Serpine1 negatively regulates Th1 cell responses in experimental autoimmune encephalomyelitis.

(Running title: Serpine1 negatively regulates Th1 cell responses in EAE)

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Abstract

Th1 cells are critical in experimental autoimmune encephalomyelitis (EAE). Serpine1 has been posited as an inhibitor of IFNγ from T cells though its role in autoimmunity remains unclear. Here, we show that *Serpine1*$^{-/-}$ mice develop EAE of enhanced severity relative to wild-type controls. *In vitro*, Serpine1 transcript and protein are upregulated in Th1 cells. *In vivo*, *Serpine1*$^{-/-}$ x 2D2 Th1 cells transfer EAE of increased severity in comparison to *Serpine1*$^{+/+}$ x 2D2 Th1 cells. Notably, polarized *Serpine1*$^{-/-}$ Th1 cells display delayed expression of the Th1-specific inhibitory receptor, Tim-3. *Serpine1*$^{-/-}$ Tim-3-Tg Th1 cells, which transgenically over-express Tim-3, showed increased expression of IFNγ and reduced expression of the checkpoint molecules Lag-3 and PD-1. Further, Serpine1 deficiency restored the EAE phenotype of Tim-3-Tg mice that normally develop mild disease. Together, we identify Serpine1 as a negative regulator of Th1 cells.

Key points

- Serpine1 inhibits EAE in a T cell-dependent manner.
- Serpine1 is upregulated in Th1 cells and inhibits their pathogenicity.
- Serpine1 promotes expression and function of Th1-specific inhibitory receptor Tim-3.

48

49 **Introduction**

50 Effector CD4$^+$ Th1 cells are characterized by IFNγ production, recruit macrophages and
51 CD8$^+$ T cells to sites of infection and inflammation and are potent initiators and propagators of
52 autoimmune diseases such as in the T cell-driven EAE[1] model of MS[2] (1). Th1 cells express a
53 lineage-specific inhibitory receptor, Tim-3[3], that resolves inflammatory responses when triggered
54 at sites of inflammation (2-4). The Tim-3 pathway is crucial to repressing EAE pathology (2, 5,
55 6). Understanding the processes regulating Tim-3 expression and function might permit us to
56 develop strategies to curb Th1-mediated inflammation in self-tissue.

57 Serpine1[4], or PAI-1[5], represses the conversion of the plasminogen proenzyme into mature
58 plasmin and was previously suggested to regulate IFNγ-driven T cell responses (7, 8). Here, we
59 show that Serpine1 restrains EAE pathogenicity via its inhibitory effects on Th1 cells, and that
60 Serpine1 is required for optimal expression of Tim-3 by Th1 cells.

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62

63

64 **Materials and Methods**

65

66 *Mice*

67 Tim-3-Tg (5) are described. B6[6] (*Serpine1*$^{+/+}$; stock #000664), *Serpine1*$^{-/-}$ (B6.129S2-
68 *Serpine1*^{tm1Mlg}/J; #002507), 2D2-Tg (C57BL/6-Tg (Tcra2D2,Tcrb2D2)1KuchJ; #006912) and
69 *Rag1*$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}/J; #002216) mice were obtained from Jackson Labs. Experimental
70 and control animals were co-housed at the animal facilities of CRCHU de Québec-Université
71 Laval or Brigham & Women's Hospital. All procedures were authorized by the Animal Care
72 Committee of Université Laval (CPAUL-3) or the Institutional Animal Care and Use Committee
73 (IACUC) of Harvard University.

74

75 *T helper cell differentiation*

76 CD4^{+} T cells were enriched from B6 spleens using anti-CD4 Microbeads (Miltenyi), purified as
77 CD4+CD62L^{hi} using a FACSAria (BD) high-speed cell sorter and cultured in supplemented T cell
78 media as described (3). They were differentiated (9) for 2 days with plate bound anti-CD3 and
79 anti-CD28 (2 μg mL^{-1} each; BioXcell) into Th1 cells – 10 ng mL^{-1} rmIL-12 (R&D Biosystems)
80 plus anti-IL-4 (10 μg mL^{-1} , BioXcell), Th17 – rhTGFβ (3 ng mL^{-1}, Miltenyi) + rmIL-6 (20 ng
81 mL^{-1}, Miltenyi) + anti-IFNγ (10 μg mL^{-1} , BioXcell), Treg[7] – rhTGFβ (3 ng mL^{-1}). Cells were
82 then transferred to uncoated tissue culture plates and cultured for an additional 3 days, with rmIL-
83 2 (10 ng mL^{-1}, Miltenyi) added to Th1, and rmIL-23 (20 ng mL^{-1}, R&D Biosystems) added to
84 Th17. Tiplaxtinin (25 μM, Tocris) or equivalent volume of DMSO were maintained for 5 days of
85 culture where indicated. Serpine1 cDNA was cloned into pMIG[8] vector and RV[9] gene transduction
86 of Th1 or Th17 cells was conducted using our described spin-infection protocol (10).

