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ARTICLE

The CD19 signalling molecule is elevated in NOD mice and controls type 1 diabetes development

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Abstract
Aims/hypothesis Type 1 diabetes is characterised by early peri-islet insulitis and insulin autoantibodies, followed by invasive insulitis and beta cell destruction. The immunological events that precipitate invasive insulitis are not well understood. We tested the hypothesis that B cells in diabetes-prone NOD mice drive invasive insulitis through elevated expression of CD19 and consequent enhanced uptake and presentation of beta cell membrane-bound antigens to islet invasive T cells.
Methods CD19 expression and signalling pathways in B cells from NOD and control mice were compared. Expansion of $CD8^+$ T cells specific for insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) were compared in CD19-deficient and wild-type NOD mice and this was correlated with insulitis severity. The therapeutic potential of anti-CD19 treatment during the period of T cell activation was assessed for its ability to block invasive insulitis.
Results CD19 expression and signalling in B cells was increased in NOD mice. CD19 deficiency significantly diminished the expansion of $CD8^+$ T cells with specificity for the membrane-bound beta cell antigen, IGRP. Conversely the reduction in $CD8^+$ T cells with specificity for the soluble beta cell antigen, insulin, was relatively small and not significant.
Conclusions/interpretation Elevated CD19 on NOD B cells promotes presentation of the membrane-bound antigen, IGRP, mediating the expansion of autoreactive T cells specific for antigens integral to beta cells, which are critical for invasive insulitis and diabetes. Downregulating the CD19 signalling pathway in insulin autoantibody-positive individuals before the development of type 1 diabetes may prevent expansion of islet-invasive T cells and preserve beta cell mass.

Keywords B cells · CD19 signalling molecule · Invasive insulitis · Membrane-bound antigen · NOD mice · T cells · Type 1 diabetes

Abbreviations

APC	Antigen-presenting cell
B6	C57BL/6 mouse
BCR	B cell receptor
DC	Dendritic cell
FO	Follicular
HEL	Hen egg lysozyme
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
LN	Lymph node
MbAg	Membrane-bound antigen

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MZ	Marginal zone
PI3K	Phosphoinositide 3-kinase
sAg	Soluble antigen
TCR	T cell receptor
Treg	Regulatory T cell

Introduction

Type 1 diabetes (hereafter called diabetes) is an autoimmune disease resulting from mononuclear infiltration of the pancreatic islets and destruction of beta cells [1]. The infiltrate includes dendritic cells (DCs), macrophages and B lymphocytes, all capable of processing and presenting antigen (Ag) to beta cell-destructive T cells [2]. DCs, known to have defects in NOD mice, are thought to be the primary antigen-presenting cell (APC) that activates autoreactive T cells [3–5]. B cells are not required for the initiation of disease since diabetes-prone NOD mice lacking B cells do develop peri-islet insulitis [6]. However, B cells are required for the development of invasive insulitis and diabetes [7–10]. Their contribution to disease progression may be through the production of autoantibodies [11] and/or through the presentation of autoantigens to diabetogenic T cells leading to the expansion of the insulitis lesion [12–14].

In the B cell-independent stage of insulitis, autoreactive T cells have a prevalent T cell receptor (TCR) specificity for the soluble antigen (sAg) proinsulin; whereas in the B cell-dependent stage of invasive insulitis that follows, the autoreactive T cell repertoire expands to include autoreactive T cells with specificities for membrane-bound antigens (MbAgs) expressed by beta cells, such as the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) [15–18]. B cells are more efficiently activated by MbAgs than by sAgs and the CD19 signalling molecule has recently been shown to be essential in this process [19–22]. The role of CD19 expression on B cells in driving the progression of insulitis from responses against secreted beta cell antigen, to responses against integral beta cell antigen, has not been assessed. We therefore tested whether B cells in NOD mice have an altered CD19 phenotype and whether this is correlated with an enhanced ability to drive the expansion of islet invasive T cells specific for the beta cell MbAg, IGRP. Finally, we tested whether the absence of CD19 during the T cell activation period could attenuate the development of invasive insulitis.

Methods

Mice NOD/Lt (NOD), NOD.*IgHEL* transgenic [23], NOD.8.3 (IGRP-specific TCR transgenic mice [17]), NODscid (from WEHI or ANU [24]), NODCD19KO (backcrossed >20 generations to NOD by Slattery, as described previously [25]), NODCD19Het, C57BL/6 (B6) and BALB/c (the latter two strains sharing H-2-D^b and -K^d alleles with NOD mice, respectively) were maintained under specific pathogen free (SPF) conditions at the Precinct Animal Centre at the Alfred Hospital (PAC, Melbourne, VIC, Australia) or the Garvan Institute (Sydney, NSW, Australia). All animal experiments were performed in compliance with the Animal Ethic committees of AMREP and Garvan Institute.

