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Enhanced responsiveness to T-cell help causes loss of B-lymphocyte tolerance to a β-cell neo-self-antigen in type 1 diabetes prone NOD mice

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Self-reactive B lymphocytes contribute to type 1 diabetes pathogenesis as APC and auto-Ab producers in NOD mice and humans. To shed light on the mechanisms responsible for the breakdown in B-lymphocyte self-tolerance to β-cell Ag, we utilised a model whereby hen-egg lysozyme (HEL)-specific Ig Tg (*IgHEL*-Tg)-Tg B lymphocytes were allowed to develop in or were transferred into mice expressing the HEL Tg under an insulin promoter (*insHEL*-Tg). *IgHEL*-Tg B lymphocytes enhanced type 1 diabetes susceptibility of *insHEL*-Tg NOD mice. A comparison of the tolerogenic activity of *IgHEL*-Tg B lymphocytes with NOD and non-autoimmune-prone C57BL/6 genetic backgrounds showed that both were rendered anergic in the presence of insHEL when competing with polyclonal B lymphocytes. Nevertheless, NOD *IgHEL*-Tg B lymphocytes transferred into *insHEL*-Tg mice were more readily susceptible to rescue from anergy than their C57BL/6 counterparts, following provision of *in vivo* T-cell help. The different tolerogenic outcomes were an intrinsic property of B lymphocytes rather than being related to the quality of T-cell help, with the defective response being at least partially controlled by genes mapping to *insulin-dependent diabetes* (*Idd*) susceptibility loci on Chromosome 1 (*Idd5*) and 4 (*Idd9/11*).

Key words: Anergy · B lymphocytes · Self-tolerance · Transgenic mice · Type 1 diabetes

Supporting Information available online

Introduction

B lymphocytes make important contributions to pancreatic β-cell pathogenesis in the NOD mouse model of type 1 diabetes (T1D): first, through secretion of auto-Ab which enhance capture of auto-Ag by DC and NK cells; second, and more importantly, through their action as efficient APC capable of activating and expanding β-cell-reactive $CD4^+$ T cells [1]. Both pathogenic functions depend on the production of self-reactive Ig by B lymphocytes [2, 3], indicating that defective B-lymphocyte self-tolerance is a mechanism that contributes to susceptibility to this disease. Several lines of evidence also point to a pathogenic role for B lymphocytes in human T1D. For example, a recently completed clinical trial has demonstrated that treatment of newly diagnosed T1D patients with the B lymphocyte depleting Ab Rituximab resulted in markedly improved preservation of β-cell function [4]. Furthermore, the fact that the presence and spectrum of β-cell-specific auto-Ab

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have predictive value in determining T1D susceptibility is consistent with the impairment of B-lymphocyte tolerance in patients developing disease [5].

Studies of models in which BCR Tg specific for natural or neo-self-Ag are expressed in the germ-line of non-autoimmune prone mouse strains have revealed a range of self-tolerance mechanisms in the B-lymphocyte compartment [6]. Which of these mechanisms operate in particular situations is determined by the avidity of the Tg BCR for its cognate self-Ag, *i.e.* a combination of Ag valency, concentration and receptor affinity [7]. Self-reactive B lymphocytes that encounter cognate Ag with high avidity modify their specificity by receptor editing or are purged from the repertoire by clonal deletion. Lower avidity encounters cause B lymphocytes to undergo maturation arrest in a non-responsive anergic state. In the event that the self-Ag is inaccessible or present at very low concentrations, self-reactive B lymphocytes remain in an "ignorant" non-tolerant state.

In the first reported analysis of mechanisms of B-lymphocyte tolerance in NOD mice, Tg encoding hen-egg lysozyme (HEL) as a ubiquitous neo-self-Ag in high avidity multivalent membrane-bound form (mHEL) or a lower avidity oligovalent-soluble form (sHEL) were introduced into NOD.*IgHEL*-Tg mice, whose B-lymphocyte repertoire is largely composed (>90%) of HEL-specific B lymphocytes [8]. HEL-specific B lymphocytes encountering mHEL on the NOD background underwent deletion and receptor editing as efficiently as those on the non-autoimmune prone C57BL/6 (B6) background. B lymphocytes from mice of both genetic backgrounds were also equally susceptible to induction of anergy following exposure to sHEL, as manifest by decreased production of Ab, down-regulation of surface IgM and loss of marginal zone (MZ) B lymphocytes. Nevertheless, two lines of evidence suggested that tolerance among HEL-specific Ig Tg (*IgHEL*-Tg) B lymphocytes in NOD mice was less complete than in those with a B6 background. First, immature NOD.*IgHEL* B lymphocytes proved to be less susceptible to deletion than B6 counterparts when exposed to sHEL. Second, the anergic state of NOD compared with B6 *IgHEL*-Tg B lymphocytes escaping deletion was more readily reversed following *in vitro* stimulation through BCR and CD40. The greater predisposition of NOD B lymphocytes to loss of self-tolerance was subsequently shown to be controlled by genes mapping to *insulin-dependent diabetes* (*Idd*) susceptibility loci on Chromosomes 1(*Idd5*) and 4 (Idd9/11), respectively [9].

To determine whether such defects in tolerance were responsible for the production of diabetogenic B lymphocytes specific for β-cell proteins *per se* in NOD mice, Thomas and colleagues generated a BCR Tg model (termed *125*-Tg) where the majority of B lymphocytes were rendered specific for insulin [10]. Surprisingly, *125*-Tg B lymphocytes seemed to be effectively tolerised in mice on both NOD and B6 backgrounds, as demonstrated by their failure to secrete anti-insulin Ab or to respond to a range of potent stimuli. However, it was not clear whether these findings were applicable to all B lymphocytes recognising insulin in NOD mice given that anti-insulin auto-Ab are regularly detected at elevated levels in WT NOD, but not B6 mice [11]. Furthermore, this Tg system did not allow comparison of tolerant B lymphocytes with cells of the same specificity that develop in the absence of insulin. We therefore adopted an alternative strategy for analysing the reactivity of B lymphocytes specific for β-cell-restricted proteins in mice with a diabetes-prone genetic background. The strategy involved generation of a different version of the *HEL/IgHEL*-Tg model where the fate of B6 and NOD HEL-specific B lymphocytes could be examined in the presence or absence of their cognate neo-self-Ag HEL expressed as a Tg under the transcriptional control of an insulin promoter (*insHEL*).

Results

Tg model of B-lymphocyte tolerance to β-cell Ag in T1D-prone and -resistant environments

To examine tolerance in B lymphocytes recognising only β-cell Ag, *IgHEL*-Tg mice were crossed onto *insHEL*-Tg mice in which mHEL is expressed under the transcriptional control of the rat insulin promoter and therefore confined to β cells. In addition to being expressed at high levels relative to other proteins on the β-cell surface, HEL is cleaved off and released into the circulation of *insHEL*-Tg mice in minute concentrations, mirroring the physiological levels of insulin (1–2 ng/mL) [12, 13]. In our hands, serum levels of HEL were similar between NOD.*insHEL* and B6.*insHEL* mice (2.2 ± 0.9 *versus* 1.4 ± 0.1 ng/mL ($n = 5$), respectively; NS, *t*-test).

To determine whether expression of the *insHEL* Tg in the presence or absence of the *IgHEL* Tg would influence development of T1D in NOD or B6 mice, the onset of disease was monitored in female progeny over a 40-wk period. B6 mice expressing *insHEL* with or without the *IgHEL* Tg did not develop diabetes (data not shown). On the other hand, NOD mice expressing the *insHEL* Tg remained T1D susceptible, albeit at a lower incidence than their non-Tg littermates (37.5 *versus* 100%, respectively; Fig. 1). Since the Tg integration site of *insHEL* is on chromosome 12 [14], which does not have a reported *Idd* susceptibility locus in NOD mice [15], genes within the congenic region surrounding the Tg are unlikely to be the cause of decreased T1D susceptibility in this strain. Rather, we expect that expression of the Tg may interfere with β-cell recognition by T cells in NOD mice, since co-expression of *insHEL* and *IgHEL* Tg in NOD mice, making most B lymphocytes specific for β-cell-restricted HEL, significantly increased the overall T1D incidence compared with *insHEL*-Tg mice (87.5%, Fig. 1). This provided direct evidence in favour of a role for B lymphocytes in the pathogenesis of T1D. The fact that the onset of disease was delayed in *IgHEL* × *insHEL* double (Dbl)-Tg NOD mice compared with non-Tg controls does not invalidate the model, presumably being a reflection of the limited diversity in the anti-self B-lymphocyte response towards β cells or the reduced efficiency with which HEL-reactive T cells are recruited into the diabetogenic response.