87

88 *EAE*

89 Active immunization was induced by s.c. injection of $MOG_{[35-55]}$[10] (CHU de Québec) in
90 incomplete Freund's adjuvant (Difco) supplemented with 5 mg mL^{-1} *Mycobacterium tuberculosis*
91 extract (Fisher). A dose of 25 μg $MOG_{[35-55]}$ per mouse was used to compare *Serpine1*$^{+/+}$ to
92 *Serpine1*$^{-/-}$ mice, while 100 μg per mouse was used in EAE experiments involving Tim-3-Tg mice.
93 In Figure 1D, passive EAE was induced by first immunizing *Serpine1*$^{+/+}$ 2D2 and *Serpine1*$^{-/-}$ 2D2
94 mice with $MOG_{[35-55]}$. Nine days later, splenocytes were collected and stimulated *ex vivo* for 48
95 hours with $MOG_{[35-55]}$ (20 μg mL^{-1}), rmIL-12 and rmIL-23. Next, $20x10^6$ splenocytes were injected
96 i.p. into unimmunized B6 recipients. Adoptive transfer of 2D2, *Serpine1*$^{-/-}$ 2D2 or RV-transduced
97 2D2 Th1 cells entailed i.v. injection ($2x10^6$) into *Rag1*$^{-/-}$ recipients at d5 of culture. In all EAE
98 experiments, mice received 200 ng pertussis toxin (List Biological Labs) i.p. at d0 and d2. Mice
99 were assessed for clinical symptoms daily as previously described (9).

100

101 *Flow cytometry*

102 Cell surface and intracellular flow cytometry were conducted as previously described (10). The
103 following Abs and dyes were used: *CD4*, clone RM4-5, ThermoFisher (TF) cats #45-0042-82, 48-
104 0042-82; CD62L, MEL-14, TF #47-0621-82, *Tim-3*, RMT3-23, Biolegend #119706; *PD-1*, J43,
105 TF #25-9985-82; *Lag-3*, eBioC9B7W, TF #12-2231-82; *IFNγ*, XMG1.2, TF # 48-7311-82; *TNFα*,
106 MP6-XT22, TF #11-7321-82, 12-7321-41, 17-7321-82; IL-2, JES6-5H4, BD Biosciences
107 #560547; IL-17, TC11-18H10.1, Biolegend #506922; Fixable Viability Dye, TF #65-0865-14; 7-
108 aminoactinomycin D, TF #A1310. Data were collected using an LSRII flow cytometer (BD
109 Biosciences) and were analyzed with FlowJo (BD). Gates were set on fluorescence minus one

110 controls and the following global strategy was used: *i)* singlets were selected based on FSC-H vs
111 FSC-A; *ii)* live $CD4^+$ T cells based on $CD4^+$ Viability Dye^{neg} events, or on $CD4^+$7-
112 aminoactinomycin D^{neg} events. RV-transduced $CD4^+$ T cells were further gated on GFP positivity
113 as indicated in Figure legends. In Figures 4C,D, live $CD4^+$ T cells were gated as $CD4^+$Tim-3^{pos} or
114 $CD4^+$Tim-3^{neg}.
115
116 *Ex vivo assessment of T cell function*
117 Splenocyte cultures from EAE mice were stimulated, or not, with 10 μg mL^{-1} $MOG_{[35-55]}$ for 48
118 hours. For proliferation studies, 1.25 μCi [^{3}H]-thymidine (Perkin-Elmer) was added to each culture
119 well for the last 16 hours. Cytokine supernatant ELISA were conducted using the following
120 capture/detection sets: *IFNγ*, clones RA-6A2/XMG1.2; *IL-2*, JES6-1412/JES6-5H4; *IL-17*, TC11-
121 18H10.1/TC11-8H4. Serpine1 protein was measured using Serpin E1/PAI-1 DuoSet ELISA (R&D
122 Biosystems).
123
124 *Analysis of Serpine1 mRNA*
125 Quantitative PCR analysis from cultured T cells was performed as previously described (10) using
126 a validated primer-probe set to murine *Serpine1* as well as to *Actb* housekeeping control.
127
128 *Statistics*
129 Two-tailed parametric tests were conducted using Prism (GraphPad). Comparisons of 2 groups
130 were made by *t*-test while comparisons of >2 groups were made by ANOVA followed by post-hoc
131 test. In EAE studies, linear regression (6, 10) or area under curve (10) analyses were conducted.
132

133

134 **Results and Discussion**

135

136 *Serpine1 inhibits the severity of EAE.*

137 Upon immunization with $MOG_{[35-55]}$, *Serpine1*$^{-/-}$ mice developed significantly more severe
138 EAE relative to *Serpine1*$^{+/+}$ controls (Figure 1A). When the data were sex-disaggregated,
139 *Serpine1*$^{-/-}$ females showed a significantly worsened disease burden as compared to *Serpine1*$^{+/+}$
140 females, while *Serpine1*$^{-/-}$ males showed a trend towards exacerbated disease relative to
141 *Serpine1*$^{+/+}$ males (Supplementary Figure 1). Notably, antigen-specific proliferation responses to
142 $MOG_{[35-55]}$ were enhanced in peripheral *Serpine1*$^{-/-}$ $CD4^{+}$ T cells prior to clinical onset (Figure
143 1B), as was secretion of IFNγ and IL-2, but not IL-17 (Figure 1C).

144 Serpine1 regulates CNS fibrinolysis (11) and thus heightened EAE severity in *Serpine1*$^{-/-}$
145 mice might be due to its absence in a non-T cell compartment. We thus crossed the *Serpine1*$^{-/-}$
146 strain to 2D2-Tg strain, which bear a $MOG_{[35-55]}$ -specific TCR (12), and immunized *Serpine1*$^{+/+}$
147 2D2 and *Serpine1*$^{-/-}$ 2D2 mice. Prior to disease onset, we isolated splenocytes and restimulated
148 them with Ag plus IL-12 and IL-23. *Serpine1*$^{-/-}$ 2D2 Ag-restimulated blasts induced EAE of
149 significantly greater severity relative to *Serpine1*$^{+/+}$ 2D2 blasts upon adoptive transfer (Figure 1D).

