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1 **Thymic B cell-mediated attack of thymic stroma precedes Type 1**
2 **Diabetes Development.**

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7

8 **Abstract**

9 Type 1 diabetes results from a co-ordinated autoimmune attack of insulin producing beta
10 cells in the pancreas by the innate and adaptive immune systems, beta cell death being
11 predominantly T cell-mediated. In addition to T cells, peripheral B cells are important in type
12 1 diabetes progression. The thymus of mice and man also contain B cells, and lately they
13 have been linked to central tolerance of T cells. The role of thymic B cells in type 1 diabetes
14 is undefined. Here we show there are abnormalities in the thymic B cell compartment prior to
15 beta cell destruction and type 1 diabetes manifestation.

16 Using non-obese diabetic (NOD) mice, we document that preceding type 1 diabetes
17 development, there is significant accumulation of thymic B cells-partly through in situ
18 development- and the putative formation of ectopic germinal centres. In addition, in NOD
19 mice we quantify thymic plasma cells and observe in situ binding of immunoglobulins to
20 undefined antigens on a significant proportion of medullary thymic epithelial cells. In
21 contrast, no ectopic germinal centres, or pronounced intrathymic autoantibodies are
22 detectable in animals not genetically predisposed to developing type 1 diabetes. Binding of
23 autoantibodies to thymic stroma correlates with apoptosis of medullary thymic epithelial
24 cells, including insulin-expressing cells. In contrast, apoptosis of medullary thymic epithelial
25 cells was decreased by 50% in B cell deficient NOD mice suggesting intrathymic
26 autoantibodies may selectively target certain medullary thymic epithelial cells for destruction.
27 Futhermore, we observe that these thymic B cell-associated events correlated with an
28 increased prevalence of premature thymic emigration of T cells.

29 Together our data suggests that the thymus may be a principal autoimmune target in type 1
30 diabetes and contributes to disease progression.

31

32 **Introduction**

33 The thymus is a primary lymphoid organ involved in shaping the T cell repertoire. Sequential
34 compartmentalisation of developing T cells into the cortical region of the thymus, and
35 subsequently the medulla enable the effective positive and negative selection events,
36 respectively, that are integral in generating an immature T cell repertoire enriched to respond
37 to pathogens but not self-tissue- termed central tolerance [1]. Central to this role for the
38 thymus, are the medullary thymic epithelial cells (mTECS) [2; 3]; capable of autoimmune
39 regulator (AIRE) driven expression of peripheral tissue specific antigens (TSAs) [4] and
40 presentation in the context of MHC class I or class II molecules, they trigger events that lead
41 to apoptosis of developing T cells bearing high affinity receptors for self-peptides.

42 Although extensive studies have documented the importance of mTECs for negative selection
43 of autoreactive T cells [5], other antigen presenting cell (APC) populations within the thymus
44 have also been shown to participate in T cell negative selection, particularly dendritic cells
45 [6]. A newer member of this family of APCs involved in negative selection are the thymic B
46 cells [7; 8], although it is still not clear how significant their role is in negative selection with
47 respect to that of mTECs and thymic DCs [9]. Thymic B cells are present both in man and
48 mice; constituting a minor population of the thymic cellular pool, they are detectable in foetal
49 through to adult mammals [10; 11]. Thymic B cells have a similar phenotype to peripheral
50 B2 cells [12; 13], and their thymic frequency is stable from birth onwards. Interestingly
51 expansion of thymic B cells in Myasthenia and Systemic Lupus Erythematosus (SLE)
52 patients [14; 15], or animal models of SLE have been linked to disease progression,
53 suggesting thymic B cells may have a potential role in breakdown of central tolerance [16].

54 Type 1 Diabetes (T1D) is an autoimmune condition where insulin secreting β cells in the
55 islets of Langerhans are destroyed through co-ordinated attack by both the innate and

56 adaptive immune systems; the final assault being perpetuated by CD8⁺ cytotoxic T cells [17;
57 18; 19]. Defects in central tolerance is linked to emergence of a β cell-specific T cell
58 repertoire [20], yet definitive understanding of the mechanisms underlying defective central
59 tolerance are unclear. Much of our understanding of the immunological events leading to β
60 cell pathology has been derived from the non-obese diabetic (NOD) mouse, a murine model
61 that spontaneously develops T1D with many similarities to those seen in man [21]. Studies in
62 NOD mice show T1D is a progressive condition, with priming of the T cell repertoire to β
63 cell antigens in early life followed by infiltration of islets with immune cells (termed
64 insulinitis), a period of regulation of the autoreactive response, but ultimately an aggressive and
65 sustained attack on the β cells. It is not clear what immunological event triggers this final
66 stage of the disease.

67 B cells, too, are known to be important in the T1D process both in man and in NOD mice;
68 abnormally high numbers of islet-infiltrating B cells is linked to rapid progression to T1D in
69 young children [22], and increasing diversity of serum antibodies for β cell antigens increase
70 substantially the risk factor of developing T1D genetically-predisposed children [23]. In
71 NOD mice, genetic or immunological ablation of B cells protects against T1D development
72 [24; 25], and in both diabetic NOD mice and diabetic patients, depletion of B cells can
73 resolve the condition albeit transiently [26; 27]. To date, the role for B cells in T1D
74 progression has been linked to their peripheral APC function- their ability to present β cell
75 antigens to β -reactive CD4⁺ T cells [28] enhances CD4⁺ T helper cell activation of CD8⁺ T
76 cells, and in islets B cells provide survival signals for activated CD8⁺ T cells enabling a
77 sustained cytotoxic attack on β cells [29].

78 Here we provide that the thymus of diabetes-prone NOD mice displays evidence of
79 autoreactivity prior to type 1 diabetes development. . We show that the post-insulitic/pre-
80 diabetic phase is characterised by abnormally high thymic B cell development, B cell

81 accumulation in follicles at the cortical-medullary junction and the emergence of ectopic
82 germinal centres. Intrathymic autoantibodies bind to undefined antigens on selective mTECs.
83 Subsequently increased mTECs apoptosis, including insulin-expressing mTECs occurs.
84 These events correlate with increased levels of peripheral T cells that have a RAG-GFP
85 phenotype akin to thymocytes that have yet to undergo negative selection, suggesting in NOD
86 mice thymic B cells may contribute to decreased efficacy of negative selection of
87 autoreactive T cells.

88 Our data provides new insights into thymic abnormalities that precede β cell destruction and
89 highlight the importance of focusing research on these unique thymic B cells as mediators of
90 this chronic condition.

91 **Methods**

92 **Mice**

93 C57BL/6 (B6), FVB.RAGp2-GFP reporter mice [30] and NOD. μ MT^{-/-} mice [25] have been
94 described elsewhere. FVB.RAGp2-GFP reporter mice were backcrossed 20 generations to
95 either non-obese diabetic (NOD) mice (NOD.RAGp2-GFP) or NOD. μ MT^{-/-} mice (NOD. μ MT^{-/-}
96 ^{-/-}.RAGp2-GFP mice). All mice used in this study were maintained under specific-pathogen free
97 conditions with a 12 hour light-dark cycle and fed normal chow. All animal experimental
98 procedures were carried out in accordance with the Animals and Scientific Procedures Act
99 1986 were approved by the University of York Animal Welfare and Ethics Review Board and
100 conducted under UK Home Office License approval conforming to ARRIVE guidelines
101 (<https://www.nc3rs.org.uk/arrive-guidelines>). Diabetes development was determined by
102 assessing urine glucose levels using Diastix (Bayer, Inc). All animals used in this study were
103 not diabetic. In addition, only female mice were used.

104

105 **Antibodies and Flow Cytometry**

106 All antibodies, unless otherwise stated, were purchased from e-Bioscience. Single-cell
107 suspensions were incubated with antibodies against CD16/32 unconjugated (93), CD3 FITC
108 (145-2C11), CD3 BV421 (17A2; Biolegend), CD4 eFluor450 (RM4-5), CD4 PE (RM4-5),
109 CD4 BV650 (GK1.5; Biolegend), CD8 α FITC (53-6.7), CD8 β PE-Cy7 (H35-17.2), CD19
110 eFluor450 (6D5), CD19 PE (6D5), CD19 BV421 (1D3; Biolegend), CD21/CD35 PE (4E3),
111 CD23 PE-Cy7 (B3B4), IgM APC (II/41), IgD eFluor450 (11-26c), IgE BV650 (R35-72; BD-
112 Biosciences), IgG PerCP-eFluor 710 (Polyclonal), IgA PE (11-44-2), biotinylated-insulin
113 (ibtsystems), CD45 PerCPCy5 (30-F11), CD45 BV510 (30-F11; Biolegend); PD1 (29F.1A12;

114 Biolegend), ICOS APC (C398.4A), CD138 BV650 (281-2; Biolegend), CD11b FITC (M1/70)
115 , CD11c PE (N418), B220 eFluor450 (RA3-6B2), BCL-6 PerCP-eFluor 710 (BCL-DWN),
116 CXCR5 PE (SPRCL5), IL-21 PE (mhalx21) and Ki67 PE (B5; BD Biosciences). Intracellular
117 labelling of Ki67 was performed using eBioscience kit following manufacturer's guidelines
118 (catalogue number 00-5523-00). Cells were acquired using a BD LSR Fortessa X-20 (BD
119 Biosciences) and data analysed using FlowJo software® (Tree Star). Doublets were excluded
120 using forward light-scatter gating (FSC-A versus FSC-W) followed by gating on cells based
121 on FSC-SSC. Dead cells were excluded by gating on LIVE/DEAD® Fixable Dead Cell
122 Staining (ThermoFisher) negative cells. The gating strategies are described in the paper in the
123 main figures and supplementary figures, explicit in the axis or described in detail in figures
124 legends. The gates were defined using fluorescence minus one and isotype controls: Rat IgG2a
125 eF450 (eBR2a), Rat IgG2a FITC (eBR2a), Rat IgG2a PE (eBR2a), Rat IgG2a PE-Cy7
126 (eBR2a), Rat IgG2a APC (eBR2a), Rat IgG2a BV421 (RTK2758, Biolegend), Rat IgG2a
127 BV650 (RTK2758, Biolegend), Rat IgG2a PerCP-eFluor 710 (eBR2a), Rat IgG2b PE (10H5),
128 Armenian Hamster IgG APC (eBio299Arm), Rat IgG1 Biotin (eBRG1) and Rat IgG1 BV650
129 (RTK2071; Biolegend).