Flow cytometry and lymphocyte isolation Single-cell suspensions were stained for CD19 (ID3), CD45R/B220 (RA3-6B2), CD21/CD35 (4E3), IgM (R6-60.2), CD23 (B3B4), CD24 (M1/69), CD4 (RM4-5) and CD8 (53–6.7) with monoclonal antibodies from BD Biosciences (North Ryde, NSW, Australia) or ebiosciene (Jomar, Kensington, SA, Australia). Tetramer staining was used for analysis of $CD8^+$ T cells specific for insulin peptide/*H-2K*^{d} (INSB15-23 [LYLVCGERL]) and IGRP/*H-2K*^{d} (IGRP206–214 [VYLKTNVFL]). TUM tetramer (TUM [KYQAVTTTL]) was used as control [15]. Data were acquired on an LSRII (BD Biosciences) and analysed using FlowJo software (www.flowjo.com/, Version 7.6.5). MACS purification of T or B cells from pooled lymph nodes (LNs) and spleen were obtained via negative selection using a pan T cell or B cell purification kit, according to the manufacturer's instructions (Miltenyi, Macquire Park, NSW, Australia). The purity of isolated populations were typically >95%.

RT-PCR Follicular (FO) B cells derived from inguinal, mesenteric, axillary and submandibular LNs of 6-week-old NOD and B6 female mice were purified using a MACS mouse B cell negative depletion kit (Miltenyi). RNA was extracted using Trizol and transcribed into cDNA using M-MuLV reverse transcriptase, according to the manufacturer's instructions. Levels of *Cd19* gene transcripts were determined by quantitative real-time PCR in a PRISM7900 HT machine (Applied Biosystem/Invitrogen, Mulgrave, VIC, Australia) using triplicate reactions containing 100 ng cDNA and 0.9 μmol/l of primers in LightCycler-RNA SYBR-Green-I Master Mix (Invitrogen). Reactions were standardised to *Hprt* housekeeping gene expression. The following primers were used: TGGAGGATAGTGGGGAGATG and AACCAGA AGTGGACCTGTGG for *Cd19*; CAATGCAAACTTT GCTTTCCC and AAATCCAACAAAGTCTGGCC for *Hprt*.

Western blots Splenic B cells were purified to >97% by negative selection. Cells were stimulated with 5 μg/ml $F(ab')_2$ goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA, USA) for 5, 15 or 30 min. Following stimulation, cells were lysed in buffer containing 1% Triton X-100; lysates were separated by SDS-PAGE

and transferred to polyvinylidene fluoride (PVDF). Blots were probed with anti-phospho-CD19(Y531), anti-phospho-Akt(Ser473), anti-Akt (Cell Signaling, Arundel, QLD, Australia), anti-tubulin (Sigma-Aldrich, Castle Hill, NSW, Australia) and fluorescence-conjugated secondary antibodies (LI-COR Biosciences, Mulgrave, VIC, Australia), then scanned using the Odyssey Infrared Imaging system (LI-COR Biosciences). The fluorescence signal intensity was quantified and converted to greyscale for figures using Odyssey Application software version 2.0 (www.licor.com/bio/blog/tag/odyssey-software).

Disease assessment and histology Mice were monitored for glycosuria by using Diastix (Bayer, Pymble, NSW, Australia) and were scored as diabetic after two positive readings 2 days apart. Mice were killed after 300 days or diabetes development. Insulitis severity was scored by blind histological analysis of haematoxylin and eosin-stained pancreatic sections taken from 80- and 200-day-old NODCD19KO and NODCD19Het female mice. Frozen sections were cut at three levels, and all (10–60) islets were scored. The proportion of islets exhibiting non-invasive or invasive insulitis was determined and expressed as a percentage of the total number of islets scored [2].

Proliferation assays Triplicate aliquots of 1×10^5 IGRP-specific T cells from adult female NOD.8.3 mice were co-cultured with 2×10^5 B cells from female NOD or NODCD19KO mice and either 100 μg/ml insulin or IGRP peptide in RPMI complete media [15, 26]. Sixty hours post-incubation, cells were pulsed with [^{3}H]thymidine (37 kBq/well) for 16 h, harvested, then assayed for [^{3}H]thymidine incorporation (cpm) using a Perkin Elmer (Melbourne, VIC, Australia) TopCountNXT.

Adoptive transfers NODscid mice either received splenocytes at 11–13 weeks or were reconstituted intraperitoneally with B cells, isolated from spleen, inguinal, axillary and submandibular LN at 5–7 weeks, then 5–6 weeks later with MACS purified naive T cells from 3-week-old NOD8.3 donors. Two weeks before transfer of T cells, mice receiving NODCD19KO and NOD co-transferred B cells were treated intravenously with 25 μg/ml α-CD19 (ID3) antibody or PBS on three consecutive days to deplete NOD B cells. Recipient NODscid mice were killed 66–70 days post T cell transfer. The indicated organs were analysed by FACS and pancreases analysed histologically.

Statistical analyses Data are expressed as mean ± SEM. CD19 levels on B cell subsets were compared by using two-way ANOVA, diabetes incidence by using the logrank (Mantel–Cox) test. Two-tailed p values were calculated by using the unpaired T test for column comparisons with In-Stat (GraphPad) software (www.graphpad.com/scientific-software/prism/, Version 5).