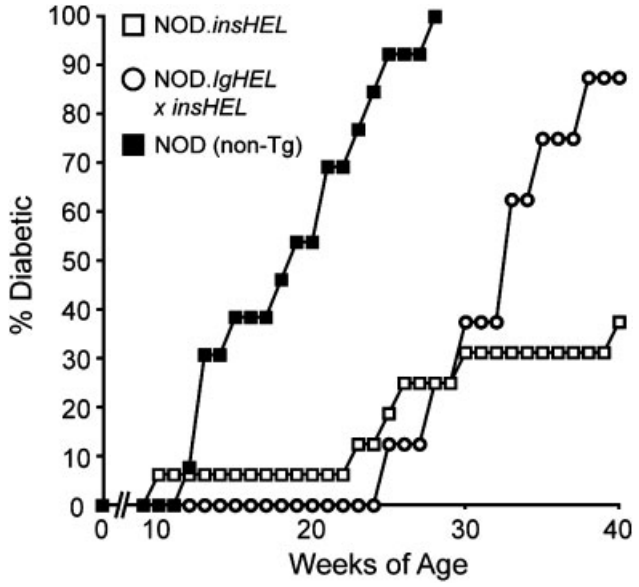

Figure 1. Reduced T1D incidence in NOD.*insHEL* mice is partly restored by co-expression of the *IgHEL* Tg. Cumulative T1D incidence for NOD.*insHEL* ($n = 16$), NOD.*IgHEL* × *insHEL* ($n = 8$) and non-Tg ($n = 13$) female littermates plotted over 40 wk of age. Incidence in all groups of mice differed significantly from each other ($p<0.05$, log-rank test).

HEL-specific B lymphocytes are not tolerised to their cognate Ag in *IgHEL* × *insHEL* Dbl-Tg mice

The next step was to compare the tolerant state of HEL-specific B lymphocytes in NOD *versus* B6 backgrounds when they developed in the presence or absence of their cognate β-cell neo-self-Ag in *IgHEL x insHEL* Dbl-Tg or *IgHEL* single (Sgl)-Tg mice, respectively. Mice were initially analysed 6–8 wk post-natally, an age associated with the early stages of T1D development in NOD mice. The proportion and numbers of HEL-specific B lymphocytes were similar in spleen, pancreatic (PLN) and inguinal LN (iLN) irrespective of whether they were obtained from Dbl- *versus* Sgl-Tg mice on NOD or B6 backgrounds (Fig. 2A and Supporting Information Fig. 1A), consistent with a lack of clonal deletion. Equivalent numbers were also detected in pancreata of Dbl and Sgl-Tg mice ($\sim 5 \times 10^5$ in both NOD groups and undetectable in B6 groups; data not shown). Similarly, there was no evidence of anergy in HEL-specific B lymphocytes from Dbl-Tg mice since the surface levels of IgM^a (Fig. 2B), distribution of B-lymphocyte sub-populations (Supporting Information Fig. 1B) and production of serum anti-HEL Ab (Fig. 2C) were comparable to those found in Sgl-Tg mice on either NOD or B6 genetic backgrounds. The absence of anergy was confirmed in two additional ways: first, proliferative responses of Dbl- and Sgl-Tg B lymphocytes cultured with various stimuli on NOD or B6 backgrounds were similar (Fig. 2D); second, B lymphocytes expressing the An1 phenotype associated with anergy ([16] and Supporting Information Fig. 1C) were virtually absent in Dbl-Tg, as they were in Sgl-Tg mice (Fig. 2E). On the other hand, B lymphocytes bearing this anergic footprint were readily detected in *IgHEL* × *sHEL* Dbl-Tg mice where the neo-self-Ag was expressed ubiquitously (Fig. 2E). On subsequent analysis of self-reactive B lymphocytes in *IgHEL* × *insHEL* Dbl-Tg mice at 16 wk of age, when disease is well established in NOD mice, there was no significant change in any of the above parameters (data not shown). Taken together, these data indicated that NOD or B6 HEL-specific B lymphocytes in *IgHEL* × *insHEL* Dbl-Tg mice are not tolerised (*i.e.* ignorant) to their cognate neo-self-Ag when its expression is confined to β cells.

Anergy induced in B lymphocytes specific for insHEL when comprising a fraction of the repertoire

A caveat to be placed on the *IgHEL* × *insHEL* Dbl-Tg model on either NOD or B6 backgrounds is that the vast majority of B lymphocytes are HEL-specific and therefore have to compete for minute quantities of the neo-self-Ag being produced by β cells. Consequently, when BCR occupancy *per* cell was measured, it was relatively low at 6.5 ± 3.6 and $4.8 \pm 2.3\%$ for splenic B lymphocytes obtained from Dbl-Tg mice on B6 and NOD backgrounds, respectively (Supporting Information Fig. 2A). This was well below the threshold of 20–25% previously shown to be required for induction of anergy in the B-lymphocyte lineage [17, 18]. Within a polyclonal repertoire, however, the frequency of self-reactive B lymphocytes would be much lower than in the Dbl-Tg model described above. Under these conditions, self-reactive B lymphocytes would not only be exposed to higher concentrations of Ag, but also to an environment where they must compete with non-autoreactive B lymphocytes for survival factors (*e.g.* BAFF) and physiological niches [19–21].

To examine B-lymphocyte tolerance to β-cell Ag within an environment where self-reactive clones comprise only a minor fraction of the total repertoire, NOD.*insHEL* and B6.*insHEL* mice as well as their non-Tg littermates were lethally irradiated and reconstituted with a mixture of BM cells from *IgHEL-Tg* and non-Tg donors of the same genetic background. Chimeras reconstituted with *IgHEL* to non-Tg BM ratios of 7:3 and 1:1 for B6 and NOD background mice, respectively, had equivalent proportions of HEL-specific B lymphocytes (mostly ranging between 10 and 20%) in BM, spleen and PLN 6 wk post-reconstitution, irrespective of whether recipients expressed *insHEL* or were non-Tg (Fig. 3A and Supporting Information Fig. 3). Thus, self-reactive B lymphocytes had not been deleted. The difference in ratio of Tg to non-Tg donor BM required to achieve equivalent levels of reconstitution suggested that NOD haematopoietic B-lymphocyte progenitors expand at a different rate to those from B6 mice, a phenomenon which has been described previously for mixed chimeras containing Tg and non-Tg T cells on the NOD background [12]. As expected, BCR occupancy of HEL on *IgHEL*-Tg B lymphocytes was increased ~3-fold in *insHEL*-Tg B6 and NOD chimeras compared with Dbl-Tg mice (17.3 ± 2.5 *versus* $18.6 \pm 4.4\%$, respectively; Supporting Information Fig. 2B).