130

131 **Detection of thymic B cells bearing insulin-specific receptors.**

132 The detection of B cells with receptors that bind insulin has been previously described [31].
133 Briefly single cell suspensions isolated from the thymus were incubated overnight at 4°C in
134 PBS supplemented with 1% foetal bovine serum, 1% anti-CD16/32 antibodies (eBiosciences)
135 and biotinylated insulin (0.1 µg/10⁶ cells, ibtsystems). Bound insulin was detected with
136 fluorochrome-labelled streptavidin Alexa6470(Invitrogen) for 30 minutes at 4°C. The cells
137 were subsequently incubated with anti- CD19 PE (6D5; eBiosciences), B220 eFluor450 (RA3-
138 6B2; eBiosciences), - CD4 BV650 (GK1.5; Biolegend), CD8β PE-Cy7 (H35-17.2;
139 eBiosciences), -CD45 PerCPCy5.5 (30-F11; eBiosciences) antibodies and LIVE/DEAD

140 Fixable Dead Cell Stains (Thermo Fisher Scientific) for 30 min at 4°C, following which the
141 cells were analysed by flow cytometry. B cell gates were defined following exclusion of dead
142 cells and T cells (dump channel). All samples were stained with insulin-biotin followed by
143 streptavidin or with streptavidin only, frequencies of B cells insulin⁺ were calculated
144 subtracting the background calculated in sample-matched streptavidin only control.

145

146 **Soluble tissue extracts and Enzyme Linked Immunosorbent Assay (ELISA)**

147 Cell-free supernatants from thymic and splenic tissue were prepared as described [32]. Briefly,
148 single cell suspension were centrifuged at 300g for 10 mins, 4 °C then 15 mins, 4 °C at 3000g.
149 Cell-free supernatants were collected and stored at -20°C until analysis. IL-2 and IL-21
150 cytokines were detected using mouse IL-2 ELISA Ready-SET-Go and mouse IL-21 ELISA
151 Ready-SET-Go ELISA kits following manufacturer guidelines (eBioscience). Isotype
152 classification of immunoglobulins in thymic cell-free supernatants or serum was achieved
153 using a rapid ELISA Mouse mAb isotyping kit (Thermofisher) following manufacturer's
154 guidelines.

155

156 **Cultures**

157 Bone marrow derived dendritic cells (BM-DCs) were prepared from the appropriate mice by
158 standard methodology. Immature DCs were pulsed for 16 hours with whole insulin (Sigma; 5
159 µg/ml), LPS (Sigma; 10ng/ml), or pro-insulin peptide pB15-23 peptide (Thermo Fisher; p4878-
160 1; 5 µg/ml). Thymocytes were prepared from mice described in the results and 1x10⁶
161 thymocytes were co-cultured in complete RPMI media (10% FCS, 50µmol/L β-
162 mercaptoethanol, L-glutamine, 50 units/ml penicillin and streptomycin (Life-Sciences)) with
163 3x10⁴ BM-DCs only, or 3x10⁴ BM-DCs pulsed with insulin or 3x10⁴ BM-DCs pulsed with
164 B15-23 peptide, or anti-CD3 (5µg/ml) (eBioscience) and anti-CD28 (2.5µg/ml) antibodies
165 (eBioscience). The co-cultures were incubated at 37°C, 5% CO₂ for 72 hrs, following which
166 cell proliferation was assessed by flow cytometry. The stimulation index was calculated

167 dividing the frequency of T cells in active proliferation (Ki67⁺) in cells following antigen
168 stimulation by the frequency of T cells in active proliferation in paired non-stimulated culture
169 (background).

170 For the detection of IL-21, single cell suspensions from the appropriate tissues were prepared
171 placed in RPMI media (as above) supplemented with 50ng/ml PMA and 1µg/ml ionomycin for
172 a total of 5 hours at 37°C, 5% CO₂. Brefeldin A (SIGMA) was added to the cultures at a
173 concentration of 0.4mg/ml 1 hr after the initiation of the culture.

174

175 **Immunofluorescence Analysis.**

176 Thymi frozen in OCT compound were sectioned (~8 µm) on a cryostat. Sections were fixed in
177 4% paraformaldehyde or ice-cold acetone then blocked in PBS supplemented with 0.5% BSA.
178 The sections were incubated with unconjugated primary antibodies rabbit-anti mouse IgG
179 (Abcam), rabbit anti-mouse insulin (Abcam) or rabbit anti-mouse cytokeratin V (Abcam)
180 overnight at 4 °C. Detection of bound antibody was achieved with goat anti-rabbit IgG-Alexa
181 647 or goat anti-rabbit Ig-Alexa488 (Invitrogen) or goat anti-rat IgG Alexa 488 (Invitrogen).
182 Anti-B220 directly conjugated with Alexa 647 was incubated for 45 minutes at room
183 temperature. For detection of apoptosis, following incubation with the secondary antibody an
184 in situ apoptosis kit was used (Click-iT™ Plus TUNEL Assay, Alexa Fluor™ 647 dye;
185 Thermofisher) according with the manufacturer instructions. Sections were counterstained with
186 DAPI (Molecular Probes) and mounted in Prolong Gold anti-fade or Prolong Diamond
187 (Invitrogen). Confocal microscopy was undertaken using Zen software on a Zeiss LSM 710
188 fitted on an Axioimager using a 63x (1.4) planApochromat or 20x (0.6) Neofluor. Binding of
189 autoreactive immunoglobulin and TUNEL in microscopy images was quantified using
190 StrataQuest V64 software. Individual nuclei were counted and the data was presented as

191 scatterplots of mean fluorescence intensity of DAPI versus mean fluorescence intensity of Ig
192 or TUNEL positive cells.

193

194 **RNA Isolation and real-time RT-PCR analysis**

195 Thymic tissues were stored at -80°C in RLT. Samples were allowed to thaw and RNA were
196 carried out using the RNeasy mini kits (Qiagen, Manchester, UK), according to the
197 manufacturer's instructions. On-column DNase digestion was carried out to remove any
198 contaminating genomic DNA using the RNase-free DNase set (Qiagen, Manchester, UK)
199 according to the manufacturer's instructions. The cDNA syntheses were performed with the
200 Superscript II reverse transcriptase system (Invitrogen), according to manufacture's
201 instructions. The qRT-PCR of *aicda* mRNA expression (*AID* gene) in total thymus was
202 performed with the Taqman qPCR Kit (Applied Biosystems, Warrington, UK)). mRNA
203 expression levels were normalized to *HPRT1* housekeeping gene using $\Delta\Delta C_t$ calculations.
204 Mean relative mRNA expression levels between control and experimental groups were
205 calculated using the $2^{-\Delta\Delta C_t}$ calculations.

206

207 **Statistical analysis**

208 Statistical analyses were performed by parametric or non-parametric tests, selected based on
209 the distribution of the raw data. The comparisons between experimental groups were performed
210 using student unpaired t-test, Mann-Whitney and one-way ANOVA as appropriate. The
211 statistical analyses for fold-changes were performed using Wilcoxon signed-rank test. All
212 analyses were conducted using GraphPad InStat (version 5) software (GraphPad).

213

214 **Results**

215 **T1D progression correlates with increased intrathymic B cell numbers in NOD mice**

216 Thymic B cells normally constitute a small population of cells within the murine and human
217 thymus in normal individuals. Abnormality in thymic B cell numbers has been linked to
218 certain autoimmune conditions [14; 15]. To determine whether thymic B cell populations
219 differ between diabetes-prone or non-prone mice, we performed time-course flow cytometric
220 studies of age-matched, sex-match NOD and control C57BL/6 (B6) mice.