150 Previous studies have been conflicting with respect to the role of Serpine1 in EAE, possibly
151 due to opposing effects on T cells and CNS repair. Treating $MOG_{[35-55]}$-immunized mice with a
152 Serpine1-derived 18mer peptide reduced disease severity; however its effects were not T cell-
153 specific (13). By contrast, a small molecule inhibitor of Serpine1, TM5484, reduced EAE severity,
154 thereby suggesting that Serpine1 augments inflammation; however, this may have been due to
155 CNS-intrinsic effects, as TM5484 can permeate the blood-brain barrier (14). Notably, in the
156 chronic/relapsing Biozzi model of EAE, *Serpine1*$^{-/-}$ mice developed EAE of delayed onset and

157 milder severity, due to superior CNS fibrinolytic capacity compared to controls (11); it is possible
158 that the fibrogenic, pathogenic, properties of Serpine1 outweigh its anti-inflammatory function in
159 this model. Here, we show that Serpine1 represses antigen-specific T cell-driven CNS
160 autoimmunity in a T cell-intrinsic manner.
161
162 *Serpine1 is expressed in Th1 cells and suppresses their function in vitro and in vivo*
163 We next found that *Serpine1* transcript expression was significantly higher in Th1 cells as
164 compared to Th0, Th17 and Treg cells (Figure 2A). Th1 cells can be driven towards a highly
165 differentiated phenotype by repeated stimulation of TCR and CD28 (2). We observed that *Serpine1*
166 mRNA was sharply upregulated in Th1 cells upon a second round of differentiation (Figure 2B).
167 Analysis of Serpine1 secretion from differentiated Th1 cells confirmed these kinetics at the protein
168 level (Figure 2C).
169 Serpine1-deficient T cells can produce increased levels of IFNγ relative *in vivo* (7, 8);
170 however, the functional role of Serpine1 in differentiated Th1 cells has never been directly
171 assessed. Upon RV OE[11] of Serpine1 in Th1 cells, we observed a downregulation in expression of
172 IFNγ and a trend towards reduced TNFα relative to control transduced cells. By contrast,
173 overexpression of Serpine1 in Th17 cells did not impact expression of either IL-17 or TNFα
174 (Figure 2D).
175 To determine whether enforced expression of Serpine1 altered Th1 cell pathogenicity, we
176 transduced 2D2 Th1 cells with Serpine1-OE or control RV and adoptively transferred these cells
177 to *Rag1*$^{-/-}$ mice (6). Interestingly, Serpine1-OE 2D2 Th1 cells induced disease of lessened severity
178 compared to control cells (Figure 2E), characterized by a reduced frequency of inflammatory
179 IFNγ^{+}IL-2^{+} T cells *in vivo* (Figure 2F).

180 We next treated Th1 cells with tiplaxtinin, a small molecule that inhibits the antiproteolytic
181 activity of Serpine1 towards the plasminogen activators urokinase and tissue plasminogen
182 activator (15, 16). Tiplaxtinin enhanced IFNγ and IL-2 production from Th1 cells (Figure 3A),
183 suggesting that the Serpine1 may downregulate Th1 responses by repressing the activation of
184 mature plasmin. We then generated Th1 cells from *Serpine1*$^{+/+}$ 2D2 and *Serpine1*$^{-/-}$ 2D2 mice and
185 found that *Serpine1*$^{-/-}$ 2D2 Th1 cells induced EAE of significantly greater severity upon adoptive
186 transfer (Figure 3B).

187 Increased IFNγ was previously observed from *Serpine1*$^{-/-}$ CD4^{+} and CD8^{+} T cells upon
188 LPS or staphylococcal enterotoxin B treatment *in vivo* (7). Further, *Serpine1*$^{-/-}$ mice are resistant
189 to nasal allergy in a Th2-mediated OVA sensitization model, with IFNγ production upregulated
190 by *Serpine1*$^{-/-}$ splenocytes upon Ag recall (8). Here, we show that Serpine1 is expressed on Th1
191 cells and that it downregulates their inflammatory cytokine production and autoimmune potential.
192

193 *Serpine1 promotes Tim-3 expression and inhibits Tim-3*$^{+}$ *Th1 cell inflammatory responses.*

194 As Serpine1 inhibits Th1-driven inflammation, we next asked whether it could augment
195 the expression of inhibitory Tim-3. Repeatedly polarized *Serpine1*$^{-/-}$ Th1 cells expressed lower
196 Tim-3, and with delayed kinetics, relative to *Serpine1*$^{+/+}$ controls (Figure 4A). Loss of Serpine1
197 signaling did not increase expression of IFNγ; however, the frequency of IFNγ^{+}Tim-3^{+} Th1 cells
198 was strikingly lower in its absence (Figure 4B).