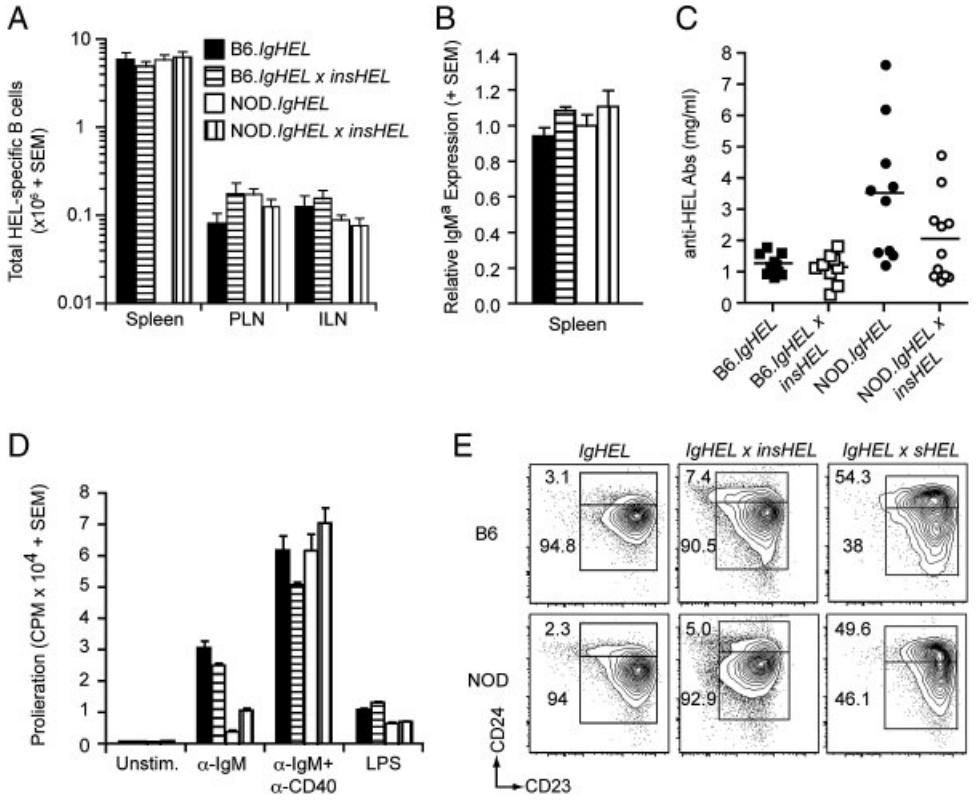

Figure 2. HEL-specific B lymphocytes in *IgHEL* × *insHEL* Dbl-Tg mice remain ignorant of their cognate β-cell neo-self-Ag in NOD and B6 mice. (A) Total numbers and (B) relative surface IgM^a expression of *IgHEL*-Tg B lymphocytes (as gated in representative FACS plots shown in Supporting Information Fig. 1A) in the indicated tissues of B6.*IgHEL* ($n = 5$), B6.*IgHEL* × *insHEL* ($n = 5$), NOD.*IgHEL* ($n = 6$), and NOD.*IgHEL* × *insHEL* ($n = 6$) mice were determined by FACS. MFI of IgM^a was normalised to the mean of the NOD.*IgHEL* group. (C) Concentration of HEL-specific Ab in the sera of indicated strains was quantified by ELISA. (D) Proliferation of 1×10^5 purified B lymphocytes cultured in triplicate in media alone, 10 μg/mL LPS, or 10 μg/mL anti-IgM F(ab′)$_2$ with or without 5 μg/mL CD40-specific Ab was determined by [^{3}H]-thymidine incorporation in the final 24 of a 72 h incubation period. Data are representative of two experiments. (E) Representative FACS plots of mice in (A), showing proportion of An1 (CD24^{hi}) and FO (CD24^{lo}) B-lymphocyte subsets (full gating strategy in Supporting Information Fig. 1C). CD93 was not used due to defective expression by NOD mice [33]. No significant differences (p>0.05, t-test) between Sgl and Dbl-Tg mice on the same genetic background in any experiment.

In contrast to *IgHEL* × *insHEL* Dbl-Tg mice, when anti-HEL Ab levels were measured in the sera of NOD and B6 chimeras, there was a marked reduction in the *insHEL* compared with non-Tg hosts, consistent with the notion that HEL-specific B lymphocytes had become tolerant on both backgrounds (Fig. 3B). Several lines of evidence supported this conclusion and pointed to the induction of anergy. First, a large proportion (~40–50%) of *IgHEL*-Tg B lymphocytes in NOD.*insHEL* and B6.*insHEL* chimeras had undergone maturation arrest at the An1 stage of B-lymphocyte development (Fig. 3C) as had occurred in the original *IgHEL* × *sHEL* Dbl-Tg model (Fig. 2E). Second, the surface levels of IgM^a were significantly diminished on HEL-specific B lymphocytes in the spleen and LN of *insHEL* compared with non-Tg chimeras on both backgrounds (Fig. 3D and Supporting Information Fig. 3). Finally, there was a substantial loss of HEL-specific MZ B lymphocytes in both NOD.*insHEL* and B6.*insHEL* chimeric mice, but not in non-Tg chimeras (Fig. 3E).

On the other hand, NOD or B6 *IgHEL*-Tg B lymphocytes exposed to insHEL in chimeras retained the capacity to up-regulate the T-cell co-stimulatory molecule CD86 (similar to naïve B lymphocytes) when stimulated through BCR alone or in combination with CD40 *ex vivo* (Fig. 3F). This capacity to increase CD86 is in contrast to other models of anergy [22], although a similar result was reported in anergic 125-Tg anti-insulin B lymphocytes [10]. Interestingly, NOD.*IgHEL* B lymphocytes from *insHEL* and non-Tg chimeras stimulated through BCR+CD40 receptor consistently showed higher levels of CD86 than B6 counterparts (Fig. 3F), suggestive of an increased capacity to act as APC for T cells.

Immunohistological analyses of the positioning of HEL-specific B lymphocytes within spleens of *insHEL*-Tg chimeras revealed another intriguing difference between NOD and B6 backgrounds. Thus, although HEL-specific B lymphocytes in the former were excluded from follicles and remained in the outer peri-arteriolar lymphoid sheath (PALS) of the T-cell zone in the presence of their cognate neo-self-Ag, their B6 counterparts were able to enter follicles as they did in non-Tg hosts (Fig. 3G). In view of the known susceptibility of NOD mice to autoimmunity, we reasoned that NOD B lymphocytes, by virtue of their localisation in the T-cell zone and increased CD86 expression, may be better positioned than B6 B lymphocytes to