Results

Higher surface levels of CD19 on B cells from diabetes-prone NOD mice B cells in NOD mice are hyper-responsive [27] and a high proportion display an activated phenotype [28]. Since CD19 signalling can lower the threshold for B cell activation, we analysed CD19 expression levels in NOD mice compared with non-autoimmune strains. B cells from LNs displayed a significantly elevated surface expression of CD19 in NOD compared with B6 mice (Fig. 1a, b); this is not influenced by the disease state of the mice, since elevated CD19 levels persist in >200 day old mice (data not shown). CD19 expression was also 20–60% higher on transitional (T), marginal zone (MZ) and FO B cells from LN and spleen of NOD mice compared with B6 mice (Fig. 1b, c). Similar differences in CD19 surface expression were noted between NOD and BALB/c B cells (data not shown). Splenic B cells in NOD.*IgHEL* and B6.*IgHEL* transgenic mice, which are mostly specific for the diabetes-irrelevant protein hen egg lysozyme (HEL), displayed a similar difference in CD19 surface levels, demonstrating that differences in CD19 expression are not due to local activation of B cells (Fig. 1d). Increased CD19 expression level was not the result of post-transcriptional or translational modulation since real-time PCR demonstrated the same increase in *Cd19* transcript expression in NOD and B6 B cells (Fig. 1e).

To test whether increased levels of CD19 in NOD B cells resulted in enhanced signalling, B cells were stimulated by B cell receptor (BCR) cross-linking, known to induce rapid CD19 phosphorylation, and signalling events were analysed (Fig. 1f). Relative to levels of the housekeeping protein tubulin, CD19 phosphorylation was enhanced in NOD B cells at the later stimulation time-points compared with B6 or BALB/c B cells (Fig. 1g). Based on the ratio of pCD19 to total CD19, the enhanced phosphorylation of CD19 is due to the increased levels of CD19 on NOD B cells (Fig. 1h), not that CD19 is a target of enhanced (Src family) kinase activity (Fig. 1i). The ratio is marginally lower in NOD B cells compared with B6 or BALB/c B cells, which may represent a mechanism to tone down signalling due to increased levels of CD19. We examined whether the enhanced CD19 phosphorylation in NOD B cells led to enhanced phosphoinositide 3-kinase (PI3K) signalling by examination of phospho-Akt, an enzyme that is directly downstream of PI3K and considered a marker of PI3K activation [29]. Indeed, Akt was hyperphosphorylated in B cells from NOD mice compared with non-autoimmune strains (Fig. 1j). These studies demonstrate that PI3K signalling in NOD B cells is enhanced, concomitant with increased expression of CD19.