199 Tim-3-Tg mice ectopically overexpress Tim-3 cDNA in a T cell-restricted manner, without
200 the need for multiple rounds of polarization under Th1 conditions (17). Reasoning that this might
201 help us uncover Serpine1-dependent differences in IFNγ expression, we crossed *Serpine1*$^{-/-}$ mice
202 to the Tim-3-Tg strain and generated Th1 cells from these and Tim-3-Tg control mice. Tim-3^{pos}

203 Th1 cells derived from *Serpine1*$^{-/-}$ Tim-3-Tg mice were strikingly more positive for IFNγ than their
204 *Serpine1*$^{+/+}$ Tim-3-Tg counterparts; notably, no differences were observed with Tim-3^{neg} Th1 cells
205 between the strains (Figure 4C). This indicated that Serpine1 represses IFNγ expression in a Tim-
206 3-dependent manner. Interestingly, there was a concomitant reduction in the expression of the T
207 cell negative regulatory receptors PD-1 and Lag-3 in *Serpine1*$^{-/-}$ Tim-3-Tg Th1 cells when
208 compared to *Serpine1*$^{+/+}$ Tim-3-Tg controls (Figure 4D).

209 Tim-3-Tg mice develop EAE of attenuated severity (5). To examine whether loss of
210 Serpine1 expression could reverse this phenotype, we actively immunized *Serpine1*$^{+/+}$,
211 *Serpine1*$^{+/+}$ Tim-3-Tg and *Serpine1*$^{-/-}$ Tim-3-Tg mice. While *Serpine1*$^{+/+}$ Tim-3-Tg mice
212 developed disease of only mild severity, *Serpine1*$^{-/-}$ Tim-3-Tg mice displayed EAE that was of
213 comparable severity to that seen in wild-type animals (Fig 4E). Our data thus demonstrate that
214 Serpine1 is required for Tim-3-mediated repression of T cell inflammation and pathogenicity in
215 EAE.

216 Tim-3 marks exhausted T cells in chronic viral infections and cancers (18-20) and is
217 functionally tractable, as concomitant blockade of Tim-3 and PD-1 causes tumor regression greater
218 degree than that with anti-PD-1 alone (20). Further, depletion of Bat3, an intracellular repressor of
219 Tim-3 signaling, causes Th1 cells to adopt an exhausted-like phenotype *in vivo* (6, 21). While
220 Serpine1-mediated upregulation of Tim-3 may be desirable in the context of autoimmunity, it
221 remains to be seen what role, if any, Serpine1 plays in T cell exhaustion.

222 Altogether, our data identify a novel Th1 cell-intrinsic regulatory mechanism. Serpine1
223 represses Th1 cell pathogenicity by inhibiting their production of inflammatory cytokines and by
224 restraining them from adopting a highly differentiated Tim-3^{+} phenotype. Strategies to augment

225 Serpine1 expression and function in Th1 cells could present an attractive therapeutic option in
226 autoimmune disease.
227
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231
232 **Author Contributions**[12]
233
234 **Data availability statement**[13]
235 **Conflicts of interest**[14]
236
237
238

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[1] Experimental autoimmune encephalomyelitis
[2] Multiple sclerosis
[3] T cell immunoglobulin and mucin domain-containing-3
[4] Serine protease inhibitor clade E1
[5] plasminogen activation inhibitor 1
[6] C57BL6/J
[7] T regulatory cell
[8] pMSCV-IRES-GFP
[9] retroviral
[10] Myelin oligodendrocyte glycoprotein, amino acids 35-55
[11] overexpression

[12] I.A. conducted experiments and managed the project. R.T., J.B., A-P.R., P.M.I.A.D and C.Z. conducted experiments. V.K.K. co-supervised the project. M.R. conducted experiments, supervised the project and wrote the manuscript.

[13] The datasets generated for this study are available from the corresponding authors upon reasonable request.

[14] V.K.K. has an ownership stake and is a member of the Scientific Advisory Board for Tizona Therapeutics. Further, he is a co-founder of, and has an ownership stake in, Celsius Therapeutics. In addition, he is an inventor on patents related to Th17 cell function. His interests are reviewed and managed by Brigham & Women's Hospital and Partners Healthcare in accordance with their conflict of interest policies.

319

320 **Figure Legends**

321

322 **Figure 1. Serpine1 inhibits the severity of EAE. A.** *Left,* Representative EAE curve of $MOG_{[35-}$
323 $_{55]}$-immunized *Serpine1*$^{+/+}$ (n=10) and *Serpine1*$^{-/-}$ (n=8) female mice were monitored for signs of
324 EAE. *Left*, mean disease course. *Middle*, linear regression curves of the representative disease
325 courses. The dashed lines indicate the 95% confidence intervals for each curve. *Right*, area-under-
326 curve (AUC) comparison of mice pooled from 3 experiments. Black filled and open circles, female
327 mice (n=12, each genotype); grey filled and open circles, male mice (n=10, each genotype). ***,
328 p<0.001; two way-ANOVA analysis of genotype as a variable. **B.** $MOG_{[35-55]}$-immunized female
329 *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice (n=3 each) were sacrificed 10 days post-immunization, and lymph
330 node cells were assessed for proliferation over 48 hours in response to $MOG_{[35-55]}$. cpm, counts per
331 minute. **, p<0.01, *t*-test. **C.** Splenocytes from immunized female *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$
332 mice (n=3 each) were restimulated with $MOG_{[35-55]}$ for 48 (IL-2) or 72 (IFNγ, IL-17) hours, and
333 secretion of the indicated cytokines was measured by ELISA. *, p<0.05; **, p<0.01, *t*-test. **D.**
334 Splenocytes from female *Serpine1*$^{+/+}$ 2D2 and *Serpine1*$^{-/-}$ 2D2 mice were restimulated with
335 $MOG_{[35-55]}$ in the presence of IL-12 and IL-23, prior to adoptive transfer to WT mice (n=5 each
336 condition) that were monitored for signs of EAE. Right graph, linear regression analysis with 95%
337 confidence intervals. ****, p<0.0001.