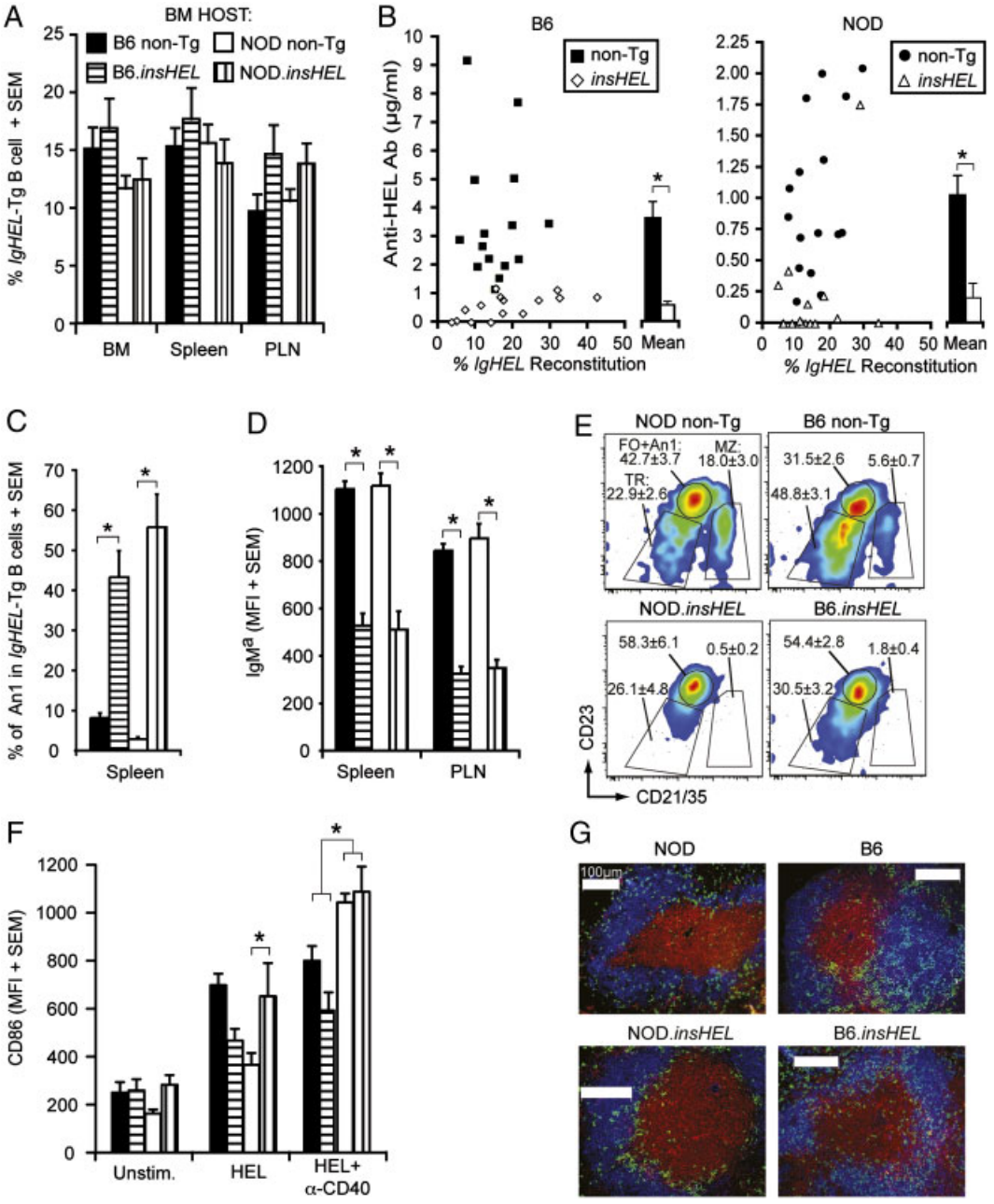

Figure 3. Diminished frequency of *IgHEL*-Tg B lymphocytes in mixed BM chimeras results in anergy to insHEL in NOD and B6 backgrounds. Lethally irradiated *insHEL* and non-Tg mice on NOD or B6 backgrounds were reconstituted with 1:1 or 7:3 mixtures of *IgHEL* to non-Tg BM cells of the same background, respectively. Tissues and sera were analysed 6–8 wk post-reconstitution. (A) Mean percentage of *IgHEL*-Tg B lymphocytes relative to the total (B220+) B-lymphocyte pool was determined for the indicated tissues of B6 ($n = 15$), B6.*insHEL* ($n = 16$), NOD ($n = 16$), NOD.*insHEL* ($n = 16$) chimeras by FACS. Representative FACS plots with gating strategy shown in Supporting Information Fig. 3. No significant differences between groups ($p > 0.05$, one-way ANOVA). (B) Concentration of anti-HEL Ab in sera of individual B6, B6.*insHEL*, NOD and NOD.*insHEL* chimeras was measured by ELISA and plotted against splenic reconstitution levels of *IgHEL*-Tg B lymphocytes. Mean concentrations shown by bars (+SEM). $^*p < 0.05$ (t-test). (C) Mean proportion of *IgHEL*-Tg B lymphocytes presenting the An1 phenotype was determined by FACS using the gating strategy in Supporting Information Fig. 1C. (D) MFI of surface IgM^a on *IgHEL*-Tg B lymphocytes residing in spleens or PLN of B6 ($n = 5$), B6.*insHEL* ($n = 7$), NOD ($n = 5$) and NOD.*insHEL* ($n = 4$) BM chimera recipients were determined by FACS. $^*p < 0.05$ (one-way ANOVA *post hoc* test). (E) Representative FACS plots of chimeras described in (A), displaying the mean proportions (±SEM) of transitional, FO+An1 and MZ subsets of *IgHEL*-Tg B lymphocytes in all recipients from each group. (F) Splenocytes from three B6, B6.*insHEL*, NOD, NOD.*insHEL* BM chimeras were independently cultured for 16 h in media alone (Unstim.) or with 100 ng/mL HEL ± 5 µg/mL of anti-CD40. Graph representative of four similar experiments showing mean MFI of CD86 on cultured *IgHEL*-Tg FO+An1 B lymphocytes (gated as in (E)) as measured by FACS. $^*p < 0.05$ (two-way ANOVA *post hoc* test). (G) Representative immunohistological staining of spleen sections from three mice of the indicated chimera groups showing B220 (marking follicles) in blue, CD4 (marking PALS) in red, and HEL-specific B lymphocytes in green. Slides were analysed under 10 × magnification. Lengths of white bars mark 100 µm.

interact with T-helper cells, resulting in reversal of their tolerant state. Such a possibility was clearly worth testing, given that self-tolerance in the T-cell compartment of NOD mice is also compromised, leading to the presence of self-reactive T cells in the outer PALS.

Adoptive transfer of HEL-specific B lymphocytes into *insHEL*-Tg mice also renders them tolerant

The proposition that defective B-lymphocyte tolerance in NOD mice may be due to enhanced sensitivity to T-cell help was not readily testable in BM chimeras for two reasons: (i) basal levels of anti-HEL Ab present in their sera (Fig. 3B) could mask *de novo* production of Ab by T-cell-activated *IgHEL-Tg* B lymphocytes and absorb immunising conjugates containing HEL; (ii) analysis of B-lymphocyte fate could be performed only 6 wk after initial exposure to Ag (*i.e.* after complete reconstitution). Consequently, to test the above hypothesis, we utilised an alternative model incorporating adoptive transfer, in which competition between the self-reactive B lymphocytes and the polyclonal repertoire was maintained and mice could be analysed within 24–36 h of exposure to the neo-self-Ag. It was initially important to determine that self-reactive B lymphocytes were subject to anergy in this model before using it to compare susceptibility of NOD *versus* B6 B lymphocytes to the breakdown of this anergy, following provision of T-cell help (see next section).

Splenocytes from B6.*IgHEL* or NOD.*IgHEL* donors (containing $\sim 1–2 \times 10^6$ HEL-specific B lymphocytes) were transferred i.v. into naïve *insHEL-Tg* or non-Tg recipients of the same background (Fig. 4A). One day after transfer, similar numbers of NOD and B6 *IgHEL*-Tg B lymphocytes (0.4–0.5% of all B lymphocytes) were found in spleens (Supporting Information Fig. 4) and LN (data not shown) of both *insHEL*-Tg and non-Tg recipients. IgM^a levels were reduced on *IgHEL*-Tg NOD and B6 B lymphocytes obtained from spleen, PLN and inguinal LN (iLN) of *insHEL* but not non-Tg recipients (Fig. 4B), indicative of encounter with the shed β-cell neo-self-Ag. This resulted in a high BCR occupancy of 88.2 ± 13.3 and 89.2 ± 11.5% on B6 and NOD backgrounds, respectively (Supporting Information Fig. 2C). NOD and B6 HEL-specific B lymphocytes transferred into non-Tg recipients persisted past day 7 as expected, whereas those transferred to *insHEL* mice were significantly depleted by day 4 and absent by day 7 in spleen and PLN (Fig. 4C and D and Supporting Information Fig. 4). Anergic B lymphocytes specific for the model neo-self-Ag were therefore effectively purged from the functional B-lymphocyte repertoire whether derived from NOD or non-autoimmune B6 donors, as had occurred in the BM chimeras.

Immunohistology of recipient spleens 1 day post-transfer yielded a similar result to the chimeras (Fig. 3G) in that B6.*IgHEL* B lymphocytes, before being eliminated, could enter follicular (FO) areas, whereas NOD.*IgHEL* B lymphocytes remained in the outer PALS of *insHEL*-Tg recipients (Fig. 4C). This difference did not appear to stem from varying exposure to insHEL in NOD *versus* B6 recipients, since the concentration of circulating self-Ag as well as the extent of IgM down-regulation (Fig. 4B) and BCR occupancy on transferred *IgHEL*-Tg B lymphocytes (Supporting Information Fig. 2C) were equivalent between strains. To determine whether positional differences were intrinsic to B lymphocytes or due to host factors such as stromal cell interactions or availability of T-cell help, purified NOD.*IgHEL* or B6.*IgHEL* B lymphocytes were transferred into common (NOD × B6)F1 hosts expressing the *insHEL* Tg. One day after transfer, NOD and B6 HEL-specific B lymphocytes could both be found residing within FO areas in spleens of (NOD × B6)F1.*insHEL* recipients (Fig. 4E), indicating that differences in their positioning within NOD.*insHEL* and B6.*insHEL* recipients were due to host intrinsic factors.

Adoptively transferred NOD B lymphocytes are susceptible to reversal of anergy with T-cell help

Having established that the adoptive transfer system mimicked the findings reported in the BM chimeras, the next step was to provide transferred HEL-specific B lymphocytes with a source of T-cell help to directly test whether tolerance could be reversed in NOD but not B6 B lymphocytes. For this purpose, T cells in potential *insHEL* or non-Tg recipients on the NOD or B6 background were initially primed with sheep RBC (SRBC). Seven days later, NOD or B6 *IgHEL-Tg* B lymphocytes were transferred into these carrier-primed recipients of the same background in combination with HEL-SRBC conjugates (Fig. 5A). In this way, *IgHEL*-Tg B lymphocytes could specifically capture conjugates *via* HEL and attract help from SRBC-primed T cells. The advantage of this strategy was that it permitted assessment of B-lymphocyte tolerance in NOD and B6 hosts independently of differences in T-cell responsiveness and tolerance to the neo-self-Ag HEL between these strains [12]. Four to seven days after provision of T-cell help, significant expansion of donor *IgHEL*-Tg B lymphocytes was observed in both NOD and B6 non-Tg recipients (Fig. 5B and C). As expected, B6.*IgHEL* B lymphocytes transferred into B6.*insHEL* mice with T-cell help underwent significantly less expansion than those in non-Tg recipients. On the contrary, expansion of NOD.*IgHEL* B lymphocytes persisted at similar levels irrespective of whether they were transferred into *insHEL* or non-Tg mice (Fig. 5B and C). A similar trend was observed when anti-HEL Ab produced by adoptively transferred B lymphocytes was measured in recipients. Thus, a significant T-dependent Ab response was mounted by NOD.*IgHEL* and B6.*IgHEL* B lymphocytes provided with T-cell help in non-Tg recipients, with the peak occurring at day 7 (Fig. 5D). When B6.*IgHEL* B lymphocytes were transferred into *insHEL* recipients with T-cell help, they consistently produced much less Ab compared with those transferred into non-Tg recipients (Fig. 5D and E). On the other hand, no difference was observed in Ab levels generated by NOD.*IgHEL* B lymphocytes, following their transfer into *insHEL* or non-Tg recipients with T-cell help (Fig. 5D and E). A five to tenfold decrease in the level of T-cell help did not significantly alter the ability of NOD B lymphocytes to be rescued from tolerance, with induction of anergy occurring only when T-cell