221 Diabetes incidence in our female NOD mouse colony is 95%, approximately 3% of mice
222 develop T1D at 12-14 weeks of age, 85% at 18-20 weeks of age with the remaining 7% of
223 females progressing to T1D by 23 weeks of age. Animals not diabetic by 23 weeks of age
224 rarely develop T1D. The data is based on a cohort of 200 animals (Supplementary Figure 1a).
225 This diabetes incidence, combined with the insulinitis score- that is the degree of immune cell
226 infiltration of islets and degree of β cell destruction- as mice age (Supplementary Figure 1b)
227 highlight that 12-14 weeks of age in our colony represents late insulitic-preultimate diabetic
228 stage, a critical time when immunoregulation of the autoreactive response starts to
229 breakdown. Thus, in our initial studies we focused on two major time points; the pre-early
230 insulitic phase (4-6 weeks) and the post-insulitic/pre-diabetic phase (12-14 weeks) to assess
231 the presence of CD19⁺ thymic B cells. Representative flow cytometry plots for the respective
232 mice are shown in Figure 1a. Although absolute numbers of CD19⁺ B cells remained
233 relatively static in the thymi of control B6 mice at the time points investigated (Figure 1b),
234 with perhaps a slight increase at 12-14 weeks of age, the absolute numbers of CD19⁺ B cells
235 increased significantly in the later age group of NOD mice in comparison to numbers seen
236 either at 4-6 week old NOD mice or 12-14 week old B6 mice. Importantly, the number of
237 thymic B cells at 4-6 weeks of age was comparable between NOD and control B6 mice.

238 This increased number of thymic B cells in 12-14 week old NOD mice was not related to
239 increased B cell development in the bone marrow, as frequencies of CD19⁺ B cells in this
240 primary lymphoid tissue was comparable between the two strains of mice at both time points
241 investigated (data not shown). These data show that inappropriate accumulation of thymic B
242 cells precedes the overt β cell destruction phase of T1D.

243

244 **Intrathymic signals trigger enhanced B cell development in NOD mice.**

245 Although previous studies have documented the ability of the thymic environment to enable
246 B cell development in non-autoimmune-prone mice, other reports suggest thymic B cells
247 accumulate via periphery B cell migration to the thymus [16; 33]. To determine whether the
248 NOD mouse thymus promotes B cell development we used recombination activation gene
249 green fluorescent protein (RAG2p-GFP) reporter mice on a non-T1D-prone FVB background
250 (hereafter called FVB-RAG-GFP), or on the NOD background (hereafter called NOD-RAG-
251 GFP). In RAG2p-GFP reporter mice, highest GFP expression occurs when RAG genes are
252 active [30]. Once recombination of the B cell receptors and T cell receptors is complete and
253 RAG activity is silenced, GFP expression decreases over a 54 hour period [30]. As such,
254 newly developed B cells can be identified from thymic resident/recirculatory B cells based on
255 the expression of GFP.

256 Since our control RAG2p-GFP transgenic mice are on a FVB background, we compared
257 thymic B cell frequencies and numbers of this alternative control murine strain to control B6
258 mice or NOD mice. Although frequencies and absolute numbers of thymic B cells in the FVB
259 strain were higher than the B6 strain, the NOD strain demonstrated significantly greater
260 thymic B cell frequencies and numbers to the FVB strain (Supplementary Figure 2a-b).

261 We performed time-course, flow cytometry studies of the two strains of mice at the ages
262 shown in Figure 1c, and quantified the number of GFP^{hi} B cells. Representative flow
263 cytometry plots showing the gating strategy for CD19⁺GFP^{hi} B cells is shown in
264 Supplementary Figure 1c. Recently developed CD19⁺GFP^{hi} B cells were readily detectable in
265 both strains of mice at all time points analysed (Figure 1c). In control FVB-GFP mice, there
266 was no significant changes in B cell development as mice aged. In the NOD strain, although
267 there was no significant change in B cell development when the two age groups were
268 compared, it was clear that thymic B cell development is enhanced as mice enter the late
269 insulitic-prediabetic phase of the T1D pathway.

270 In light of evidence that the late insulitic-prediabetic phase is characterised by increased B
271 cell development, we asked if homeostatic proliferation of thymic B cells is also affected as
272 mice enter the late insulitic-prediabetic phase. We performed comparative flow cytometric
273 studies between NOD and control B6 mice, assessing for Ki67 expression as a marker for
274 homeostatic proliferation. Interestingly for both strains of mice, the highest level of
275 homeostatic proliferation of thymic B cells is an early event, with CD19⁺Ki67⁺ B cell
276 frequencies higher in younger mice when compared to older mice (Figure 1d). Further, this
277 decrease in homeostatic proliferation in the 12-14 week old group was more pronounced in
278 NOD mice, although the decrease was not significant.

279

280 **The NOD thymic environment has ectopic germinal centre formation potentiality.**

281 To further investigate the phenotype of thymic B cells in NOD mice we assessed their surface
282 markers. B cells undergo a series of transitions from the immature stage developing follicular
283 or marginal zone properties. Thus, we qualified the phenotype of thymic B cells assessing for
284 follicular (IgM^{lo}IgD⁺CD21/35⁺,CD23⁺) versus marginal zone (IgM⁺IgD^{lo}CD23⁻CD21/35⁺).

285 We focused our studies on 11-14 week old mice due to the evidence that at this age B cell
286 development is enhanced as are thymic B cell numbers in NOD mice when compared to
287 control mice. Representative flow cytometric plots for our gating strategies are shown in
288 Supplementary Figure 2d.

289 As shown in Figure 2, the frequency of follicular B cells within the thymic B cell pool was
290 significantly higher in NOD mice compared to control B6 mice (Figure 2a). This
291 enhancement in follicular B cells in the NOD mouse thymus was recapitulated when absolute
292 number of follicular B cells was calculated (Figure 2b). In contrast, although the frequency of
293 B cells with a marginal zone phenotype were significantly decreased in the NOD mouse
294 thymus compared to control B6 mouse thymus, the absolute numbers of these cells was
295 similar between the two strains of mice.

296 The increased numbers of FO B cells in NOD mice with respect to B6 control mice led us to
297 investigate whether the thymic B cell form follicle-like structures. Immunohistochemical
298 studies revealed B cell follicle-like structures form only in NOD mice (Figure 2c). Initially B
299 cells are detectable at the cortical-medullary junction at 9 weeks of age in NOD mice (not
300 shown) with pronounced accumulation of B cells into follicle-like structures in this location
301 by 11 weeks of age. The presence and location of B cell follicle-like structures was identical
302 irrespective of whether we used anti-B220 or anti-CD19 antibodies to identify B cells (not
303 shown) confirming the accumulating B220⁺ cells are not plasmacytoid DCs. We quantified
304 the number of B cell follicle-like structures in the thymus of 9-11 week old NOD mice; of 15
305 individual sections assessed, 90% contained 1 follicle, 5% two follicles and 5% no follicles.

306 The presence of follicle-like structures in the thymus of late insulitic-pre-diabetic NOD mice,
307 but not control B6 mice, lead us to ask if the thymic environment could support germinal
308 centre formation. Of interest was the relationship between IL-2 and IL-21, the latter being a

309 key mediator of germinal centre formation; the cytokine promotes B cell somatic
310 hypermutation and class switching, and the development and maintenance of T follicular
311 helper cells (T_{fh} cells) [34]. In NOD mice IL-21 has been associated with T1D progression
312 [35; 36] and CD4⁺CD45R⁻ T cells isolated from T1D patients secrete greater quantities of IL-
313 21 than quantified from normal individuals [37]. We prepared cell-free supernatants [32]
314 from the thymi of NOD and control B6 mice at the ages shown in Figure 3a and performed
315 ELISA assays. As a comparison, we analysed cell-supernatants from the spleens of the same
316 mice. The results were tabulated as ratio of IL-21:IL-2. No differences were seen in IL-21:IL-
317 2 ratios in splenic preparations from the two strains of mice. However, the NOD mouse
318 thymus had a significant bias in IL-21 concentrations in comparison to B6 mice.

319 In light of this IL-21 bias, we quantified the frequency and absolute numbers of CD4SP cells
320 that expressed a T_{fh} cell phenotype in the thymus of the two strains of mice. As shown in
321 Figure 3b-c, NOD mice exhibited a significant increase in frequencies and absolute numbers
322 of CD4SPPD1^{hi}ICOS⁺ T cells, and these cells also expressed transcription factor Bcl-6
323 (Figure 3d) and CxCR5 (Supplementary Figure 3a). In addition, approximately 5% of NOD
324 putative thymic T_{fh} cells secreted IL-21, a frequency that was comparable to that seen for
325 splenic T_{fh} cells from the same mice (Supplementary Figure 3b). Furthermore, this increase
326 in thymic T_{fh} cells in NOD mice in comparison to B6 control mice correlated with an
327 increased number of CD4⁻CD8⁻B220^{low/-}CD138⁺ plasma cells in the NOD mouse thymus,
328 although this increased number was not significant (Figure 3e, Supplementary Figure 3c).
329 Together, these data suggested that ectopic germinal centres could be present in the NOD
330 mouse thymus, but absent in control B6 mouse thymus. To support this hypothesis we looked
331 for a bone-fide germinal centre marker; the enzyme activation-induced cytidine deaminase
332 (AID). RNA was prepared from thymi isolated from NOD mice or control B6 mice and
333 quantitative real-time RT-PCR performed. As an additional control we included thymic

334 mRNA isolated from age-matched, sex-matched NOD- μ MT^{-/-} mice. The relative expression
335 of transcripts for AID in NOD mice was normalised to control B6 mice. As shown in Figure
336 3f, the NOD mouse thymi has enhanced AID expression in comparison to control B6 mice.
337 Thus, ectopic germinal centre formation is likely a feature of the NOD thymus and precedes
338 the preultimate β cell destruction phase of T1D.

