6.g7 mice. Cells are gated on singlet$_+$, $CD3^+$,$B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$, $InsB_{10\text{-}23r3}$:$I\text{-}A^{g7}$ PE^+ and $InsB_{10\text{-}23r3}$:$I\text{-}A^{g7}$ APC^+. Non-diabetic NOD data at 3 weeks (n=9), 5 weeks (n=13), 14 weeks (n=13), and 20 weeks (n=17). Diabetic NOD data is from six mice. Data from B6.g7 mice at 3 weeks (n=4), 5 weeks (n=7), and 20 weeks (n=8). (**B**) Cells from (A) are gated on $CD44^{high}$.