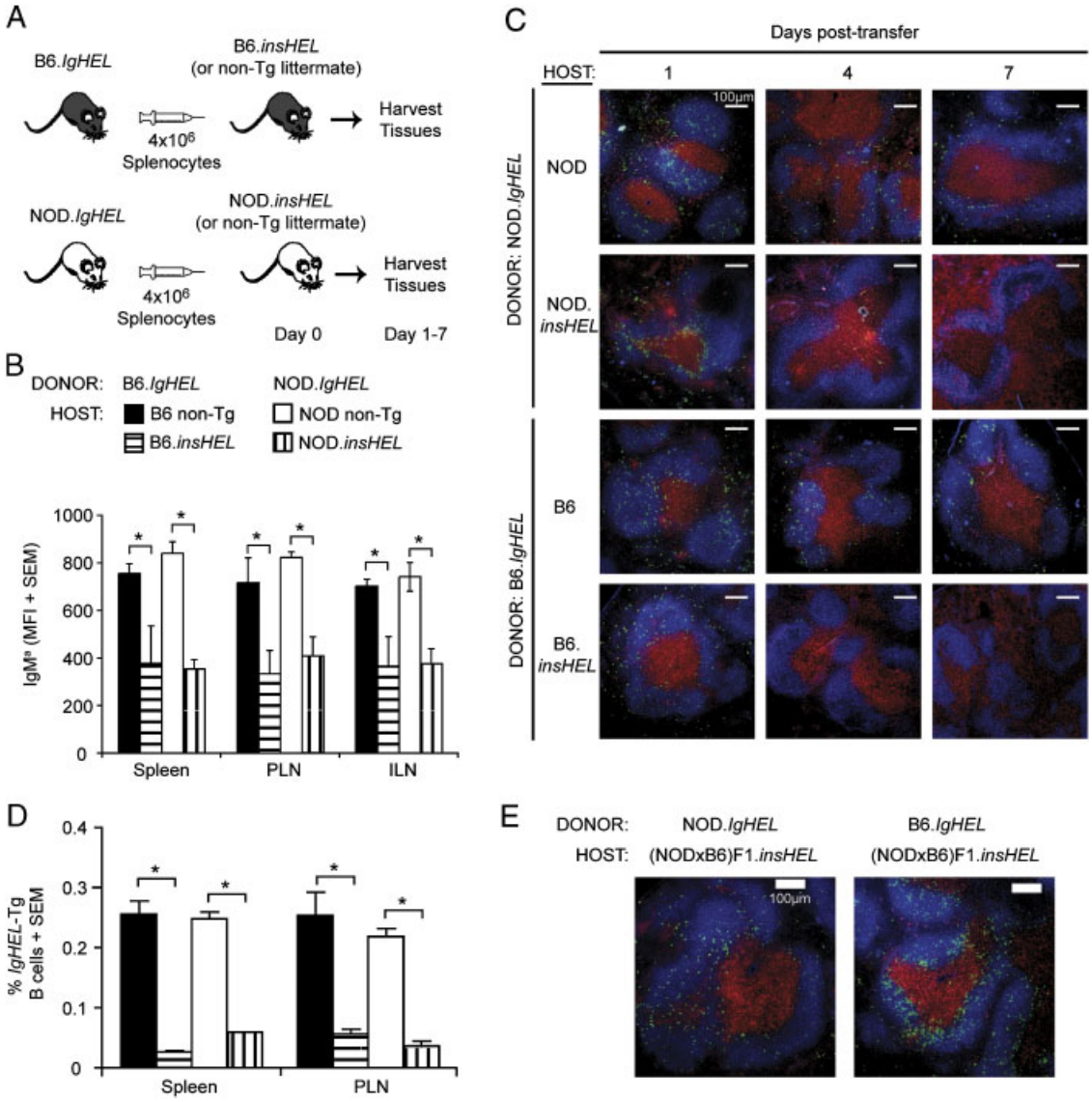

Figure 4. HEL-specific B lymphocytes are effectively tolerised on NOD and B6 backgrounds upon adoptive transfer into *insHEL*-Tg recipients. (A) Protocol for adoptive transfer of NOD.*IgHEL* or B6.*IgHEL* Tg B lymphocytes into *insHEL* and non-Tg mice of the same background. (B) MFI of surface IgM^a on *IgHEL*-Tg B lymphocytes (gated as shown in Supporting Information Fig. 4) from the indicated tissues of three *insHEL* and non-Tg recipients on NOD and B6 backgrounds were determined one day post-transfer by FACS. (C) Representative immunohistological staining of spleens from three recipient mice of the indicated groups, 1, 4 or 7 days post-transfer. B220 staining (marking follicles) shown in blue, CD4 (marking PALS) in red, and HEL-specific B lymphocytes in green. Slides were analysed under 10 × magnification. Lengths of white bars mark 100 μm. (D) Mean percentage of donor *IgHEL*-Tg B lymphocytes remaining in the indicated organs of recipients ($n = 3$–5) 7 days post-transfer was determined by FACS. Representative FACS plots for days 1, 4 and 7 are shown in Supporting Information Fig. 4. Graph representative of two experiments. $^*p<0.05$ *insHEL versus* non-Tg recipients of the same background (*t*-test). (E) Representative immunohistological staining of spleen sections from three (NODxB6)F1.*insHEL*-Tg recipients 1 day after transfer of 2×10^6-purified B lymphocytes from NOD.*IgHEL* or B6.*IgHEL* donors. Sections were analysed as in (C).

help was reduced 50-fold (Supporting Information Fig. 5A). Similar results were observed in experiments where donor *IgHEL*-Tg B lymphocytes were transferred from LN containing predominantly FO B lymphocytes (Supporting Information Fig. 5B), indicating that the disparity in the tolerogenic fate of self-reactive NOD *versus* B6 B lymphocytes was not due to strain differences in spleen-resident MZ B lymphocytes (Supporting Information Fig. 1B). Together, these data confirmed the hypothesis that NOD self-reactive B lymphocytes are peculiarly susceptible to being rescued from anergy, following activation by T-cell signals.

B-lymphocyte intrinsic factors govern susceptibility to the breakdown of tolerance

The failure of B lymphocytes on the NOD background to maintain anergy to a β-cell neo-self-Ag when provided with T-cell help raised the question of whether this was an intrinsic property of transferred B lymphocytes or due to extrinsic host factors like those controlling B-lymphocyte positioning (Fig. 4E) or the quality of T-cell help. To investigate this question, we utilised the same adoptive transfer system as above except that purified