339

340 **Thymic immunoglobulins binding selective mTECs correlates with mTEC apoptosis.**

341 The presence of AID and enhanced plasma cell frequencies in the NOD thymus with respect
342 to control B6 mice, made us query the immunoglobulin isotype of the thymic B cells and
343 secreted antibodies. Since we previously had investigated the IgM⁺ B cell thymic subtype
344 (Figure 2), this time we focused on class-switched IgM⁻ cells. The number of IgM⁻IgD⁻IgA⁺
345 and IgM⁻IgD⁻IgG⁺ B cells were similar in the thymus of both NOD and control B6 mice as
346 determined by flow cytometry (Figure 4a). In contrast, the number of IgM⁻IgD⁻IgE⁺ B cells
347 were significantly increased in the NOD mouse thymus with respect to control mice.

348 Interesting, a unique population of IgM⁻IgD⁺ B cells (similar to those reported in T1D
349 patients [31]) was detectable in the thymic tissue. These IgM⁻IgD⁻ B cells dually expressed
350 IgG, IgE or IgA with the number of dual expressing IgD⁺IgA⁺ and IgD⁺IgG⁺ B cells being
351 significantly higher in the NOD mouse thymus than the B6 control mouse thymus, the most
352 significant being the IgD⁺IgG⁺ isotype (Figure 4b, Supplementary Figure 4a). In contrast, no
353 differences in IgD⁺IgE⁺ B cell numbers were seen between the two strains of mice. We next
354 assessed the isotype of soluble thymic immunoglobulin by ELISA, in comparison to serum
355 immunoglobulin. Only the IgG1 and IgA immunoglobulin isotypes were enhanced in the
356 thymi of NOD mice in comparison to control B6 mice (Figures 4c,e). In contrast, IgG2a,
357 IgG2b and IgM antibody levels were similar in both strains of mice, with IgG3 antibody

358 levels being slightly lower in NOD mice than in the thymus of B6 control mice. Interestingly,
359 in NOD mice thymus, B cells predominately used the kappa light chain, there being a
360 significant decrease in the presence of lambda light chains when compared to B6 control
361 mice (Supplementary Figure 4c). In addition, this isotype pattern documented in the NOD
362 mouse thymus seemed unique for this tissue, as similar ELISA-based isotyping of
363 immunoglobulins in the serum of the two strains of mice revealed little difference in levels of
364 each isotype assessed (Figure 4d, f). However, similar to the thymus, in the serum there was a
365 significant decrease in lambda light chain usage in NOD mice in comparison to B6 controls
366 (Supplementary Figure 4b). Quantification of the thymic Ig isotypes supported the data that
367 IgG1 and IgA are significantly greater in the thymus of NOD mice with respect to control B6
368 mice (Supplementary Figure 5a).

369 We decided to explore further these thymic B cells to determine whether they harboured
370 receptors specific for islet autoantigens, focusing on their specificity for insulin [31].
371 Representative gating strategy for identifying insulin-reactive B cells is shown in
372 Supplementary Figure 5b. Although the frequency of cells bearing receptors specific for
373 insulin is significantly less in the thymus of NOD mice with respect to control B6 mice
374 within the B cell fraction, absolute numbers of insulin reactive B cells was similar in NOD
375 mice and B6 control mice (Supplementary Figure 5c). Thus, insulin-reactive B cell numbers
376 do not correlate with T1D susceptibility at this time point. Due to this finding, we decided to
377 ask whether thymic B cells produce antibodies that target, as yet, undefined antigens on
378 thymic stroma. Thymic tissue sections from 11 week old NOD mice were incubated with
379 anti-mouse antibodies that would detect any mouse immunoglobulin bound to thymic stroma
380 in situ and bound antibodies were detected by confocal microscopy. To qualify whether any
381 immunoglobulins that bound to thymic stroma interacted with mTECs, we included mTEC-
382 binding anti-cytokeratin V antibodies in the assay. As shown in Figure 5a, there was

383 detectable binding of murine immunoglobulins to thymic stroma, suggesting these cells had
384 murine immunoglobulins bound to them in situ. Interestingly, intrathymic immunoglobulins
385 were bound almost exclusively to cytokeratin V⁺ mTECs and it appeared that only a
386 proportion of mTECs were being targeted by the immunoglobulins. In contrast to NOD mice,
387 there was substantially less intrathymic immunoglobulin in control B6 mice interacted with
388 thymic stroma, particularly cytokeratin V⁺ mTECs (Figure 5b). Further, there was no
389 evidence of intrathymic immunoglobulins bound to thymic stroma, including cytokeratin V⁺
390 mTECs in B cell deficient NOD- μ MT^{-/-} mice confirming the specificity of the anti-mouse
391 antibodies for mouse immunoglobulins (Supplementary Figure 6). We quantified the
392 frequency of cells with murine immunoglobulin bound in the thymus of 11-14 week old NOD
393 and B6 mice. We selected images that had comparative frequencies of cytokeratin V⁺ mTECs
394 and counted $2-3 \times 10^4$ DAPI⁺ cells/mm². As shown in Figure 5c, approximately 7% of cells
395 had bound murine immunoglobulin (Ig) in the NOD mouse thymus. In contrast, the
396 frequency of cells bound by murine immunoglobulins in B6 mice was so low as to be
397 undetectable.

398 Finally we queried the significance of in situ binding of thymic stroma by immunoglobulins,
399 particularly the potential that a selective number of mTECs underwent apoptosis. In this
400 regard, we incubated thymic tissue sections from 11 week old, female NOD mice with
401 antibodies specific to cytokeratin V⁺ and assessed for apoptosis by confocal microscopy
402 following TUNEL staining (Figure 6a). As controls, we similarly analysed thymic tissue
403 sections from control B6 mice and NOD- μ MT^{-/-} mice. The inclusion of NOD- μ MT^{-/-} mice
404 was important to determine whether the diabetes-associated MHC class II molecules unique
405 to NOD mice was sufficient to trigger mTEC apoptosis via non-B cell-mediated mechanisms.
406 Thymic tissue sections from NOD mice had clear evidence of apoptosis, and such apoptotic
407 cells were almost exclusively cytokeratin V⁺ mTECs. Apoptosis of cytokeratin V⁺ mTECs

408 was also evident in NOD- μ MT^{-/-} mice, although the proportion of apoptotic cells seemed
409 lower than that for B cell sufficient NOD mice. In contrast to the NOD strains, we could not
410 see any apoptotic cells in the B6 control mouse thymic tissue section. We quantified the
411 frequency of apoptotic cells in the thymic sections of the respective strains of mice (Figure
412 6). We counted a total of 4×10^4 DAPI⁺ cells/mm² per section, ascertaining similar frequencies
413 of cytokeratin V⁺ mTECs for each tissue sections examined. As shown in Figure 6b, ~6% of
414 DAPI⁺ cells were apoptotic in NOD mice. This frequency of apoptosis was two-fold higher
415 than seen for NOD- μ MT^{-/-} mice (~3%). In contrast to the NOD strains, <1% of cells were
416 apoptotic in control B6 mice. We were curious to determine if apoptotic cytokeratin V⁺
417 mTECs in NOD mice expressed insulin. Thymic tissue sections from 11 week old female
418 NOD mice were incubated with anti-cytokeratin V⁺ and anti-insulin antibodies and apoptosis
419 determined by TUNEL staining as before. As a control, we similarly analysed thymic tissue
420 sections from age-matched, female B6 mice. Interestingly, within the apoptotic cytokeratin
421 V⁺ mTEC pool in NOD mice resided cytokeratin V⁺ mTECs that expressed insulin, although
422 it is important to note that some insulin⁺ cytokeratin V⁺ mTECs were not apoptotic
423 suggesting there is not a complete loss of insulin⁺ cytokeratin V⁺ mTECs but a reduction in
424 their numbers. Similarly, some apoptotic mTECs did not express insulin (Supplementary
425 Figure 7).

426 Taken together, these data suggest that B cell-mediated autoimmune targeting of cytokeratin
427 V⁺ mTECs results in the loss of a distinct population of cytokeratin V⁺ mTECs, some of
428 which express insulin, and this key feature occurs before sustained autoimmune attack in the
429 pancreas.