338

339 **Figure 2. Serpine1 is expressed in Th1 cells and suppresses Th1-driven EAE. A.** Naïve
340 $CD4^{+}CD62L^{hi}$ T cells were isolated from female B6 mouse spleen and were differentiated under
341 Th0, Th1, Th17 and T_R conditions. Expression of *Serpine1* was assessed by qPCR after 5 days
342 from distinct triplicate cultures. **, p<0.01, Tukey's multiple comparisons test after one-way

343 ANOVA. **B.** Naïve splenic female B6 T cells were differentiated under Th1 conditions over 5
344 days, and expression of *Serpine1* mRNA was assessed by qPCR at the indicated timepoints. ****,
345 $p<0.0001$, one way ANOVA. Representative of 2 experiments. **C**. Naïve splenic female B6 T cells
346 were differentiated under Th1 conditions over 5 days, and secretion of Serpine1 protein was
347 measured in culture supernatant by ELISA. *, $p<0.05$, one way ANOVA. Representative of 2
348 experiments. **D.** Female B6 Th1 or Th17 cells were transduced with control or Serpine1-OE RV.
349 Cells were analyzed for production of the indicated cytokines after 5 days. Gated on GFP-positive
350 live events. Quantitation represents paired *t*-test analysis of 3 independent experiments. *, $p<0.05$.
351 **E.** Female 2D2 Th1 cells were transduced with control- (n=5) or Serpine1-OE (n=4) RV. After 5
352 days of stimulation, cells were adoptively transferred to *Rag1*$^{-/-}$ recipients who were monitored for
353 signs of EAE. *Bottom*, linear regression curves and 95% confidence intervals, for the disease
354 courses. **F.** At disease endpoint (d35), splenic CD4^{+} T cells from mice in (**E**) were assessed for
355 production of IFNγ and IL-2 by flow cytometry. Gated on live CD4^{+} events. **, $p<0.01$, *t*-test.
356
357 **Figure 3. Loss of Serpine1 function or expression exacerbates Th1 responses. A.** Female B6
358 Th1 cells were treated with DMSO (control) or 25 μM tiplaxtinin for 5 days, at which point IFNγ
359 and IL-2 were measured by flow cytometry. *, $p<0.05$, t-test; 3 independent observations. **B.**
360 Female *Serpine1*$^{+/+}$ 2D2 and *Serpine1*$^{-/-}$ 2D2 CD4^{+} Th1 cells were transferred i.v. to *Rag1*$^{-/-}$
361 recipients ($2x10^{6}$ cells/mouse). n=4, *Serpine1*$^{+/+}$ 2D2; n=5, *Serpine1*$^{-/-}$ 2D2. Mice were
362 subsequently assessed for signs of EAE. Left graph, linear regression curves of the disease courses.
363
364 **Figure 4. Serpine1 promotes Tim-3 expression and inhibits Tim-3^{+} Th1 cell inflammatory**
365 **responses. A, B.** Female *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ T cells were subjected to 4 successive rounds

366 of Th1 polarization, and Tim-3 (**A,B**) and IFNγ (**B**) were assessed by flow cytometry. **(A)**
367 Quantification of Tim-3 expression at the end of each round. **, p<0.01, one-way ANOVA. **B.**
368 Expression of Tim-3 versus IFNγ after 20 days of total culture (end of round 4). Gated on live
369 $CD4^+$ events. Representative of 4 experiments. **C.** Splenic T $CD4^+$ T cells from male *Serpine1*$^{+/+}$
370 Tim-3-Tg and *Serpine1*$^{-/-}$ Tim-3-Tg mice (n=6 independent cultures from each) were differentiated
371 under Th1 conditions for 5 days. CD4, Tim-3 and IFNγ expression were assessed by flow
372 cytometry, with cells first gated on live $CD4^+Tim\text{-}3^{pos}$ or live $CD4^+Tim\text{-}3^{neg}$. ***, p<0.001; *t*-test.
373 **D.** Splenic T $CD4^+$ T cells from male *Serpine1*$^{+/+}$ Tim-3-Tg and *Serpine1*$^{-/-}$ Tim-3-Tg mice (n=3
374 independent cultures from each) were differentiated under Th1 conditions for 5 days. Lag-3 and
375 PD-1 expression were assessed by flow cytometry. *, p<0.05; *t*-test. Gated on live $CD4^+Tim\text{-}3^+$
376 events. **E.** Female *Serpine1*$^{+/+}$ (n=4), *Serpine1*$^{-/-}$ Tim-3-Tg (n=14) and *Serpine1*$^{+/+}$ Tim-3-Tg (n=5)
377 mice were immunized with $MOG_{[35\text{-}55]}$ and were monitored for signs of EAE. *Right graph*, linear
378 regression analysis of the disease curves with Bonferroni's correction applied. Representative of
379 3 immunizations.
380
381 **Supplementary Figure 1.** AUC comparison of $MOG_{[35\text{-}55]}$-immunized Serpine1$^{+/+}$ vs *Serpine1*$^{-/-}$
382 females (right) and males (left). **, p<0.01, *t*-test.
383