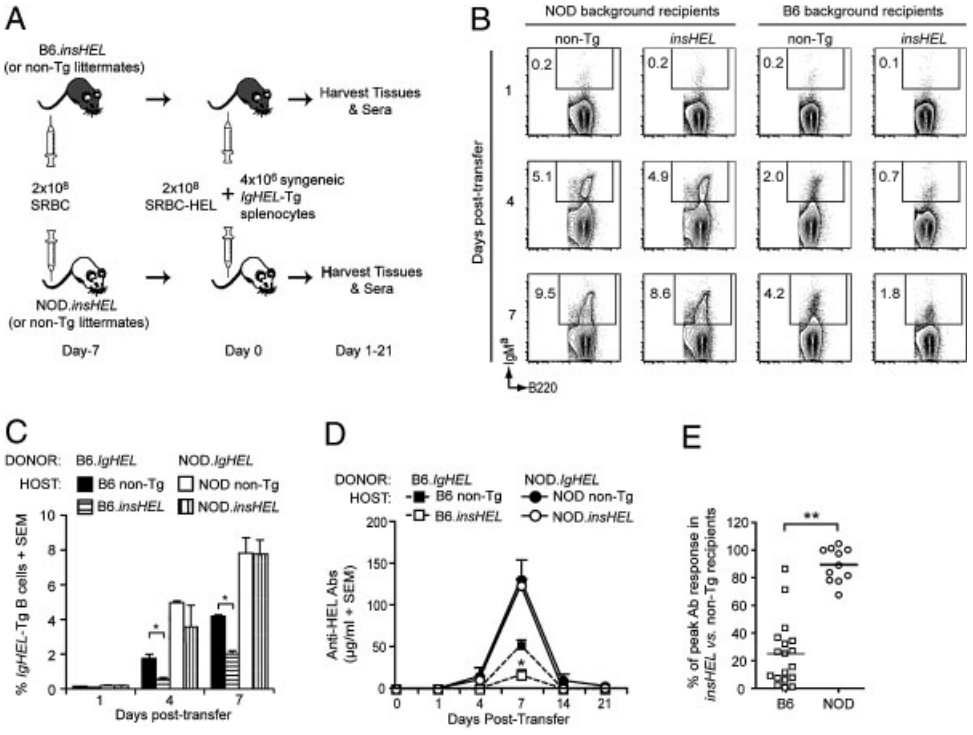

Figure 5. Induction of B-lymphocyte tolerance to insHEL can be reversed by provision of T-cell help in the NOD but not the B6 genetic background. (A) Protocol for adoptive transfer of NOD.*IgHEL* or B6.*IgHEL* Tg B lymphocytes into *insHEL* or non-Tg mice of the same background with T-cell help. (B) Representative FACS plots and (C) group means of B lymphocytes of *IgHEL*-Tg donor origin (B220+IgM^a+) in spleens of B6 non-Tg, B6.*insHEL*, NOD non-Tg and NOD.*insHEL* recipient mice 1, 4 or 7 days post-transfer ($n = 3$–5/timepoint). *$p<0.05$ *insHEL versus* non-Tg groups on the same background (one-way ANOVA, *post hoc* test). (D) Representative of six ELISA experiments showing mean concentrations of anti-HEL Ab in the sera of seven B6 non-Tg, five B6.*insHEL*, three NOD non-Tg and three NOD.*insHEL* recipient mice at various time points before (day 0) and after transfer of *IgHEL*-Tg splenocytes with SRBC-HEL. *$p<0.05$ *insHEL versus* non-Tg recipient groups given the same donor splenocytes at a specific time point (*t*-test). (E) Comparison of anti-HEL Ab levels produced by NOD *versus* B6 *IgHEL*-Tg B lymphocytes in individual *insHEL* recipients at the peak of response (day 7), presented as a percentage of the mean anti-HEL Ab levels in non-Tg control recipients injected with the same donor splenocytes and SRBC/SRBC-HEL preparations in all six experiments. **$p<0.001$ (*t*-test).

NOD.*IgHEL* or B6.*IgHEL* B lymphocytes were injected together with SRBC-HEL into SRBC-primed *insHEL* or non-Tg recipients on a common (NOD × B6)F1 background (Fig. 6A). Consistent with susceptibility to reversal of tolerance induction being an intrinsic property of B lymphocytes, the Ab response of B6.*IgHEL* B lymphocytes transferred with T-cell help into (NOD × B6)F1. *insHEL* recipients was significantly reduced compared with those in non-Tg littermates, whereas NOD.*IgHEL* B lymphocytes receiving help in the same host environment produced robust Ab responses whether or not *insHEL* was expressed (Fig. 6B).

Idd5 and *Idd9/11* loci contribute to the T-cell-mediated breakdown of tolerance in NOD B lymphocytes

A previous study from our laboratories indicated that introgression of *Idd5* or *Idd9/11* T1D-resistance loci into the NOD genetic background restored the anergic state of self-reactive B lymphocytes from the *IgHEL* × *sHEL* Dbl-Tg model [9]. We therefore set out to confirm whether these particular *Idd* loci were involved in the T-cell-mediated breakdown of B-lymphocyte tolerance to the β-cell neo-self-Ag in NOD.*insHEL* recipients. This was done by giving SRBC-primed NOD.*insHEL* or non-Tg littermates purified *IgHEL*-Tg B lymphocytes from NOD mice containing either a congenic region of C57BL/10 (B10)-derived resistance alleles at *Idd5* (NOD.*Idd5*^{B10}), non-obese resistant (NOR)-derived resistance alleles at *Idd9/11* (NOD.*Idd9/11*^{NOR}) or both (Fig. 7A). Upon challenge with SRBC-HEL, the anti-HEL Ab response produced by NOD.*Idd5*^{B10} or NOD.*Idd9/11*^{NOR} HEL-specific B lymphocytes following provision of T-cell help at day 7 was comparable in *insHEL* and non-Tg recipients (Fig. 7B), as occurred in the previous experiments using NOD.*IgHEL* B lymphocytes (Fig. 5D and E). Nevertheless, there was a marked reduction in the T-dependent Ab response of HEL-specific B lymphocytes from NOD mice containing both *Idd5*^{B10} and *Idd9/11*^{NOR} congenic regions following transfer into *insHEL* compared with non-Tg recipients (Fig. 7B), reminiscent of experiments performed with B6.*IgHEL* B lymphocytes (Fig. 5D and E). Hence, contributions from B10- and NOR-derived genes located within both *Idd5* and *Idd9/11* regions were required to prevent T-cell-induced breakdown of B-lymphocyte self-tolerance in NOD mice. Since only the transferred *IgHEL*-Tg B lymphocytes expressed different alleles of *Idd5* and *Idd9/11* genes, this result confirms that the breakdown of tolerance to insHEL in the

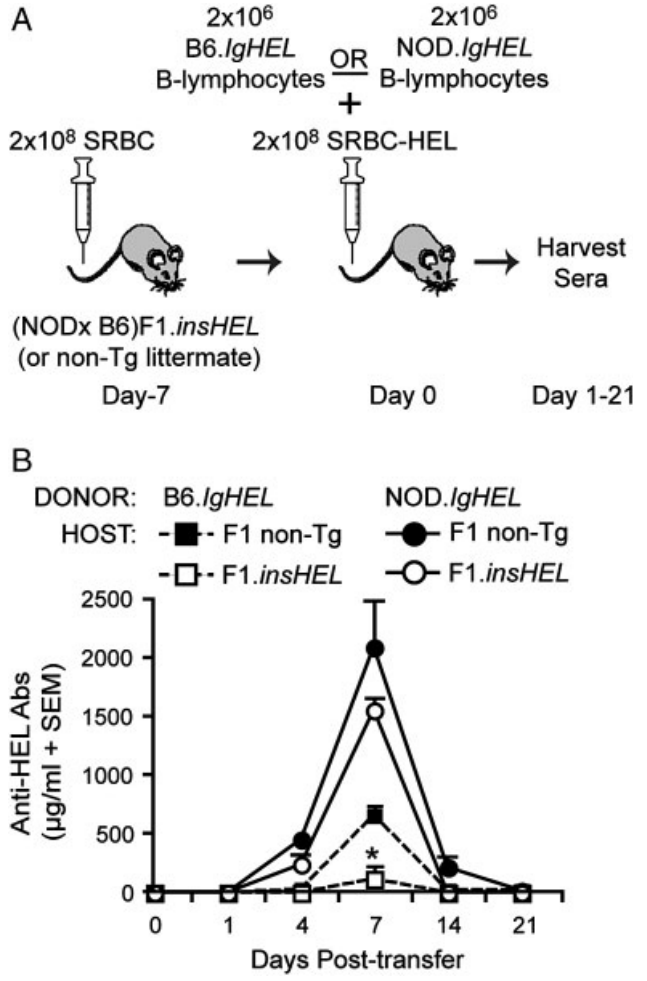

Figure 6. B-lymphocyte intrinsic factors govern the breakdown of tolerance to insHEL following provision of T-cell help. (A) Protocol for adoptive transfer of purified NOD.*IgHEL* or B6.*IgHEL* Tg B lymphocytes into *insHEL* and non-Tg recipients of the (NOD × B6)F1 background with T-cell help. (B) Concentration of serum anti-HEL Ab on the indicated days post-transfer in (NOD × B6)F1 *insHEL*-Tg and non-Tg recipients ($n = 6$/group) receiving purified B6.*IgHEL* or NOD.*IgHEL* B lymphocytes were measured by ELISA. Representative of two experiments. $^*p<0.05$ *insHEL versus* non-Tg recipient groups injected with the same donor B lymphocytes at a specific time point (t-test).

presence of T-cell help is dependent on factors intrinsic to B lymphocytes.