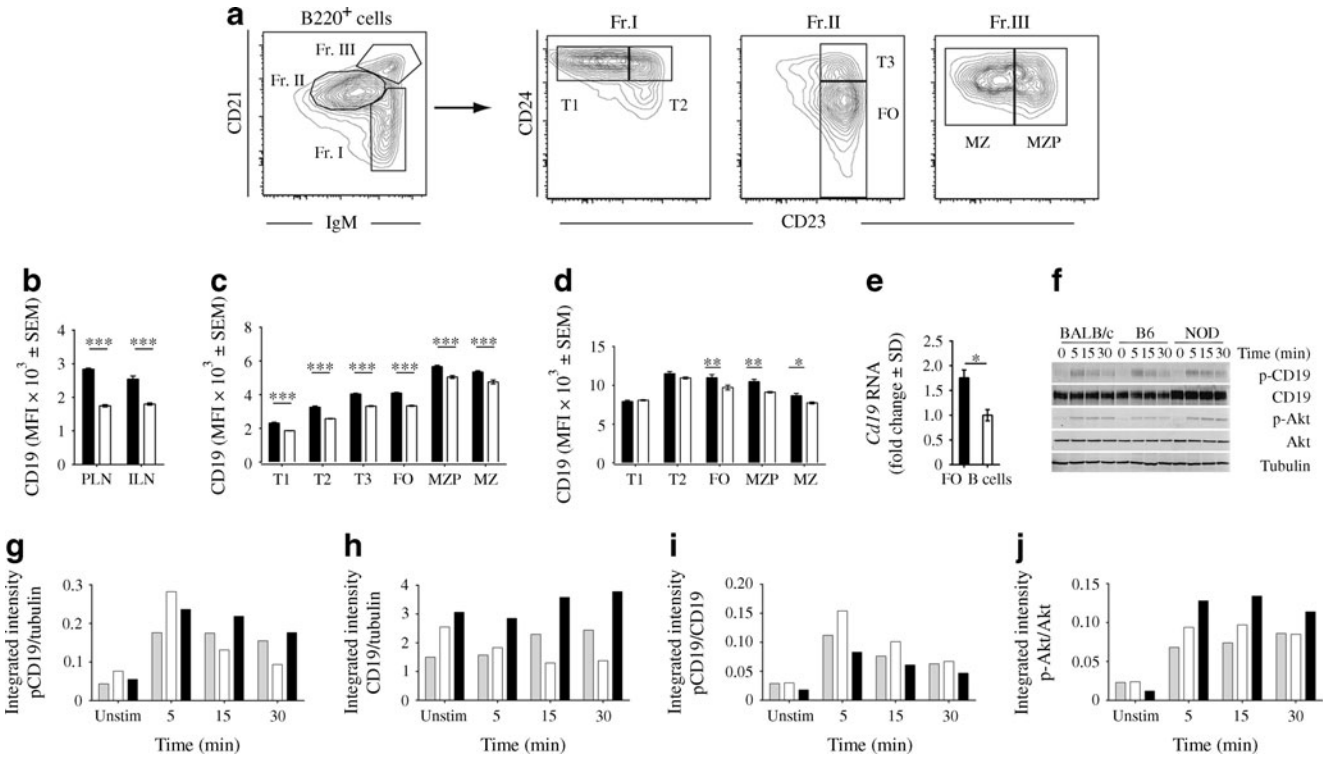

Fig. 1 CD19 expression is elevated in NOD B cells. (**a**) Representative gating strategy (Fr. I, fraction 1; Fr. II, fraction 2; Fr. III, fraction 3) for transitional (T1)–T3, FO, MZP and MZ B cells in spleen. (**b**) Mean expression (MFI) of CD19 in $B220^+$ B cells from pancreatic (PLN) and inguinal (ILN) LNs of NOD ($n=5$, black bars) and B6 mice ($n=5$, white bars) at 6 weeks of age (***$p<0.001$). (**c**, **d**) Mean CD19 expression in the gated splenic B cell subsets of either NOD (black bars) and B6 (white bars) mice (**c**; $n=5$, ***$p<0.001$) or transgenic NOD.*IgHEL* (black bars) and B6.*IgHEL* (white bars) mice (**d**; $n=3$, *$p<0.05$, **$p<0.01$,) determined by flow cytometry. (**e**) Mean expression of *Cd19* transcripts in FO B cells from NOD (black bar) and B6 (white bar) mice was determined by real-time PCR. Expression is presented as fold change relative to the B6 B cell group (*$p<0.05$). (**f**) Representative western blot ($n=2$) of purified splenic B cells from BALB/c, B6 and NOD mice stimulated in the presence of 5 μg/ml of $F(ab')_2$ anti-IgM for the indicated time periods. Blots were probed with antibodies against phospho-CD19 (Y531) and phospho-Akt (Ser473), then stripped and reprobed with antibodies specific for CD19, Akt and tubulin as house-keeping protein. (**g**–**j**) Graphical representation of the data in (**f**); NOD B cells (black bars), assessed relative to levels of the housekeeping protein tubulin, show increased phospho-CD19 (**g**) and CD19 levels (**h**) compared with B6 (white bars) and BALB/c (grey bars) counterparts. (**i**) Levels of p-CD19 relative to surface levels were normal in NOD B cells. (**j**) NOD B cells exhibit enhanced Akt signalling. Unstim, unstimulated

CD19-deficiency protects against diabetes To investigate whether CD19 is important in the development of diabetes, we generated NOD mice lacking CD19 expression (hereafter called NODCD19KO). NODCD19KO mice showed similar defects to B6CD19KO mice (ESM Fig. 1) and other CD19KO strains [25]. The cumulative diabetes incidence was assessed in female NODCD19KO and NODCD19Het littermate mice. NODCD19KO mice were significantly protected against diabetes compared with NODCD19Het littermates (9/41 vs 17/27 mice developed diabetes by 300 days of age, respectively; Fig. 2) thus demonstrating an essential role for CD19 in diabetes pathogenesis.

CD19 expression on B cells is a key driver of the switch from non-invasive to invasive insulitis To understand how CD19 expression contributes to the development of diabetes we tracked the point at which disease progression was arrested in NODCD19KO mice. Histological sections of pancreases from NODCD19Het and NODCD19KO mice were scored for the degree of non-invasive vs invasive insulitis at 80 and 200 days of age. While no significant difference was seen at 80 days, there was a significant decrease in invasive insulitis at 200 days in NODCD19KO mice (Fig. 3a). The decrease in islet infiltration seen in older NODCD19KO mice suggests that CD19 signalling is crucial in either the expansion of islet-specific T cells or in blocking the immunoregulation of these cells. We analysed the pancreatic LNs for regulatory T cells (Treg, $CD4^+CD25^+FoxP3^+$) and found no significant difference in their percentage in 200-day-old NODCD19KO vs NODCD19Het mice (data not shown), suggesting that expression of CD19 did not contribute to diabetes susceptibility by dampening immunoregulation. To determine whether CD19 deficiency resulted in a diminished ability of B cells to present antigen or provide co-stimulatory signals to T cells, we compared NOD and NODCD19KO B cells in an in vitro T cell proliferation assay. No difference in T cell proliferation was

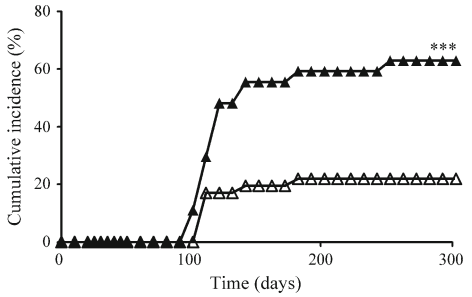

Fig. 2 CD19 deficiency protects NOD mice against diabetes. The cumulative incidence of diabetes in female NODCD19KO mice (n=41, white triangles) and NODCD19Het littermates (n=27, black triangles) was assessed for 300 days (see Methods). The incidence of diabetes in NODCD19KO mice was significantly lower than that in NODCD19Het mice (***p<0.001, Mantel–Cox test)

observed from cultures of NOD or NODCD19KO B cells with peptide and α-CD28 or α-CD3 stimulation (data not shown). Neither was there any difference when purified NOD.8.3 T cells were co-cultured with either NOD or NODCD19KO B cells with peptide in the absence of artificial stimulation (Fig. 3b). CD19 signalling is therefore not required for efficient B cell antigen presentation but probably