430

431

432 **Thymic B cells enhance premature egress of T cells from the thymus.**

433 The evidence that thymic stroma had bound autoantibodies and the presence of these
434 autoantibodies correlated with increased apoptosis of thymic stroma, including some insulin⁺
435 mTECs, we investigated the impact this may have on thymocytes capable of responding to
436 islet antigen, particularly insulin. We isolated the thymocytes from NOD mice thymi and
437 cultured the cells in the presence of bone-marrow derived dendritic cells and either whole
438 insulin or proinsulin peptide15:23 [38]. The proliferative response to the CD4SP and CD8SP
439 thymocytes to the respective stimulants was assessed by flow cytometric analysis of Ki67
440 (Figure 7, Supplementary Figure 8a). As controls we included B6 mice stimulated with whole
441 insulin, and B cell-deficient NOD- μ MT^{-/-} mice stimulated with whole insulin or proinsulin
442 peptide 15:23. For CD4SP cells only those isolated from B cell sufficient NOD mice
443 responded to both whole insulin, although the response was not significant in comparison to
444 control mice. (Figure 7a). The responses to whole insulin for thymocytes from B6 and NOD-
445 μ MT^{-/-} mice being close to baseline. In contrast, CD4SP thymocytes from NOD mice
446 exhibited a significantly increased response to proinsulin P15:23 with respect to NOD- μ MT^{-/-}
447 mice. The responses of CD8SP thymocytes was slightly different; whereas thymocytes
448 isolated from NOD mice responded to the whole insulin molecule, the responses for
449 individual mice was quite diverse- some responded well, others' response close to baseline
450 levels for B6 control mice (Figure 7b). Similarly, CD8SP thymocytes from NOD- μ MT^{-/-} mice
451 had some diversity in responsiveness to whole insulin, although it was noted that even the
452 best responders still responded weaker than that seen for NOD mice. In contrast, CD8SP
453 thymocytes from NOD mice responded far better to proinsulin P15:23, than those from NOD-
454 μ MT^{-/-} mice, although the response was not significantly enhanced. In these same mice the
455 responses to the proinsulin peptide were less diverse and above baseline levels.

456 We initially wondered whether this increased response for insulin and proinsulin peptide by
457 NOD thymocytes was representative of increased survival of autoreactive T cells, and thus a
458 breakdown in negative selection. In particular, we queried whether thymocytes that had
459 very recently rearranged their TcR escaped from the thymus before completing negative
460 selection. If this held true, we expected an increase in RAG-GFP^{hi} T cells in the blood; RAG-
461 GFP levels normally fall during negative selection due to the time to complete the process
462 and as such, peripheral T cells are usually RAG-GFP^{int} [30]. To test this hypothesis we
463 performed flow cytometry analysis of total GFP levels of T cells in the peripheral blood of
464 NOD-RAG-GFP mice in comparison to control FVB-RAG-GFP mice and B cell deficient
465 NOD- μ MT^{-/-}-RAG-GFP mice. Representative flow cytometry plots showing the gating
466 strategy is shown in Supplementary Figure 8b. As shown in Figure 7c, in the NOD murine
467 strains the frequency of total GFP⁺ T cells in peripheral blood was greater than seen for the
468 control FVB strain, for NOD mice this increase being significant. Furthermore, this increased
469 frequency of GFP⁺ T cells in the peripheral blood of the NOD strains was almost entirely due
470 to GFP^{hi} cells, as GFP^{int} cells were only slightly increased in frequency in comparison to
471 control FVB-RAG-GFP mice, again NOD mice showing a significant increase. Importantly,
472 although not significant, it was clear that the frequency of RAG-GFP^{hi} cells in B cell
473 sufficient NOD-RAG-GFP mice was higher than in B cell deficient NOD- μ MT^{-/-}-RAG-GFP
474 highlighting the importance of B cells in the early release of T cells from the thymus prior to
475 negative selection.

476 **Discussion**

477 Ablation of efficient purging of autoreactive T cells in the thymus and the role of B cells in
478 T1D seem two distinct entities in understanding how immunological tolerance is broken in
479 this chronic autoimmune condition. Here, we establish that inappropriate accumulation of B
480 cells in the NOD mouse thymus is a unique feature of the disease process, and these thymic B
481 cells may play a role in the egress of pre-negatively selected T cells.

482 T1D progression in both man and NOD mice occurs over time. The initial stages of T1D,
483 where priming of the immune response to islet antigen occurs but not overt β cell destruction,
484 is characterised by autoantibodies to β antigens [39]. It is accepted that following priming of
485 the autoreactive T cell repertoire to β cell antigens, the activity of the autoreactive T cells is
486 kept in check by regulatory mechanisms. Ultimately, such regulation fails, and leading to β
487 cell destruction. Little is known as to why regulation of autoreactive T cells fails over time,
488 although paucity of, or dysfunction of, T regulatory cells is speculated to contribute to the
489 phenomenon [40; 41; 42]. Our data adds a new dimension to our understanding of the
490 immunological changes that occur at the late insulitic- pre-diabetic phase that may tip the
491 autoreactive T cell response in favour of β cell destruction; targeted thymic B cell
492 autoimmune attack of thymic stroma expressing β cell antigens.

493 B cells are present in the thymus of mammals from fetal age to adulthood, their numbers
494 remaining relatively static in ontogeny and equating to those of thymic dendritic cells [5; 11;
495 13]. Previous studies in NOD mouse strains documented B cell accumulation in the thymus
496 of aged mice [43; 44]. Here we extended on these early studies showing that in NOD mice,
497 thymic B cell numbers are not static, their numbers significantly increase at the late insulitic-
498 pre-diabetic phase suggesting the restricted B cell niche normally present expands. This
499 change in B cell numbers occurs at the same time as increased numbers of RAG⁺ B cells are

500 detected in the thymus, but decreased homeostatic proliferation. Together these findings
501 suggest that permissiveness of B cell development that can normally occur within the thymus
502 [33; 45; 46; 47] is enhanced in NOD mice as they age, and the increase in B cell numbers
503 potentially reflects this increased rate of development rather than in situ proliferation.
504 Although we cannot exclusively discount that peripheral B cells migrating to the thymus
505 contribute to the thymic B cell population, we, like others, have found peripheral B cells have
506 little propensity to traffic to the thymus (data not shown, [47]). Future studies in how the
507 NOD mouse thymic environment potentially nurtures B cell development and retention will be
508 informative.

509 The phenotype of thymic B cells in NOD mice resembles that of thymic B cells in non-
510 autoimmune strains of mice; they predominantly express B2 follicular cell markers, and have
511 a predominantly activated phenotype with high MHC and costimulatory molecule expression
512 (not shown, [45; 48]). The location of thymic B cells in NOD mice is also reminiscent of
513 reports in other murine strains- positioned predominantly at the cortico-medullary junction-
514 but in contrast to non-autoimmune prone mice, large B cell follicles form and this is age-
515 dependent. Furthermore, the hallmarks of germinal centres are readily detectable in the NOD
516 thymus; IL-21 and T follicular helper (T_{fh}) cells. Abnormalities in levels of IL-21 and T_{fh}
517 cells in peripheral tissues, and blood, have been strongly associated with T1D [37; 49]. Here,
518 we show similar abnormalities exist in the thymus occurring specifically at the late insulitic-
519 pre-diabetic phase of the T1D condition. In addition the thymus of NOD mice has enhanced
520 levels of AID mRNA transcripts, suggesting increased in situ somatic hypermutation and
521 class switching of the B cell repertoire activity. Plasma cells are also increased in the thymus
522 of NOD mice with respect to control animals which taken all this information together
523 implies ectopic germinal centres are a feature of the NOD thymus, not just their pancreas
524 [50]. Our evidence that the NOD mouse thymus is populated with significantly increased

525 numbers of B cells with IgG, IgA and IgE receptors with respect to non-autoimmune prone
526 mice, as well as enhanced levels of soluble IgG1 and IgA antibodies supports our rationale of
527 ectopic germinal centre formation in this primary lymphoid tissue.

528 The significance of these unique changes in the NOD mouse thymus as mice progress along
529 the T1D pathway, we believe, is that they have the potential to impact on the capacity of
530 negative selection of autoreactive T cells to occur effectively. The importance of mTEC
531 expression of TSAs for efficient deletion of developing T cells bearing autoreactive T cell
532 receptors is well established [51]. Our evidence that a selective population of mTECs have
533 autoantibodies bound in situ, and in the presence of thymic B cells a proportion of mTECs
534 undergo apoptosis, a number of which express insulin, is likely to have implications on
535 negative selection of islet-reactive T cells. The antigenic specificity of the intrathymic
536 autoantibodies target is unknown, and we do not believe that they must recognise insulin to
537 impact of T1D progression. It is possible that the intrathymic autoantibodies recognise and
538 promote apoptosis of particular mTECs that express certain TSAs that are associated with
539 other autoimmune conditions NOD mice develop [52; 53; 54]. It follows that reduction in
540 insulin expressing mTECs may happen inadvertently. Alternatively, or in addition, it is
541 possible that thymic cognate B-T cell interactions promote survival of developing
542 autoreactive T cells as opposed to their deletion [55].

543 Our data is supportive of the rationale that pre-negatively selected T cells are potentially
544 released from the thymus prematurely. Two photon microscopy has documented that
545 developing T cells reside in medulla for 3-5 days to complete negative selection [56]. In
546 RAG2p-GFP reporter mice, this duration in the medulla equates to decreased GFP intensity
547 due to the 54 hour half-life of the molecule [30]. Our evidence that in NOD mice CD3⁺GFP^{hi}
548 cells are significantly enhanced in peripheral blood with respect to non-autoimmune prone
549 mice suggests an aborted time, or failed entry into, the medulla of GFP^{hi} T cells, and as a

550 consequence failed negative selection. It follows that the increased export of non-negatively
551 selected T cells could overpower waning regulatory mechanisms in the islets leading to the
552 final sustained attack of the β cells.