Figure 1

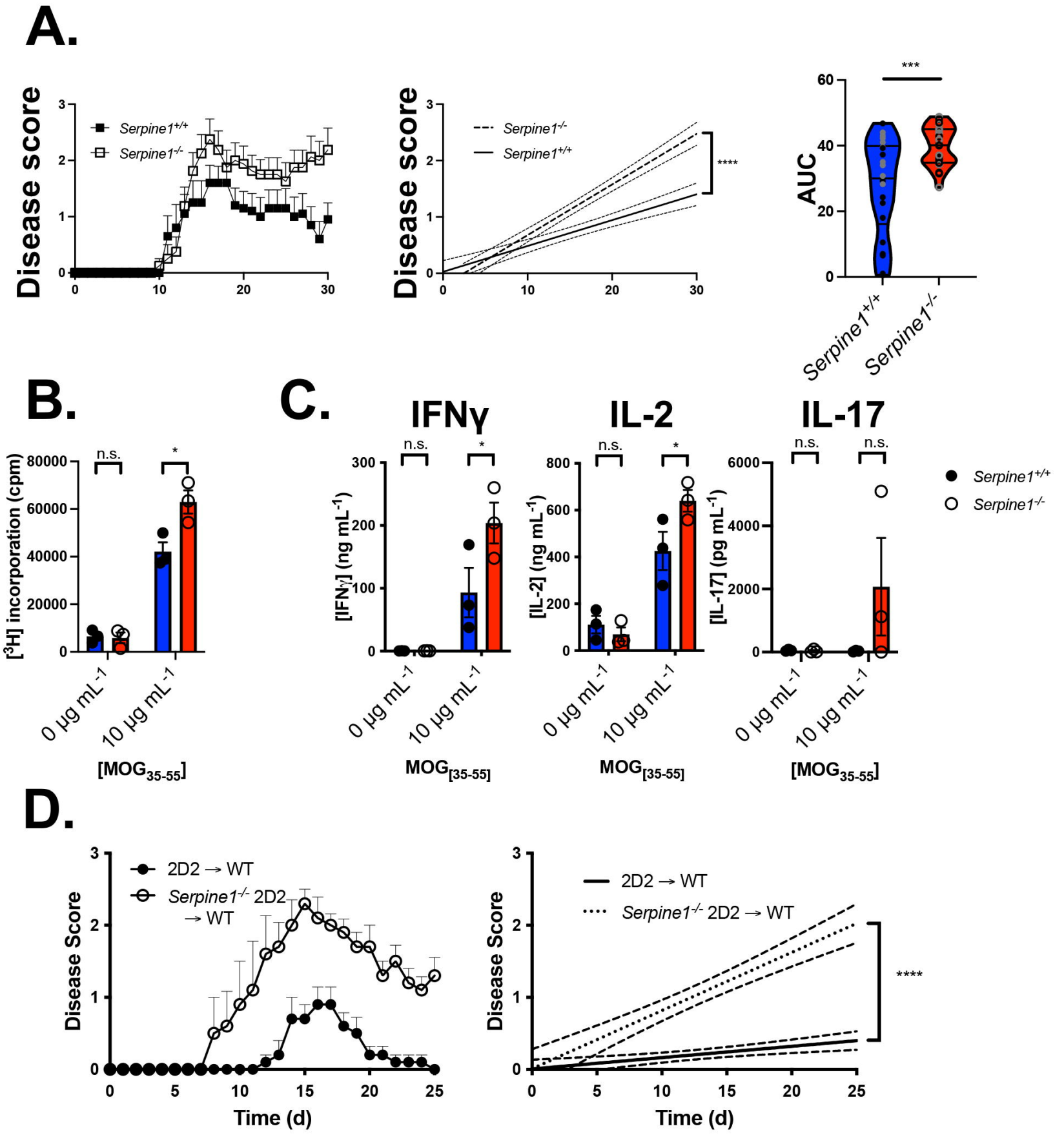

A.
Disease score
Serpine1+/+
Serpine1-/-
Time (d)
Disease score
Serpine1-/-
Serpine1+/+
****
AUC
***
Serpine1+/+
Serpine1-/-
B.
[3H] incorporation (cpm)
n.s.
*
0 µg mL-1
10 µg mL-1
[MOG35-55]
C.
IFNγ
IL-2
IL-17
[IFNγ] (ng mL-1)
[IL-2] (ng mL-1)
[IL-17] (pg mL-1)
n.s.
*
MOG[35-55]
Serpine1+/+
Serpine1-/-
D.
Disease Score
2D2 → WT
Serpine1-/- 2D2 → WT
Time (d)
****