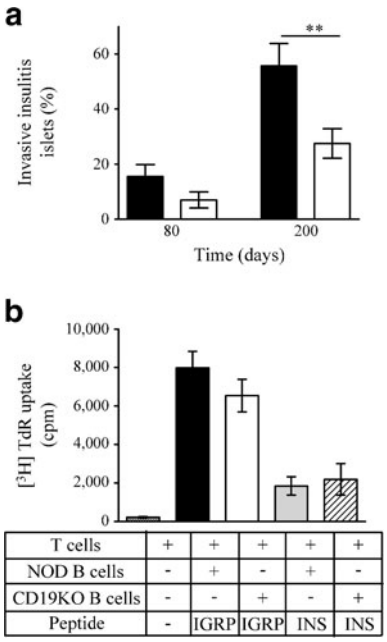

Fig. 3 CD19 expression influences the development of invasive insulitis. (**a**) Pancreases of 80- and 200-day-old female NODCD19Het (n=16/15, black bars) and NODCD19KO (n=11/18, white bars) mice were scored for invasive insulitis. The mean ± SEM of the percentage of islets with invasive insulitis is shown. At day 200, there was a significant decrease in invasive insulitis in NODCD19KO compared with NODCD19Het mice (**p<0.01). (**b**) Purified NOD.8.3 T cells were incubated in triplicate either alone or with NOD or NODCD19KO B lymphocytes. Cells were cultured for 66 h in the presence of IGRP or insulin (INS) peptide (n=3) and then pulsed with [^{3}H]thymidine for an additional 16 h. Data represent the mean cpm ± SEM of triplicate cultures

contributes to the availability of islet antigen to autoreactive T cells through altered uptake and/or processing.

CD19 expression is required to drive the expansion of IGRP-specific CD8$^+$ T cells The switch from non-invasive to invasive insulitis correlates with an increased heterogeneity in the repertoire of autoreactive T cell antigen specificities. The earliest autoreactive T cell responses are directed towards the sAg, insulin, and the later responses include those with specificities for MbAg targets [15, 17]. CD19 has recently been shown to be important in the uptake of MbAg but not sAg [21]. We therefore analysed the frequency of CD8$^+$ T cells in the pancreatic LNs of 200-day-old mice and found a significantly reduced percentage of CD8$^+$ T cells with specificity for the MbAg, IGRP, in NODCD19KO mice compared with NOD mice. In contrast, the reduction in percentage of insulin-specific CD8$^+$ T cells in NODCD19KO mice was relatively small and not significant (Fig. 4). These data support the hypothesis that CD19 on B cells contributes to the pathogenesis of diabetes predominantly by driving expansion of CD8$^+$ T cells specific for islet MbAg, thereby initiating invasive insulitis and beta cell destruction.

CD19 expression on B cells promotes the activation of IGRP-specific CD8$^+$ T cells Although it is well established that DCs have a primary role in normal T cell activation, we wished to ascertain whether elevated CD19 expression by B cells in NOD mice contributes to autoimmune activation of IGRP-specific T cells. We have previously shown that activated T cells from young (5-week-old) NOD mice can transfer diabetes to NODscid recipients in the absence of B cells, indicating that the requirement for B cells in driving the expansion of islet invasive T cells occurs at an earlier stage [30]. To test whether B cells were required at the time of T cell activation, naive NOD.8.3 T cells were transferred into NODscid mice and insulitis assessed 9–10 weeks later. Although naive NOD.8.3 T cells within a population of splenocytes transferred disease to NODscid recipients, the same naive population of T cells, without the co-transfer of other splenocytes, failed to efficiently transfer insulitis or diabetes, and this deficiency was restored by prior engraftment with NOD B cells (Fig. 5a). This indicates that B cells are crucial for the development of invasive insulitis at the time of, or immediately after, T cell activation. However, when NODCD19KO B cells were used for prior engraftment of NODscid recipients, the T cells were unable to transfer invasive insulitis (Fig. 5a). Moreover, the percentage of IGRP$^+$ CD8$^+$ T cells in the pancreatic LNs of NODscid mice engrafted with NOD B cells was significantly higher than that found in mice engrafted with NODCD19KO B cells (Fig. 5b) whereas the numbers of CD4$^+$ T cells were not significantly different (data not shown), supporting the recent finding that NOD B cells can cross-present antigens to CD8$^+$ T cells [14].