553 The fledgling field of thymic B cell research is starting to unravel the importance of this
554 unique population of cells in the immune system. Our data highlight a new relationship
555 between thymic B cells and type 1 diabetes development. Future studies that define the in situ
556 developmental pathway and receptor specificity of thymic B cells will be important for
557 identifying key therapeutic strategies for type 1 diabetes and other autoimmune conditions in
558 which thymic B cells make a contribution.

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564 **Authors contributions:**

565 AP, JS, MK, KH and EAG performed the experiments and analysed data; AP and EAG wrote
566 the paper.

567 **Conflict of Interest Statement**

568 All authors declare there was no conflict of interest.

569 **References**

- 570 [1] J.W. Kappler, N. Roehm, and P. Marrack, T cell tolerance by clonal elimination in the
571 thymus. *Cell* 49 (1987) 273-80.
- 572 [2] A.J. Bonito, C. Aloman, M.I. Fiel, N.M. Danzl, S. Cha, E.G. Weinstein, S. Jeong, Y. Choi,
573 M.C. Walsh, and K. Alexandropoulos, Medullary thymic epithelial cell depletion leads
574 to autoimmune hepatitis. *Journal of Clinical Investigation* 123 (2013) 3510-3524.
- 575 [3] B. Kyewski, J. Derbinski, J. Gotter, and L. Klein, Promiscuous gene expression and central
576 T-cell tolerance: more than meets the eye. *Trends in Immunology* 23 (2002) 364-371.
- 577 [4] M.S. Anderson, E.S. Venanzi, L. Klein, Z. Chen, S.P. Berzins, S.J. Turley, H. von Boehmer,
578 R. Bronson, A. Dierich, C. Benoist, and D. Mathis, Projection of an immunological self
579 shadow within the thymus by the aire protein. *Science* 298 (2002) 1395-401.
- 580 [5] L. Klein, B. Kyewski, P.M. Allen, and K.A. Hogquist, Positive and negative selection of
581 the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* 14 (2014)
582 377-91.
- 583 [6] J. Oh, and J.S. Shin, The Role of Dendritic Cells in Central Tolerance. *Immune Netw* 15
584 (2015) 111-20.
- 585 [7] F. Frommer, and A. Waisman, B Cells Participate in Thymic Negative Selection of Murine
586 Auto-reactive CD4(+) T Cells. *Plos One* 5 (2010).
- 587 [8] T. Yamano, J. Nedjic, M. Hinterberger, M. Steinert, S. Koser, S. Pinto, N. Gerdes, E.
588 Lutgens, N. Ishimaru, M. Busslinger, B. Brors, B. Kyewski, and L. Klein, Thymic B
589 Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction.
590 *Immunity* 42 (2015) 1048-1061.
- 591 [9] P. Kleindienst, I. Chretien, T. Winkler, and T. Brocker, Functional comparison of thymic
592 B cells and dendritic cells in vivo. *Blood* 95 (2000) 2610-6.
- 593 [10] K. Nango, M. Inaba, K. Inaba, Y. Adachi, S. Than, T. Ishida, T. Kumamoto, M. Uyama,
594 and S. Ikehara, ONTOGENY OF THYMIC B-CELLS IN NORMAL MICE. *Cellular*
595 *Immunology* 133 (1991) 109-115.
- 596 [11] P.G. Isaacson, A.J. Norton, and B.J. Addis, The human thymus contains a novel population
597 of B lymphocytes. *Lancet* 2 (1987) 1488-91.
- 598 [12] J. Spencer, M. Choy, T. Hussell, L. Papadaki, J.P. Kington, and P.G. Isaacson,
599 PROPERTIES OF HUMAN THYMIC B-CELLS. *Immunology* 75 (1992) 596-600.

- 600 [13] J.L. Andreu-Sanchez, J. Faro, J.M. Alonso, C.J. Paige, C. Martinez, and M.A. Marcos,
601 Ontogenic characterization of thymic B lymphocytes. Analysis in different mouse
602 strains. *Eur J Immunol* 20 (1990) 1767-73.
- 603 [14] B. Christensson, P. Biberfeld, and G. Matell, B-cell compartment in the thymus of patients
604 with myasthenia gravis and control subjects. *Ann N Y Acad Sci* 540 (1988) 293-7.
- 605 [15] I.R. Mackay, M. Masel, and F.M. Burnet, Thymic Abnormality in Systemic Lupus
606 Erythematosus. *Australas Ann Med* 13 (1964) 5-14.
- 607 [16] T. Sato, S. Ishikawa, K. Akadegawa, T. Ito, H. Yurino, M. Kitabatake, H. Yoneyama, and
608 K. Matsushima, Aberrant B1 cell migration into the thymus results in activation of CD4
609 T cells through its potent antigen-presenting activity in the development of murine
610 lupus. *Eur J Immunol* 34 (2004) 3346-58.
- 611 [17] D.L. Kaufman, M. Clare-Salzler, J. Tian, T. Forsthuber, G.S. Ting, P. Robinson, M.A.
612 Atkinson, E.E. Sercarz, A.J. Tobin, and P.V. Lehmann, Spontaneous loss of T-cell
613 tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature*
614 366 (1993) 69-72.
- 615 [18] R. Tisch, X.D. Yang, S.M. Singer, R.S. Liblau, L. Fugger, and H.O. McDevitt, Immune
616 response to glutamic acid decarboxylase correlates with insulinitis in non-obese diabetic
617 mice. *Nature* 366 (1993) 72-5.
- 618 [19] S.M. Lieberman, A.M. Evans, B. Han, T. Takaki, Y. Vinnitskaya, J.A. Caldwell, D.V.
619 Serreze, J. Shabanowitz, D.F. Hunt, S.G. Nathenson, P. Santamaria, and T.P.
620 DiLorenzo, Identification of the beta cell antigen targeted by a prevalent population of
621 pathogenic CD8+ T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A* 100 (2003)
622 8384-8.
- 623 [20] Q. He, Y.M. Morillon, 2nd, N.A. Spidale, C.J. Kroger, B. Liu, R.B. Sartor, B. Wang, and
624 R. Tisch, Thymic development of autoreactive T cells in NOD mice is regulated in an
625 age-dependent manner. *J Immunol* 191 (2013) 5858-66.
- 626 [21] K. Kachapati, D. Adams, K. Bednar, and W.M. Ridgway, The non-obese diabetic (NOD)
627 mouse as a model of human type 1 diabetes. *Methods Mol Biol* 933 (2012) 3-16.
- 628 [22] P. Leete, A. Willcox, L. Krogvold, K. Dahl-Jorgensen, A.K. Foulis, S.J. Richardson, and
629 N.G. Morgan, Differential Insulitic Profiles Determine the Extent of beta-Cell
630 Destruction and the Age at Onset of Type 1 Diabetes. *Diabetes* 65 (2016) 1362-9.
- 631 [23] V. Parikka, K. Nanto-Salonen, M. Saarinen, T. Simell, J. Ilonen, H. Hyoty, R. Veijola, M.
632 Knip, and O. Simell, Early seroconversion and rapidly increasing autoantibody

633 concentrations predict prepubertal manifestation of type 1 diabetes in children at
634 genetic risk. *Diabetologia* 55 (2012) 1926-36.

635 [24] H. Noorchashm, Y.K. Lieu, N. Noorchashm, S.Y. Rostami, S.A. Greeley, A.
636 Schlachterman, H.K. Song, L.E. Noto, A.M. Jevnikar, C.F. Barker, and A. Naji, I-Ag7-
637 mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint
638 in T cell tolerance to islet beta cells of nonobese diabetic mice. *J Immunol* 163 (1999)
639 743-50.

640 [25] D.V. Serreze, H.D. Chapman, D.S. Varnum, M.S. Hanson, P.C. Reifsnnyder, S.D. Richard,
641 S.A. Fleming, E.H. Leiter, and L.D. Shultz, B lymphocytes are essential for the
642 initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic"
643 stock of NOD.Ig mu null mice. *J Exp Med* 184 (1996) 2049-53.

644 [26] C.Y. Hu, D. Rodriguez-Pinto, W. Du, A. Ahuja, O. Henegariu, F.S. Wong, M.J.
645 Shlomchik, and L. Wen, Treatment with CD20-specific antibody prevents and reverses
646 autoimmune diabetes in mice. *J Clin Invest* 117 (2007) 3857-67.

647 [27] M.D. Pescovitz, C.J. Greenbaum, H. Krause-Steinrauf, D.J. Becker, S.E. Gitelman, R.
648 Goland, P.A. Gottlieb, J.B. Marks, P.F. McGee, A.M. Moran, P. Raskin, H. Rodriguez,
649 D.A. Schatz, D. Wherrett, D.M. Wilson, J.M. Lachin, J.S. Skyler, and C.D.S.G. Type
650 1 Diabetes TrialNet Anti, Rituximab, B-lymphocyte depletion, and preservation of
651 beta-cell function. *N Engl J Med* 361 (2009) 2143-52.