Discussion

According to our previous studies in which *IgHEL*-Tg B lymphocytes were placed in an environment where HEL was expressed as a ubiquitous neo-self-Ag, the loss of self-tolerance in the B-lymphocyte repertoire of NOD mice occurred in peripheral lymphoid tissues, whereas central tolerance in the BM, manifest by deletion and receptor editing, remained intact [8]. Nevertheless, these studies did not tell us how autoimmune B-lymphocyte responses to β-cell-specific Ag *per se* occur. The solution we adopted was to express HEL as a neo-self-Ag specifically on β cells through introduction of the *insHEL* Tg. Interestingly, B lymphocytes remained non-tolerant of the β-cell-restricted Ag when *insHEL*-Tg mice were crossed with *IgHEL*-Tg mice (Fig. 2), as had been demonstrated previously when the expression of HEL was confined to another tissue specific site, the thyroid [23]. Ignorance in our model could not be explained simply in terms of Ag concentration since soluble cleaved HEL was present in the circulation at similar levels to insulin, which normally confers tolerance [24]. Moreover, insufficient affinity for self-Ag was an equally unlikely explanation given that the BCR on *IgHEL*-Tg B lymphocytes has an exceptionally high affinity for HEL ($2 \times 10^{10}\,M^{-1}$, [25]). Consequently, it was logical to surmise that BCR occupancy was inadequate to trigger the *IgHEL*-Tg B lymphocytes due to their high frequency in *IgHEL* × *insHEL* Dbl-Tg mice. Indeed receptor occupancy in these mice (Supporting Information Fig. 2A) was well below the threshold of 20–25% previously shown to be required for activation and induction of anergy to soluble Ag in the B-lymphocyte repertoire [17, 18].

The initial strategy we used to reduce the frequency of self-reactive B lymphocytes to more physiological levels was to generate BM chimeras. By doing so, the frequency of HEL-specific B lymphocytes was indeed reduced (Fig. 3A), which meant that they had to compete for survival factors and environmental niches with the polyclonal B-lymphocyte repertoire. Moreover, there was a marked increase in BCR occupancy by insHEL (Supporting Information Fig. 2B), resulting in induction of B-lymphocyte self-tolerance in the form of anergy not only in non-autoimmune B6 chimeras, but also unexpectedly, on the NOD background. B lymphocytes from both backgrounds displayed the typical anergic characteristics of surface IgM down-regulation (Fig. 3D), arrest in the An1 compartment (Fig. 3C), loss of the MZ population (Fig. 3E) and a reduction in Ab-producing capacity (Fig. 3B). A similar result was obtained in the adoptive transfer model, which also revealed that clones recognising insHEL on either background exhibited a significantly shortened lifespan when competing with polyclonal B lymphocytes (Fig. 4B and C). These features, while well-recognised markers of anergy in most relevant models [22], did not all apply to endogenous insulin-specific B lymphocytes in the *125*-Tg model [10]. Hence, despite a reduction in anti-insulin Ab production and *in vitro* responsiveness to various stimuli by NOD and B6 *125*-Tg B lymphocytes, surface IgM expression remained normal and the MZ subset was increased rather than decreased. Since the frequency of self-Ag-specific B lymphocytes in this Tg model was high (>90%), the explanation for the apparent discrepancy in results could be related either to the ability of insulin to act as a "molten globule" whose configuration is altered by molecular interactions with Ab [26] or to the fact that BCR occupancy was just sufficient to affect Ab production and responsiveness without interfering with other aspects of B-lymphocyte function. Interestingly, an unusual feature of anergic B lymphocytes in both our model and theirs was the retention of the ability to up-regulate co-stimulatory molecules such as CD86 (Fig. 3F and [10]), suggesting that clones specific

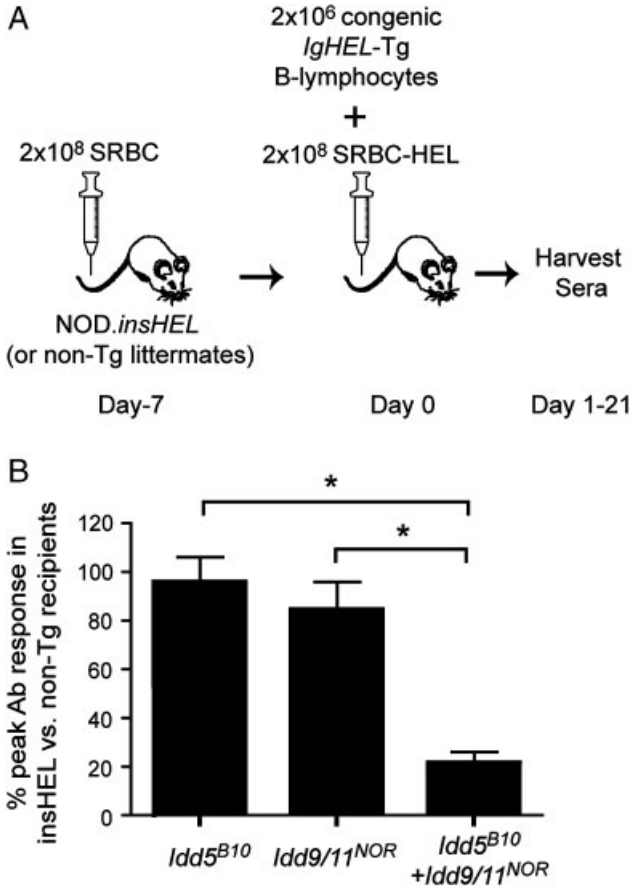

Figure 7. *Idd5* and *Idd9/11* diabetes susceptibility loci contribute to the breakdown of B-lymphocyte tolerance to insHEL following provision of T-cell help. (A) Protocol for adoptive transfer of purified B lymphocytes from NOD.*IgHEL* mice containing $Idd5^{B10}$, $Idd9/11^{NOR}$ or both congenic regions into *insHEL* or non-Tg recipients of the NOD background. (B) Serum anti-HEL Ab concentration in *insHEL* recipients ($n = 6$–8/group) at the peak of response (day 7) as determined by ELISA are displayed as the mean percentage (+SEM) of that detected in non-Tg recipients ($n = 6$–8/group) injected with the same donor B lymphocytes and SRBC/SRBC-HEL preparations in two independent experiments. $^*p<0.001$ between indicated groups (t-test).

for β-cell Ag may possess an enhanced capacity to act as APC for T cells.

In contrast to the *125*-Tg model, where no variation was detected between the tolerogenic outcomes of NOD and B6 self-reactive B lymphocytes [10], our studies did reveal two intriguing differences. First, B6 and NOD *IgHEL*-Tg B lymphocytes recognising insHEL in a competitive environment localised to follicles and T-cell zones, respectively (Figs. 3G and 4B). However, despite their positional differences, self-reactive B lymphocytes were effectively tolerised and eliminated from the repertoire on both backgrounds (Figs. 3B–D, G and 4B–D), consistent with the report of Ekland *et al.* [27], showing that exclusion from FO areas is not a prerequisite for competitive elimination. Nevertheless, the observation led us to speculate that NOD HEL-specific B lymphocytes were better positioned to receive T-cell help; especially in mice on a genetic background where self-tolerance in the T-cell compartment is known to be defective, resulting in the presence of autoreactive T cells capable of providing help [28]. Consistent with this hypothesis, the second difference between self-reactive B lymphocytes on NOD and B6 backgrounds was the enhanced susceptibility of the former to loss of anergy following provision of help (Fig. 5). This difference supported the previous study from our laboratories in which we had shown that *in vitro* stimulation of purified HEL-specific B lymphocytes derived from *IgHEL* × *sHEL* Dbl-Tg donors with anti-IgM and anti-CD40 Ab (mimicking Ag and T-cell encounter), resulted in reversal of anergy in those cells when derived from donors with the NOD, but not the B6 background [8]. These *in vitro* experiments also suggested that anergic B lymphocytes on the NOD background were intrinsically more susceptible to a breakdown in self-tolerance upon provision of T-cell help than anergic B lymphocytes from B6 mice. Confirmation that NOD B lymphocytes specific for β-cell Ag were indeed intrinsically more susceptible to activation *in vivo* was obtained by transferring purified NOD or B6 HEL-specific B lymphocytes into (NOD × B6)F1.*insHEL* recipients, where they were provided with a common source of T-cell help. Here, B6 HEL-specific B lymphocytes remained anergic producing only low levels of Ab, whereas NOD B lymphocytes produced comparable levels of Ab to those transferred into control recipients not expressing the β-cell neo-self-Ag (Fig. 6). Thus properties of NOD B lymphocytes *per se* were the cause of the reversal of anergy rather than differences in T-helper cell function. Moreover, HEL-specific clones from NOD and B6 backgrounds were both capable of entering follicles when transferred into (NOD × B6)F1.*insHEL* recipients (Fig. 4E), thereby de-linking variations in positioning of anergic B lymphocytes from the factors causing differential responsiveness to T-cell help.