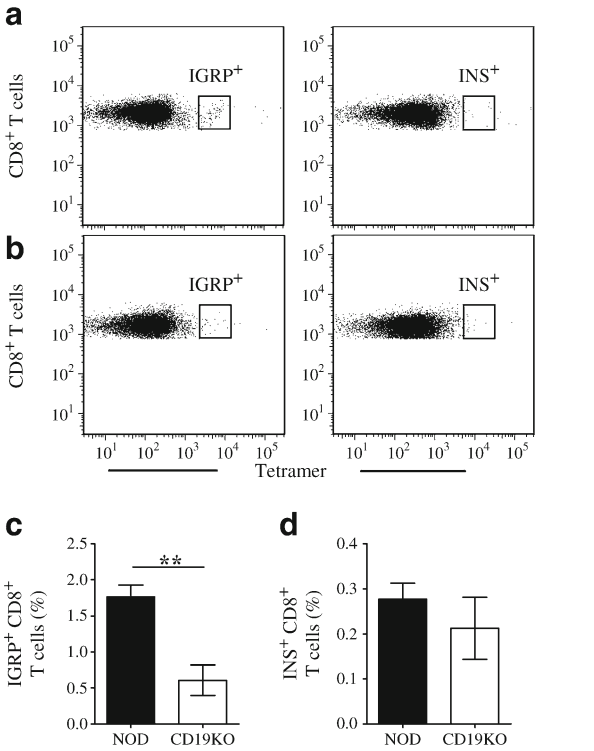

Fig. 4 CD19 expression is required for expansion of IGRP-specific T cells. (**a**, **b**) Representative tetramer staining patterns of NOD (**a**) or NODCD19KO cells (**b**). (**c**, **d**) Mean percentage ± SEM of IGRP$^+$ (**c**) or insulin (INS)$^+$ (**d**) CD8$^+$ T cells from LNs of 200-day-old female NOD (n=6, black bars) and NODCD19KO (n=4, white bars) non-diabetic mice. NODCD19KO mice showed a significantly reduced percentage of CD8$^+$ T cells specific for the MbAg IGRP compared with NOD mice (**p<0.01) whereas there was no significant difference in the percentage of insulin-specific CD8$^+$ T cells between the groups

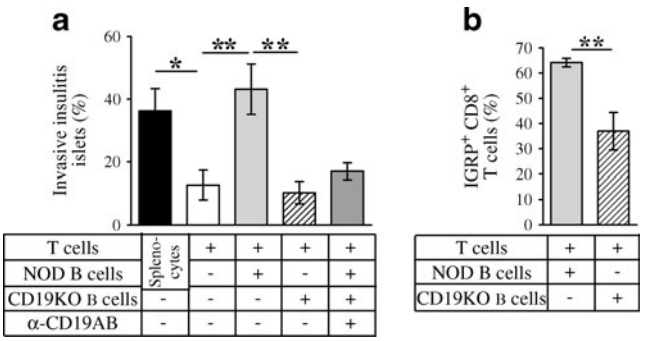

Fig. 5 Invasive insulitis requires CD19$^+$ B cells. (**a**) NODscid mice were reconstituted with either splenocytes (black bar) containing 2.5×10^6 T cells (n=8) or 2.5×10^6 purified naive 8.3 T cells, 5–6 weeks after receiving either no B cells (n=6, white bar), 5×10^6 NOD B cells (n=6, light-grey bar), 5×10^6 NODCD19KO B cells (n=7, hatched bar) or co-transferred 2.5×10^6 NOD and 2.5×10^6 NODCD19KO B cells that received α-CD19 monoclonal antibody to deplete NOD B cells (n=3, dark-grey bar). Pancreatic tissue sections were scored for mean% of islets ± SEM with invasive insulitis 66–70 days post T cell transfer. Mice reconstituted with either splenocytes or with NOD B cells plus naive T cells developed invasive insulitis, whereas mice reconstituted with naive T cells alone, with NODCD19KO B cells plus naive T cells or with co-transferred NOD and NODCD19KO B cells treated with α-CD19 monoclonal antibody did not develop severe invasive insulitis (*p<0.5, **p<0.01). (**b**) FACS analysis of pancreatic LNs from recipient mice that received NODCD19KO (n=6, hatched bar) B cells before T cell transfer revealed a significant reduction in the percentage of IGRP$^+$ CD8$^+$ T cells compared with those that received NOD (n=6, grey bar) B cells (**p<0.01). The mean ± SEM of the percentage of IGRP$^+$ CD8$^+$ T cells is shown

These data demonstrate a direct role for CD19 on B cells in driving availability of the islet MbAg, IGRP, for cross-presentation to CD8$^+$ T cells.

CD19 deficiency protects from diabetes through a B cell intrinsic mechanism In addition to their role as APCs, B cells also contribute to diabetes pathogenesis through the production of autoantibodies [12]. However, since serum from diabetic NOD mice transferred to B cell-deficient NOD mice failed to restore diabetes, the contribution of autoantibodies to disease is likely to be secondary to the intrinsic role of B cells [13]. Because deletion of CD19 leads to the inability of B cells to undergo efficient class switching [31], we assessed whether a deficiency in autoantibody, either maternally transmitted or endogenously generated, was critical for diabetes protection in NODCD19KO mice. CD19 deficiency-mediated protection was not due to a maternal deficiency of autoantibody transmission during their pre-weaning life since protection from diabetes was observed in NODCD19KO offspring of diabetes-prone NODCD19Het dams capable of generating autoantibodies (Fig. 6a). A potential role for endogenously produced autoantibodies was assessed by co-transfer of NODCD19KO B cells together with NOD B cells as a source of normal IgG production, into NODscid recipients. Before transfer of naive T cells, recipient mice were transiently depleted of CD19$^+$ B cells using α-CD19 monoclonal antibody resulting in <6% of total B cells expressing CD19 compared with 30% in control mice. At the completion of the experiment IgG was reconstituted in α-CD19-treated mice and the percentage of CD19$^+$ NOD B cells had returned to pre-depletion levels (Fig. 6b). However, expansion of IGRP-specific CD8$^+$ T cells (Fig. 6c) and also invasive insulitis (Fig. 5a) were reduced in recipients depleted of CD19$^+$ B cells during the T cell engraftment period. These results demonstrate that: (1) the restoration of antibodies in the absence of NOD B cells is not sufficient to promote activation of autoreactive IGRP-specific CD8$^+$ T cells and (2) if the early exposure of naive 8.3 T cells is with CD19-deficient B cells, then the autoreactive IGRP-specific CD8$^+$ T cells fail to expand sufficiently to cause disease. Taken together, these data support the hypothesis that CD19 on B cells is important for enhancing the availability of the MbAg, IGRP, for presentation to CD8$^+$ T cells and for the development of invasive insulitis.