652 [28] D.V. Serreze, S.A. Fleming, H.D. Chapman, S.D. Richard, E.H. Leiter, and R.M. Tisch,
653 B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated
654 autoimmune diabetes in nonobese diabetic mice. *Journal of Immunology* 161 (1998)
655 3912-3918.

656 [29] G.M. Brodie, M. Wallberg, P. Santamaria, F.S. Wong, and E.A. Green, B-cells promote
657 intra-islet CD8+ cytotoxic T-cell survival to enhance type 1 diabetes. *Diabetes* 57
658 (2008) 909-17.

659 [30] T.M. McCaughtry, M.S. Wilken, and K.A. Hogquist, Thymic emigration revisited. *Journal*
660 *of Experimental Medicine* 204 (2007) 2513-2520.

661 [31] M.J. Smith, T.A. Packard, S.K. O'Neill, C.J.H. Dunand, M. Huang, L. Fitzgerald-Miller,
662 D. Stowell, R.M. Hinman, P.C. Wilson, P.A. Gottlieb, and J.C. Cambier, Loss of
663 Anergic B Cells in Prediabetic and New-Onset Type 1 Diabetic Patients. *Diabetes* 64
664 (2015) 1703-1712.

665 [32] J.H. Li, E. Lu, T.S. Yi, and J.G. Cyster, EB12 augments Tfh cell fate by promoting
666 interaction with IL-2-queenching dendritic cells. *Nature* 533 (2016) 110-+.

- 667 [33] K. Akashi, L.I. Richie, T. Miyamoto, W.H. Carr, and I.L. Weissman, B lymphopoiesis in
668 the thymus. *J Immunol* 164 (2000) 5221-6.
- 669 [34] D. Zotos, J.M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L.M. Corcoran, D.I.
670 Godfrey, K.M. Toellner, M.J. Smyth, S.L. Nutt, and D.M. Tarlinton, IL-21 regulates
671 germinal center B cell differentiation and proliferation through a B cell-intrinsic
672 mechanism. *J Exp Med* 207 (2010) 365-78.
- 673 [35] H.M. McGuire, A. Vogelzang, N. Hill, M. Flodstrom-Tullberg, J. Sprent, and C. King,
674 Loss of parity between IL-2 and IL-21 in the NOD Idd3 locus. *Proc Natl Acad Sci U S*
675 *A* 106 (2009) 19438-43.
- 676 [36] A.P. Sutherland, T. Van Belle, A.L. Wurster, A. Suto, M. Michaud, D. Zhang, M.J.
677 Grusby, and M. von Herrath, Interleukin-21 is required for the development of type 1
678 diabetes in NOD mice. *Diabetes* 58 (2009) 1144-55.
- 679 [37] R.C. Ferreira, H.Z. Simons, W.S. Thompson, A.J. Cutler, X.C. Dopico, D.J. Smyth, M.
680 Mashar, H. Schuilenburg, N.M. Walker, D.B. Dunger, C. Wallace, J.A. Todd, L.S.
681 Wicker, and M.L. Pekalski, IL-21 production by CD4(+) effector T cells and frequency
682 of circulating follicular helper T cells are increased in type 1 diabetes patients.
683 *Diabetologia* 58 (2015) 781-790.
- 684 [38] F.S. Wong, and L. Wen, Innate and adaptive immune responses are highly interconnected
685 at many levels. *Curr Mol Med* 9 (2009) 1-3.
- 686 [39] S.E. Regnell, and A. Lernmark, Early prediction of autoimmune (type 1) diabetes.
687 *Diabetologia* 60 (2017) 1370-1381.
- 688 [40] C. Ferreira, Y. Singh, A.L. Furmanski, F.S. Wong, O.A. Garden, and J. Dyson, Non-obese
689 diabetic mice select a low-diversity repertoire of natural regulatory T cells. *Proc Natl*
690 *Acad Sci U S A* 106 (2009) 8320-5.
- 691 [41] Y. Zhang, E. Bandala-Sanchez, and L.C. Harrison, Revisiting regulatory T cells in type 1
692 diabetes. *Curr Opin Endocrinol Diabetes Obes* 19 (2012) 271-8.
- 693 [42] A. Kukreja, G. Cost, J. Marker, C. Zhang, Z. Sun, K. Lin-Su, S. Ten, M. Sanz, M. Exley,
694 B. Wilson, S. Porcelli, and N. Maclaren, Multiple immuno-regulatory defects in type-
695 1 diabetes. *J Clin Invest* 109 (2002) 131-40.
- 696 [43] L.A. O'Reilly, D. Healey, E. Simpson, P. Chandler, T. Lund, M.A. Ritter, and A. Cooke,
697 Studies on the thymus of non-obese diabetic (NOD) mice: effect of transgene
698 expression. *Immunology* 82 (1994) 275-86.
- 699 [44] N.M. Parish, P. Chandler, R. Quartey-Papafio, E. Simpson, and A. Cooke, The effect of
700 bone marrow and thymus chimerism between non-obese diabetic (NOD) and NOD-E

701 transgenic mice, on the expression and prevention of diabetes. *Eur J Immunol* 23 (1993)
702 2667-75.

703 [45] I. Ferrero, F. Anjuere, P. Martin, G. Martinez del Hoyo, M.L. Fraga, N. Wright, R. Varona,
704 G. Marquez, and C. Ardavin, Functional and phenotypic analysis of thymic B cells: role
705 in the induction of T cell negative selection. *Eur J Immunol* 29 (1999) 1598-609.

706 [46] S. Mori, M. Inaba, A. Sugihara, S. Taketani, H. Doi, Y. Fukuba, Y. Yamamoto, Y. Adachi,
707 K. Inaba, S. Fukuhara, and S. Ikehara, Presence of B cell progenitors in the thymus. *J*
708 *Immunol* 158 (1997) 4193-9.

709 [47] J. Perera, and H. Huang, The development and function of thymic B cells. *Cell Mol Life*
710 *Sci* 72 (2015) 2657-63.

711 [48] M. Inaba, K. Inaba, Y. Adachi, K. Nango, H. Ogata, S. Muramatsu, and S. Ikehara,
712 Functional analyses of thymic CD5+ B cells. Responsiveness to major
713 histocompatibility complex class II-restricted T blasts but not to lipopolysaccharide or
714 anti-IgM plus interleukin 4. *J Exp Med* 171 (1990) 321-6.

715 [49] T. Viisanen, E.L. Ihantola, K. Nanto-Salonen, H. Hyoty, N. Nurminen, J. Selvenius, A.
716 Juutilainen, L. Moilanen, J. Pihlajamaki, R. Veijola, J. Toppari, M. Knip, J. Ilonen, and
717 T. Kinnunen, Circulating CXCR5+PD-1+ICOS+ Follicular T Helper Cells Are
718 Increased Close to the Diagnosis of Type 1 Diabetes in Children With Multiple
719 Autoantibodies. *Diabetes* 66 (2017) 437-447.

720 [50] E. Astorri, M. Bombardieri, S. Gabba, M. Peakman, P. Pozzilli, and C. Pitzalis, Evolution
721 of ectopic lymphoid neogenesis and in situ autoantibody production in autoimmune
722 nonobese diabetic mice: cellular and molecular characterization of tertiary lymphoid
723 structures in pancreatic islets. *J Immunol* 185 (2010) 3359-68.

724 [51] Y. Takahama, I. Ohigashi, S. Baik, and G. Anderson, Generation of diversity in thymic
725 epithelial cells. *Nat Rev Immunol* 17 (2017) 295-305.

726 [52] N.F. Bernard, F. Ertug, and H. Margolese, High incidence of thyroiditis and anti-thyroid
727 autoantibodies in NOD mice. *Diabetes* 41 (1992) 40-6.

728 [53] C. Thompson, H. Jacobsen, D. Pomeranz Krummel, K. Nagai, and A. Cooke, Non-
729 depleting anti-CD4 antibody not only prevents onset but resolves sialadenitis in NOD
730 mice. *Autoimmunity* 37 (2004) 549-54.

731 [54] M. Nakahara, Y. Nagayama, T. Ichikawa, L. Yu, G.S. Eisenbarth, and N. Abiru, The effect
732 of regulatory T-cell depletion on the spectrum of organ-specific autoimmune diseases
733 in nonobese diabetic mice at different ages. *Autoimmunity* 44 (2011) 504-10.