Further evidence indicating that T-cell-induced loss of B-lymphocyte tolerance to β cell Ag is B-lymphocyte intrinsic and relevant to the development of T1D came from data, showing that this phenotype could be controlled by genes located within *Idd5* and *Idd9/11* diabetes susceptibility loci when exclusively expressed by these lymphocytes (Fig. 7). This experiment also demonstrated that differences in activation of anergic NOD B lymphocytes were not necessarily due to the presentation of distinct peptides by the unique $H2\text{-}A^{g7}$ MHC class II molecule, since B lymphocytes from NOD and Dbl congenic mice express the same haplotype. Interestingly, both *Idd5* and *Idd9/11* resistance loci were required to maintain *in vivo* tolerance of NOD B lymphocytes to insHEL upon provision of T-cell help, whereas either resistance locus was sufficient to restore anergy in the *IgHEL* × *sHEL* Dbl-Tg model when B lymphocytes were activated *in vitro* through BCR and CD40 [9]. The need for both genetic loci to maintain tolerance in the *in vivo* model may result from the more stringent demands faced by self-reactive B lymphocytes when competing with non-autoreactive cells for T-cell help, survival factors and niches within lymphoid microenvironments. We are currently in the process of identifying those genes within *Idd5* or *Idd9/11* loci with the potential to regulate the integrity of B-lymphocyte tolerance. Genes within *Idd5* and *Idd9/11* loci

may underlie molecular aberrations previously identified in NOD B lymphocytes which include increases in the expression of co-stimulatory molecules such as CD40, CD80 and CD86 in addition to the hyper-responsive state of their NF-κB pathway compared with that of non-autoimmune prone strains [29, 30]. Consistent with these findings were our observations in BM chimeras of greater up-regulation of CD86 on anergic self-reactive B lymphocytes from NOD *versus* B6 donors, following stimulation through BCR and CD40 (Fig. 3F). Such molecular alterations in NOD B lymphocytes may permit stronger interactions with T cells, thereby rendering them more susceptible to rescue from an anergic state.

In summary, our studies in the β-cell neo-self-Ag-specific Tg model have demonstrated that B-lymphocyte anergy can be reversed in mice with the T1D-prone NOD genetic background, following exposure to T-cell help. Once autoreactive B lymphocytes are switched on, they can presumably act as diabetogenic APC, leading to activation and expansion of autoreactive $CD4^+$ T cells, thereby setting off a self-perpetuating cycle leading to β-cell destruction and T1D. In addition to increasing our understanding of B-lymphocyte tolerance to β-cell auto-Ag, the *IgHEL/insHEL*-Tg model has the potential to be used as a valuable tool for testing genes and gene networks responsible for generating autoreactive B lymphocytes in a diabetes-prone environment, ultimately shedding new light on molecular aberrations that may be the targets of future therapies aimed at preventing or treating T1D.

Materials and methods

Mice

Mice were housed in an SPF barrier facility. NOD and B6 mice carrying *IgHEL* or *sHEL* Tg, as well as B6.*insHEL* and NOD.$H2^k$.*insHEL* mice, have been described previously [3, 8, 12, 13, 31]. NOD.$H2^k$.*insHEL* mice were backcrossed to NOD/Lt mice to replace the $H2^k$ congenic region with the $H2^{g7}$ haplotype. NOD.*IgHEL* mice containing B10-derived *Idd5* (R444 line) or the NOR-derived *Idd9/11* congenic region have also been characterised [9] and were intercrossed to produce a Dbl congenic strain. All experiments were performed on female mice and were approved by the Garvan Institute/St. Vincent's Hospital Animal Ethics Committee.

Flow cytometry

Cell suspensions from the indicated tissues were washed in PBS containing 1% BSA and 0.1% NaN_3 (Sigma-Aldrich) and stained with the following fluorochrome-conjugated mAb: anti-B220 (RA36B2), anti-CD24 (M1-69), anti-IgM (II/41) and anti-CD86 (GL1) from BD Biosciences, plus anti-CD21/35 (eBio8D9) and anti-CD23 (B3B4) from eBioscience. *IgHEL*-Tg B lymphocytes on NOD and B6 backgrounds were distinguished from endogenous (IgM^b/IgD^b) counterparts using IgM^a/IgD^a allotype-specific mAb (DS-1 and AMS9.1, BD Biosciences). Stained cells were run on FACSCanto I/II flow cytometers (BD Biosciences) and analysis was performed on FlowJo software (Tree Star).

T1D development

T1D development was assessed weekly in mice by measuring glycosuric values with Keto-Diastix (Bayer). Glucose concentrations of >200 mg/L for two consecutive readings within a week were considered indicative of T1D onset.

Proliferation assays

B lymphocytes were purified from pooled spleens of the indicated strains using the MACS mouse B-cell isolation kit followed by cell filtration through an autoMACS separator (Miltenyi Biotec). This method routinely achieved $>95\%$ purity of B lymphocytes. Triplicate aliquots of 1×10^5 B lymphocytes were seeded into 96-well plates in 200 μL complete RPMI alone or containing 10 μg/mL anti-IgM $F(ab')_2$ (Jackson ImmunoResearch) with or without 5 μg/mL anti-CD40 mAb (HM40-3, BD Biosciences) or 10 μg/mL LPS (Sigma-Aldrich). Proliferation of cells in the final 24 of a 72-h culture was assessed by [^{3}H]-thymidine (GE Healthcare) incorporation as described previously [8].

BM chimeras

Cohorts of *insHEL* and non-Tg littermate mice on NOD or B6 genetic backgrounds were lethally irradiated with a ^{137}Cs source (0.9 and 1.2Gy, respectively) and injected i.v. with a total of 10×10^6 BM cells. *IgHEL* to non-Tg BM ratios of 1:1 and 7:3 were used to generate the NOD and B6 chimeras, respectively. Tissue and sera from chimeras were harvested at 6–8 wk post-reconstitution.

Adoptive transfers

Four million splenocytes or 2×10^6 MACS-purified B lymphocytes from *IgHEL*-Tg mice were injected i.v. into intact *insHEL* or non-Tg mice. Tissues were harvested for analysis 1–7 days post-transfer. To provide *in vivo* T-cell help, recipient *insHEL* or non-Tg mice were primed 7 days prior to splenocyte or B-lymphocyte transfer with 2×10^8 SRBC (IMVS veterinary services) to expand the SRBC-specific $CD4^+$ T-cell population. On day 0, recipients were bled and then injected i.v. with 4×10^6 splenocytes or 2×10^6 B lymphocytes from *IgHEL*-Tg mice admixed with 2×10^8 HEL-SRBC conjugates. HEL was covalently conjugated to SRBC with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma-Aldrich) as described previously [31]. Sera or tissues from recipients were harvested for analysis 1–21 days post-transfer.

Quantitation of HEL and anti-HEL Ab

HEL and anti-HEL Ab levels in sera were quantified by ELISA using previously described methods [31, 32]. Absorbance at 405 nm was measured on a Spectra ELISA Reader (Tecan) and the concentrations calculated from standard curves using the HyHEL10 anti-HEL IgM^a mAb [21] or HEL (Sigma-Aldrich) standards.

Immunohistology

Sections (5–6 μm) of snap-frozen spleens were fixed in cold acetone and blocked with 30% normal horse serum. *IgHEL*-Tg B lymphocytes were detected by coating sections with 100 ng/mL HEL followed by staining with polyclonal rabbit anti-HEL sera (Rockland) and sheep anti-rabbit IgG-FITC (Southern Biotech). PALS were visualised with an anti-rat CD4 mAb (GK1.5, BD Biosciences) followed by goat anti-rat IgG-TexasRed (Caltag Laboratories). FO areas were subsequently revealed by blocking with 5% goat serum and subsequently staining with a rat anti-B220-biotin mAb (RA3-6B2, BD Biosciences) and streptavidin-FluoroBlue (Biomeda). Slides were analysed on a Zeiss Axiovert 200 M microscope and Adobe Photoshop software.

Statistical analysis

Differences in T1D onset between experimental groups were assessed using a log-rank test. In other experiments, differences between two groups were scrutinised by Student's *t*-test and multiple groups by one or two-way ANOVA.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **B6:** C57BL/6 · **B10:** C57BL/10 · **Dbl:** double · **FO:** follicular · **HEL:** hen-egg lysozyme · **Idd:** insulin-dependent diabetes · **IgHEL:** HEL-specific Ig Tg · **insHEL:** HEL Tg under an insulin promoter · **mHEL:** membrane-bound HEL · **MZ:** marginal zone · **NOR:** non-obese resistant · **PALS:** peri-arteriolar lymphoid sheath · **PLN:** pancreatic LN · **Sgl:** single · **sHEL:** soluble HEL · **SRBC:** sheep RBC · **T1D:** type 1 diabetes

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