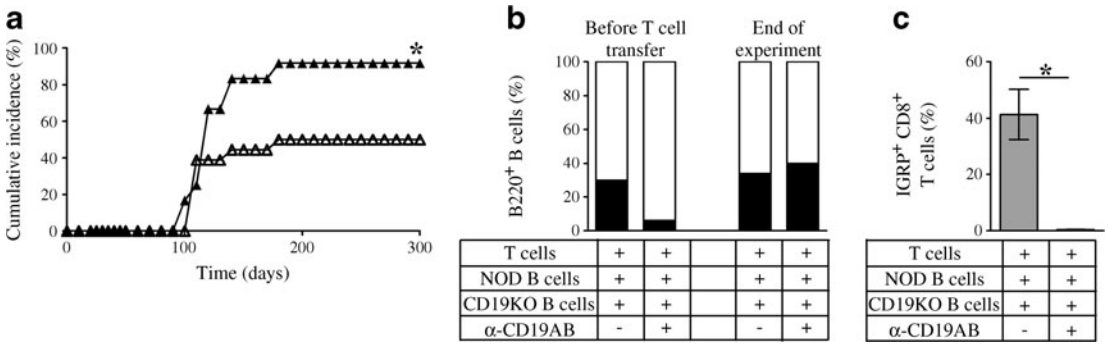

Fig. 6 The role of autoantibodies in CD19-deficient protection from diabetes. (**a**) In offspring of diabetes-prone NODCD19Het dams the cumulative diabetes incidence of NODCD19Het ($n=12$, black triangles) was significantly reduced compared with littermate NODCD19KO ($n=18$, white triangle) mice (*$p<0.5$, Mantel–Cox test), indicating that CD19-mediated protection was not due to maternal CD19 deficiency or autoantibody production. (**b**) NODscid mice received co-transfer of 2.5×10^6 NOD and 2.5×10^6 NODCD19KO B cells ($n=15$). A subset of co-transferred recipients received α-CD19 monoclonal antibody ($n=6$) to deplete NOD B cells; the control group received PBS ($n=9$). B cell reconstitution and successful depletion of $CD19^+$ B cells was assessed ($n=3$; white bars, $CD19^-$; black bars, $CD19^+$) and showed that α-CD19 monoclonal antibody treatment resulted in an 80% reduction of $CD19^+$ B cells compared with the control group ($n=3$). Two weeks later 2.5×10^6 purified naive 8.3 T cells were transferred to α-CD19 monoclonal antibody-treated and control mice. At the end of the experiment NOD B cells returned to pre-depletion levels and no significant difference between the percentage of $CD19^+$ B cells was detected between groups. (**c**) FACS analysis of pancreatic LNs revealed that there was a significant reduction in the percentage of $IGRP^+$ $CD8^+$ T cells in α-CD19 monoclonal antibody-treated mice compared with control mice (*$p<0.5$). The mean ± SEM of the percentage of $IGRP^+$ $CD8^+$ T cells is shown

Discussion

The role of B cells in the development of diabetes has been controversial with reports in both humans and NOD mice that diabetes can develop in the absence of B cells [9, 13, 32] and other reports demonstrating that there is significant protection from diabetes in mice lacking B cells [8, 10]. Since these early reports, several large studies have confirmed that although mild insulitis persists in the absence of B cells, NOD mice lacking B cells exhibit profound resistance to the development of diabetes. In support of an important role for B cells in human disease, therapeutic targeting of B cells with anti-CD20 antibody was found to preserve beta cell mass in recently diagnosed patients [33]. However, unsurprisingly patients depleted of B cells had reduced IgM levels indicating immunosuppression. A mechanistic understanding of the role of B cells in the switch from non-invasive insulitis to overt diabetes remains to be elucidated and is required for more targeted therapeutic intervention in insulin-autoantibody-positive individuals at risk of developing diabetes. Our findings demonstrate a key role for the B cell signalling molecule, and potential therapeutic target, CD19, in the switch from non-invasive to invasive insulitis in NOD mice. These findings are potentially of great significance for individuals at risk of diabetes since the *CD19* locus falls within the 16p11.2 region recently identified to be associated with susceptibility to diabetes in humans [34].