Figure 2

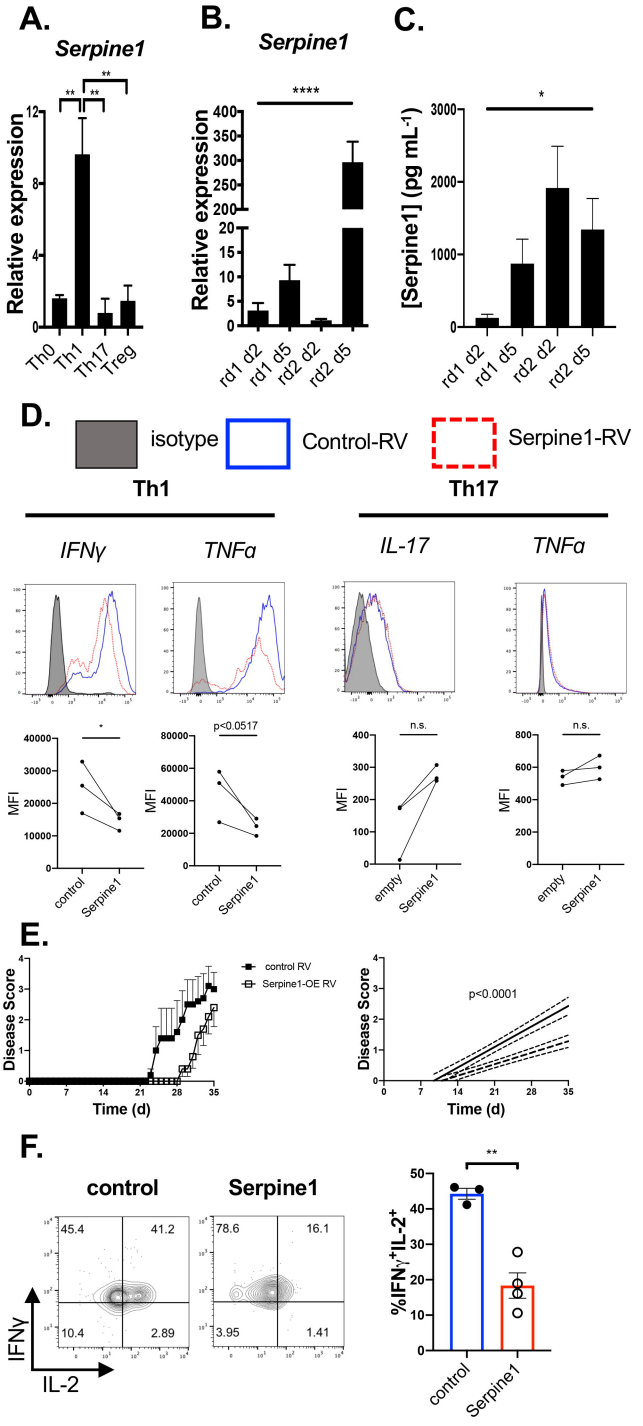

A.
Serpine1
Relative expression
Th0
Th1
Th17
Treg
B.
Serpine1
Relative expression
rd1 d2
rd1 d5
rd2 d2
rd2 d5
C.
[Serpine1] (pg mL-1)
rd1 d2
rd1 d5
rd2 d2
rd2 d5
D.
isotype
Control-RV
Serpine1-RV
Th1
Th17
IFNγ
TNFα
IL-17
TNFα
MFI
control
Serpine1
p<0.0517
n.s.
empty
E.
Disease Score
Time (d)
control RV
Serpine1-OE RV
p<0.0001
F.
control
Serpine1
45.4
41.2
10.4
2.89
78.6
16.1
3.95
1.41
IFNγ
IL-2
%IFNγ+IL-2+
control
Serpine1