- 734 [55] F. Frommer, and A. Waisman, B cells participate in thymic negative selection of murine
735 auto-reactive CD4+ T cells. PLoS One 5 (2010) e15372.
- 736 [56] M. Le Borgne, E. Ladi, I. Dzhagalov, P. Herzmark, Y.F. Liao, A.K. Chakraborty, and E.A.
737 Robey, The impact of negative selection on thymocyte migration in the medulla. Nat
738 Immunol 10 (2009) 823-30.
- 739

740 **Figure Legends**

741 **Figure 1. Intrathymic B cell accumulation precedes β cell destruction.** Single cell
742 suspensions were prepared from the respective thymi and all data analyzed on a single cell, live
743 gate. (a) Representative dot plots of thymic CD19⁺ cells: (I) Isotype control for CD19 antibody;
744 (II) 12 week old female B6 mouse; and (III) 12 week old female NOD mouse. (b) Number of
745 B cells in the thymus of 4-6 week old female B6 mice (n= 11), 12-14 week old female B6 mice
746 (n=25), 4-6 week old female NOD mice (n= 13) and 12-14 week old female NOD mice (n=
747 25). (c) Number of RAG-GFP^{hi} B cells in the thymus of 4-6 week old female FVB-RAG-GFP
748 mice (n=3), 12-14 week old female FVB-RAG-GFP mice (n=8), 4-6 week old female NOD-
749 RAG-GFP mice (n= 4) and 12-14 week old female NOD (n= 8). (d) Frequency of Ki67⁺ B cells
750 in the thymus of 4-6 week old female B6 mice (n= 10), 12-14 week old female B6 mice (n=10),
751 4-6 week old female NOD (n= 9) and 12-14 week old female NOD mice (n= 8). Data presented
752 as scatter plot, each dot equating to a mouse, the bar representing the mean value. Statistical
753 significance determined using the non-parametric Mann-Whitney U-test, significant P values
754 are shown, ns= not significant.

755

756 **Figure 2. B cells form follicle-like structures at the cortical-medullary junction in NOD**
757 **thymi.** Flow cytometric analysis of the (a) frequency of B cells displaying a Follicular (FO) or
758 Marginal-zone (MZ) phenotype in the thymus of B6 (n=10) or NOD mice (n=13) and (b)
759 absolute number of B cells displaying FO or MZ phenotypes in the thymus of B6 (n=5) or
760 NOD mice (n=6). Comparisons made between aged-matched, female 11-14 week old mice in a
761 single cell, live gate. Data acquired from at least two independent experiments and is presented
762 as scatter plot; P values were calculated using the Mann-Whitney U test analysis, ns= not
763 significant. (c) Representative confocal immunofluorescence microscopy images of thymi

764 sections examined for B220 (yellow), cytokeratin V (green) expression and the DNA-
765 intercalating dye DAPI identified nuclei (blue) from 11 week old female NOD or B6 mice. A
766 total of 14 sections from eight NOD mice and a total of six section from three B6 mice were
767 analyzed, and there was consistency in the data obtained from the appropriate strains of mice.
768 Confocal fluorescent images were obtained with a Plan-Apochromat 20x objective.

769

770 **Figure 3. The NOD thymus has the hallmarks of ectopic GC development.** (a) Evaluation
771 of IL-2/IL-21 ratio in cell-free supernatants from spleen and thymic tissue from 11-15 week
772 old B6 (n=5) or NOD (n=4) mice. The data shown is representative of two individual
773 experiments showing similar results. (b) Frequency of CD4⁺ T follicular helper cells (TfH) in
774 the thymus of B6 (n=12) and NOD mice (n=17). (c) Number of CD4⁺ T follicular helper cells
775 in the thymus of B6 (n=10) and NOD mice (n=11). (d) Representative histogram of BCL-6
776 expression in CD8⁻ CD4⁺ PD1^{hi} ICOS⁺ cells in thymus of NOD (n=5) and B6 (n=5) mice. (e)
777 Number of plasma cells in the thymus of B6 (n=15) and NOD mice (n=15). For (b-e)
778 comparisons made between female, age-matched 10-14 week old B6 and NOD mice. The
779 analysis was performed on a single cell, live gate, and the data is presented as a scatterplot,
780 each dot equating to a mouse, the bar represents mean value. P values were calculated using
781 the Mann-Whitney U test analysis and are shown in the figure, ns= not significant. The data is
782 pooled from at least two independent experiments giving similar results. (f) Quantitative PCR
783 (qPCR) analysis of AID mRNA (*aicda* expression) levels in the whole thymus. Data was
784 normalized to HPRT mRNA as described in methods, and fold change in NOD mice AID
785 mRNA when compared to normalised AID mRNA levels for B6 mice. All mice were 11-14
786 weeks of age, a total of 5 female B6 mice were compared to 5 female NOD mice. One thymic
787 sample from a female B cell-deficient NOD μ MT^{-/-} mouse was used as a negative control. The
788 data is pooled from two independent experiments and is presented mean \pm standard error mean

789 (SEM). P values were calculated using the Mann-Whitney U-test and are shown in the figure,
790 ns= not significant.

791

792 **Figure 4. The NOD thymus harbours a unique pattern of immunoglobulin isotypes** (a)
793 Number of IgM⁻ IgD⁻ IgA⁺, IgM⁻ IgD⁻ IgE⁺, IgM⁻ IgD⁻ IgG⁺ B cells in the thymus of 11-14 week
794 old female B6 (n=10) or female NOD mice (n=10). (b) Number of IgM⁻ IgD⁺ IgA⁺, IgM⁻
795 IgD⁺IgE⁺, IgM⁻ IgD⁺ IgG⁺ B cells in the thymus of 11-14 week old female B6 (n=10) and
796 female NOD mice (n=10). (c-f) Optical density (OD) values of the respective immunoglobulins
797 in cell free tissue supernatants (c,e) or serum (d,f). A total of 6 female B6 and 6 female NOD
798 mice were assessed in two independent experiments. Data is presented as scatter plot, each dot
799 equating to one mouse and bar representing the mean. P values were calculated using the Mann-
800 Whitney U-test analysis and are shown in the figure, ns= not significant.

801

802 **Figure 5. IgGs bind to thymic stromal components in NOD mice.** (a and b) Representative
803 confocal immunofluorescence microscopy images of thymi sections of NOD (aI-III) and B6
804 mice (bI-II) examined for cytokeratin V (red), murine IgG (green), and the DNA-intercalating
805 dye DAPI (white). A total of six 11 week old NOD mice and five 11 week old B6 mice, two
806 sections per mouse were examined. (a)I,II and II are derived from different NOD mice. The
807 confocal fluorescent image in AI was obtained with a Plan-Apochromat 20x objective to give
808 a broader view of the extent of immunoglobulin bound to thymic stroma, arrows indicating
809 some of the cells co-positive for cytokeratin V and mouse IgG. The confocal fluorescent images
810 in AII and AIII were obtained with a Plan-Apochromat 63x objective. For (b) the confocal
811 fluorescent image was obtained using a Plan-Apochromat 20x objective. (c) Quantification of
812 murine Ig-bound to stromal cells of age-matched 11 week old, female NOD or B6 mice.

813 Confocal immunofluorescence microscopy images were subjected to StrataQuest V64 analysis,
814 a total of $2-3 \times 10^4$ DAPI⁺ cells/mm² were counted and the mean fluorescence intensity of DAPI⁺
815 cells versus mean fluorescence intensity of anti-Ig is presented as a scattergram. The data
816 shown, is representative of two independent mice examined giving similar results.

817

818 **Figure 6. Increase in thymic B cells was associated to increased apoptosis of stromal cells.**

819 (a) Representative confocal immunofluorescence microscopy images of thymi sections from
820 9-14 week old female NOD, and NOD- μ MT^{-/-} and B6 mice examined for cytokeratin V
821 (yellow), apoptosis (red), and the DNA-intercalating dye DAPI (white) expression. The data is
822 representative of similar data acquired from 6 female NOD, 6 female NOD- μ MT^{-/-} and 4
823 female B6 mice, three sections per mouse were examined. In all cases, the confocal fluorescent
824 images were obtained with a Plan-Apochromat 63x objective. Bar represents 20 μ m. (b)
825 Quantification of TUNEL⁺ stromal cells of age-matched 11 week old, female NOD, NOD- μ MT^{-/-}
826 or B6 mice. Confocal immunofluorescence microscopy images were subjected to StrataQuest
827 V64 analysis, a total of 4×10^4 DAPI⁺ cells/mm² were counted and the mean fluorescence
828 intensity of DAPI⁺ cells versus mean fluorescence intensity of TUNEL is presented as a
829 scattergram.

830

831 **Figure 7. B cells promote premature thymic-release of T cells prior to negative selection.**

832 (a-b) Thymocytes from 11-12 week old B6, NOD and NOD NOD- μ MTKO mice were
833 stimulated with insulin or B15:23 peptide (NOD and NOD- μ MTKO mice, only) for 72 hours
834 and Ki67 expression in CD4SP (a) or CD8SP (b) cells as a measure of proliferation was
835 determined by flow cytometry. The frequency of Ki67⁺ cells for stimulated samples was
836 normalized against the frequency of Ki67⁺ cells in unstimulated samples. (c) Frequency of

837 RTEs (RAG-GFP^{hi}) in peripheral blood of 11-12 week old FVB-GFP (n=8), NOD-GFP (n=8)
838 or NOD- μ MT^{-/-}-GFP (n=5) mice. Data is pooled from two independent experiments and cells
839 were analysed on a live, single, CD3⁺ T cell gate. The data is presented as scatter plot, the bar
840 representing the mean value;P values were calculated using the two-way Anova followed by
841 Tukey multi comparison test and are shown the figure, ns= not significant.