The density of CD19 on B cells has been shown to establish a basal signal transduction threshold by acting as a positive response regulator that augments BCR signals. While CD19-deficient mice have been shown to be hyporesponsive to transmembrane signals [25, 31], mice that overexpress CD19 are hyper-responsive [31, 35], indicating that even small changes in CD19 expression levels have a substantial influence on B cell function. CD19 therefore plays a critical role in the amplification of B cells during their development and activation and its dysregulation can lead to elevated autoantibody production and the initiation of antibody-mediated autoimmunity [36–38]. However, the role of elevated CD19 in driving T cell-mediated autoimmune diseases such as diabetes has not been reported. Our results clearly demonstrate that CD19 is elevated on the surface of FO, MZ and immature transitional B cell subsets of NOD mice compared with non-autoimmune strains. Elevated surface CD19 was shown to have functional consequences since BCR cross-linking resulted in enhanced Akt signalling in NOD mice compared with B6 and BALB/c mice. Whether increased CD19 expression and signalling is due to genetic variation in the *CD19* gene itself or is a consequence of variation in an upstream regulator of CD19 is not known. However, we determined that CD19 elevation is unlikely to be a consequence of chronic inflammation since it was observed in non-pancreas draining LNs and in NOD.*IgHEL* mice, which develop a significantly delayed incidence of diabetes. Our data demonstrate that the hyper-responsive phenotype of B cells found in NOD mice [27] is a consequence of increased CD19 expression causing enhanced BCR signalling.

Whether enhanced B cell signalling through CD19 could explain the crucial contribution of B cells to the switch from non-invasive to invasive insulitis was tested in NOD mice lacking CD19 expression and in T cell-reconstituted NODscid recipients of CD19-deficient B cells. In both models there was a significant reduction in the development of invasive insulitis and diabetes confirming an important role for CD19 on B cells

in the development of diabetes. Furthermore, we found the presence and numbers of T cells specific for the sAg insulin were not significantly affected by CD19 deficiency whereas the expansion of T cells specific for the MbAg IGRP was critically dependent on CD19 expression and this expansion correlated with the development of invasive insulitis.

The requirement for B cells in driving the expansion of islet invasive T cells is early in the immunopathogenic process since activated T cells from young NOD mice can transfer diabetes to NODscid recipients in the absence of B cells [30]. It is well established that DCs play an important role in the activation of naive T cells [39]. However, recent evidence indicates that full activation of effector $CD8^+$ T cells requires a second phase of T-to-T cell interactions that is independent of direct DC contact [40]. Although B cells were not assessed in the study by Gerard et al [40], we suggest they may also interact with $CD8^+$ T cells after initial DC priming since naive T cells, transferred to DC-competent NODscid recipient mice, were not fully activated in the absence of recipient B cells. These data indicate a crucial role for B cells for complete activation, without which the IGRP-specific $CD8^+$ T cells fail to expand and invade the islets. Importantly, we show this process is CD19 dependent, since CD19KO B cells could not provide the secondary activation signals in the absence of wild-type B cells.

There are a number of pathways through which CD19 could control the development of invasive insulitis. It has been proposed that autoantibodies, present in susceptible mice and humans, have a causal role in diabetes pathogenesis [41]. High levels of circulating autoantibodies are likely to promote FcR-mediated uptake of autoantigen by macrophages and NK cells. The former drive presentation of antigen to specific T cells and both release beta cell toxic mediators that promote expansion of non-antigen-specific T cells [42]. While autoantibodies have a role in diabetes development, we demonstrated that the protection from diabetes mediated by CD19 deficiency was not due to a deficiency in maternally transmitted, or endogenously produced, immunoglobulins. These data indicate that the primary CD19-dependent mechanism by which B cells contribute to diabetes is intrinsic to B cells and not, as is the case for other autoimmune diseases associated with elevated CD19 [43], due to the antibodies they secrete.

Another explanation for reduced insulitis in NODCD19KO mice is the loss of B1 or MZ B cells [26]. However, evidence suggests that this is not the case: (1) Gagnerault et al [44] have shown that the loss of MZ B cells by complete splenectomy failed to protect NOD mice from diabetes; (2) selective targeting of FO, but not MZ or B1, B cells with anti-CD20 antibodies in NOD mice protected from diabetes [44–47] and (3) purified splenic and LN B cells (which have very few B1 B cells) from NOD but not NODCD19KO mice, transferred diabetes. It is therefore unlikely that a reduction in B1 or MZ B cell numbers could account for the protection from diabetes observed in CD19-deficient NOD mice.

The sAg insulin is thought to be the primary antigen to which autoreactive B and T lymphocytes respond. However, without artificial activation in vitro, insulin-specific T cells cannot drive invasive insulitis or diabetes [48, 49]. Our data suggest that the early response to insulin can occur independently of CD19 expression. However, immune responses to IGRP, and the development of invasive insulitis and diabetes, are critically dependent on CD19. Since recent reports have shown that the process whereby MbAg is internalised by B cells is CD19 dependent [21, 50], we propose that elevated CD19 expression and signalling drives the B cell presentation of MbAg to recently primed $CD8^+$ T cells. We propose that B cell presentation of MbAg to $CD8^+$ T cells drives their expansion, islet invasion and attack of beta cells. Given our data in the NOD mouse model, we propose that human *CD19* may be a strong susceptibility candidate gene within the 16p11.2 region. If confirmed, this raises the exciting possibility that the CD19 signalling pathway may be a target for early immune intervention strategies in autoantibody-positive individuals at risk of developing diabetes.

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