Figure 3

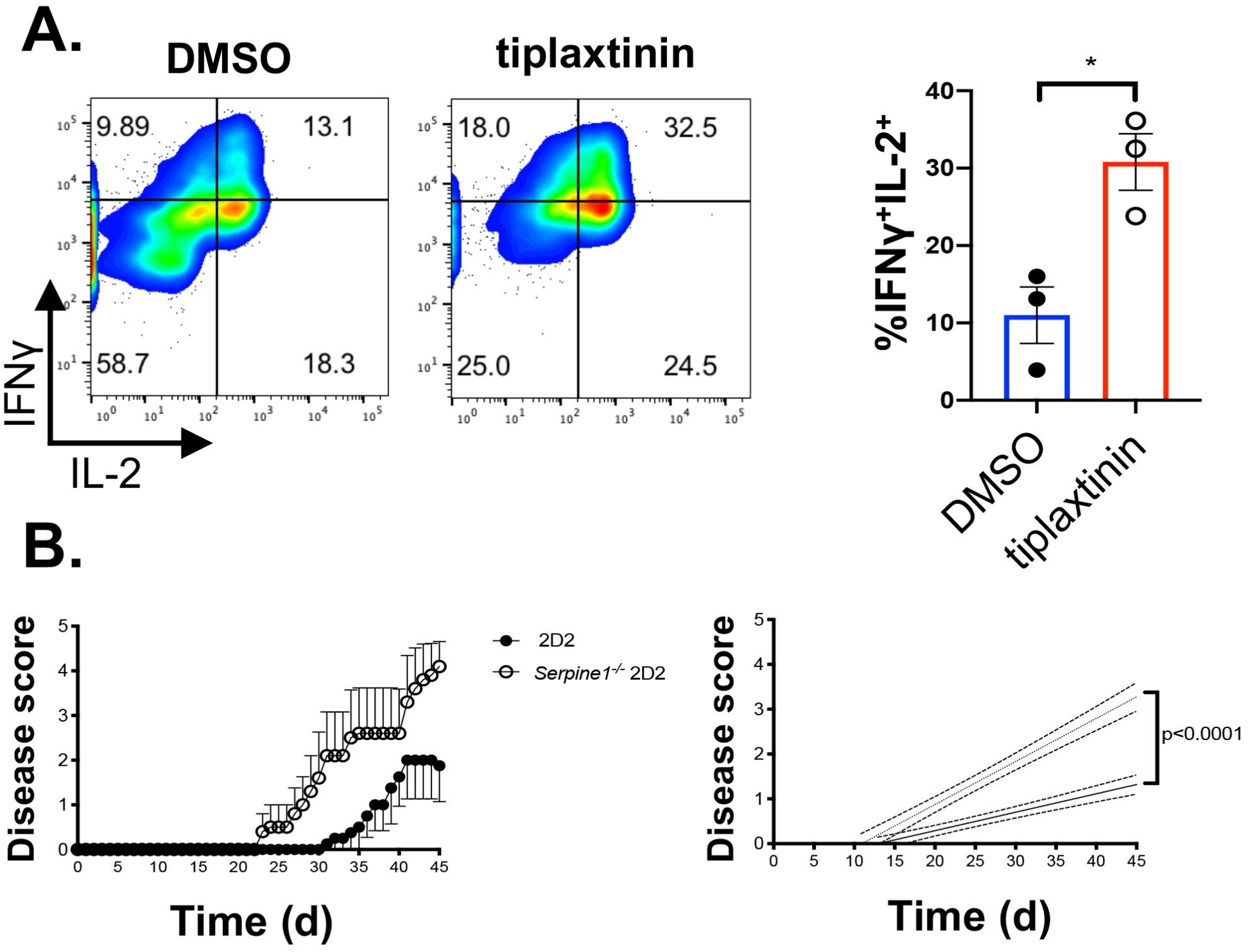
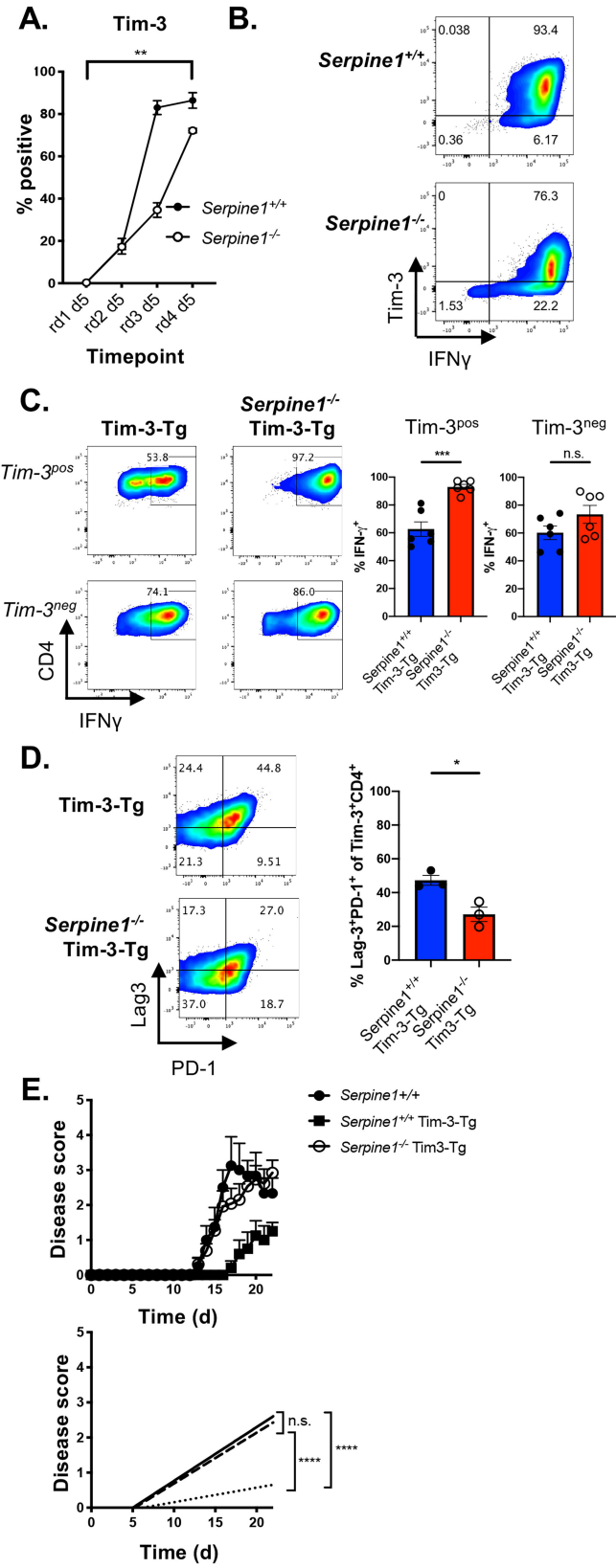

A.
DMSO
tiplaxtinin
9.89
13.1
58.7
18.3
18.0
32.5
25.0
24.5
IFNγ
IL-2
%IFNγ+IL-2+
*
DMSO
tiplaxtinin
B.
Disease score
Time (d)
2D2
Serpine1-/- 2D2
p<0.0001

Figure 4


A.
Tim-3
**
% positive
Serpine1+/+
Serpine1-/-
rd1 d5
rd2 d5
rd3 d5
rd4 d5
Timepoint
B.
Serpine1+/+
0.038
93.4
0.36
6.17
Serpine1-/-
0
76.3
1.53
22.2
Tim-3
IFNγ
C.
Tim-3-Tg
Serpine1-/- Tim-3-Tg
Tim-3pos
Tim-3neg
53.8
97.2
74.1
86.0
CD4
IFNγ
Tim-3pos
Tim-3neg
***
n.s.
% IFN-γ+
Serpine1+/+ Tim-3-Tg
Serpine1-/- Tim3-Tg
D.
Tim-3-Tg
Serpine1-/- Tim-3-Tg
24.4
44.8
21.3
9.51
17.3
27.0
37.0
18.7
Lag3
PD-1
*
% Lag-3+PD-1+ of Tim-3+CD4+
Serpine1+/+ Tim-3-Tg
Serpine1-/- Tim3-Tg
E.
Serpine1+/+
Serpine1+/+ Tim-3-Tg
Serpine1-/- Tim3-Tg
Disease score
Time (d)
n.s.
****
****