

ORIGINAL ARTICLE

Contribution of autoallergy to the pathogenesis in the NOD mice

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Abstract

The immunoglobulin isotype IgE is commonly associated with allergy. However, its involvement in autoimmune disease in general, and Type 1 diabetes (T1D) in particular, is still not completely clarified, nonetheless IgE has been observed in patients with T1D. In this article, we aimed to elucidate the contribution of IgE in the pathogenesis of the disease in a spontaneous model for T1D, i.e. the NOD mouse. We observed increased levels of IgE in splenic, lymph node and peripheral blood B cells in the NOD mice compared to the control C57BL/6 (B6) mice. No correlation was found between the IgE levels on B cells and those in the sera of these mice, indicating a B cell intrinsic property mediating IgE capture in NOD. Functionally, the B cells from NOD were similar to B6 in rescuing the IgE-mediated immune response via the low affinity receptor CD23 in a transgenic adoptive transfer system. However, the involvement of IgE in diabetes development was clearly demonstrated, as treatment with anti-IgE antibodies delayed the incidence of the diabetes in the NOD mice compared to the PBS treated group. Pancreas sections from a 13-week-old NOD revealed the presence of tertiary lymphoid structures with T cells, B cells, germinal centers and IgE suggesting the presence of autoantigen specific IgE. Our study provides an insight to the commonly overlooked immunoglobulin IgE and its potential role in autoimmunity.

Keywords

Autoallergy, B lymphocytes, IgE, NOD mouse, Type 1 diabetes

History

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Introduction

Autoimmunity and allergy have thought to be mutually exclusive, an idea that is now challenged by current research. The immunoglobulin IgE that is unanimously associated with allergy has also been shown to play a role in autoimmune diseases, a concept referred to as autoallergy [1]. Patients with autoimmune disease such as systemic lupus erythematosus [2,3] that display IgE autoantibodies have poor disease prognosis. Moreover, in several other autoimmune diseases, including rheumatoid arthritis [4], autoimmune thyroiditis [5], bullous pemphigoid [6] and T1D, presence of autoantigen specific IgE has been established [7]. In two independent cases of bullous pemphigoid, removal of IgE using the anti-IgE antibody Omalizumab has proven to be remarkably effective, demonstrating the involvement of IgE in the pathogenesis [6].

T1D is an autoimmune disease characterized by the destruction of pancreatic β cells leading to hyperglycemia. Although, T cells are the primary effector cells in T1D, the role of B cells has also been investigated, based on the fact that autoantibodies are detected up to a decade before disease onset [8]. B cells involved in antigen presentation, has also been shown to contribute to T1D [9–11]. Moreover, a clinical trial with anti-CD20 treatment (Rituximab/Mabthera) has

clearly pointed to the importance of B cells, as treatment improves β cell function in these patients that is measured by the changes in C-peptide levels [12,13]. It has been proposed that Rituximab depletes the existing pool of B cells including memory B cells and autoreactive clones causing the bone marrow to replace the B cells hence altering the immune response [1].

In addition, B cells are known to contribute to the disease with autoantibodies against islet antigens including insulin and GAD65 [15], and the autoantibodies are used as diagnostic adjunct for these patients. Previous studies have shown that autoantibodies are the first clinically detectable sign of autoimmunity [8] and the number of antigens towards which autoantibodies are present, has been thought to be predictive for development to overt diabetes [16]. Interestingly, IgE autoantibodies towards GAD65 has been observed in some patients with T1D, and T1D siblings with this trait are considered at higher risk for progressing to T1D [7]. However, the mechanism of the autoantibodies as a pathogenic factor is still to be fully elucidated. Interestingly, also anti-idiotypic antibodies have been observed in patients with autoimmune diseases and has been suggested to reflect a correction mechanism for the autoantibodies, highlighting the protective role of B cells [17].

T1D has been more thoroughly explored in the non-obese diabetic (NOD) mouse strain that develops the disease spontaneously, similar to humans [18]. It has been demonstrated that B cells in the NOD are aberrant as to anomalies in

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natural antibody production, antigen presentation, immune response patterns and surface accumulation of immunoglobulins [9–11,19–22]. As in humans, anti-CD20 treatment in pre-diabetic NOD mice protects them from disease development [23]. All of these fortify the pathogenic role of B cells in NOD.

The immunoglobulins, depending on their isotype, provide feedback to the B cell to either enhance or suppress the immune response [24]. Immunoglobulins are crucial contributors to several other effector mechanisms in autoimmunity, although the role of IgE is still ambiguous. In this study, we explore the role of IgE in the NOD mouse to identify its contribution to the pathogenesis of the disease.

Materials and methods

Mice

All mice used in this study were bred and maintained at specific pathogen free animal facilities at Umeå University. NOD/ShiLtJ (NOD) and C57BL/6 (B6) mice were originally obtained from Bomholtgaard, Denmark. All the experimental procedures were performed in compliance with relevant institutional and Swedish laws and guidelines approved by the Umeå research animal ethic committee (A44-12). The CD23 knockout mice on BALB/c background and DO11.10 TCR transgenic mice were a kind gift from Professor Birgitta Heyman, Uppsala University, Sweden. Spontaneous diabetes in NOD females occurs at 40 weeks of age to 53% of them in our colony. Age of the mice used has been mentioned in figure legends.

Antibodies for flow cytometry, immunohistochemistry and ELISA

The following antibodies were used for this study in appropriate pre-determined concentrations. Anti B220-FITC (RA3-6B2), anti-CD19-Alexa 647, anti-CD19 PE-Cy7 (1D3), anti-IgM^b FITC (AF6-78), anti-IgE-biotin (R32-75), anti-CD23-PE (B3B4), anti-CD21-FITC (7G6), anti-CD4-PE and anti-CD4-biotin (GK1.5), TCR-APC (H57-597), Streptavidin alkaline phosphatase conjugated, Streptavidin horse-radish peroxidase (HRP) were all purchased from BD Biosciences (Franklin Lakes, NJ). Anti-CD19-Alexa 700 (6D5) was purchased from Biolegend (San Diego, CA). Anti-IgE-biotin (23G3) and goat anti-mouse IgE-biotin polyclonal were purchased from Southern Biotechnology (Birmingham, AL). Anti-B220-biotin (RA3-6B2), Streptavidin-APC, GL7-Biotin and anti-DO11.10 TCR biotin (KJ1-26) were purchased from eBioscience (San Diego, CA). Streptavidin-Alexa750 was purchased from Invitrogen (Carlsbad, CA). Purified anti-CD16/32 (FcBlock; 2.4G2) was a kind gift from Prof. Per-Arne Oldenborg, Umeå University.

Single cell preparation and flow cytometry

Cell suspensions were prepared from spleens by pressing them between two glass slides or using a 70 µm nylon cell strainer (BD Biosciences). The erythrocytes were isotonicallly lysed using Gey's solution (7.0 g l⁻¹ NH₄Cl, 0.37 g l⁻¹ KCl, 0.3 g l⁻¹ Na₂HPO₄·12 H₂O, 0.024 g l⁻¹ KH₂PO₄, 1 g l⁻¹ glucose, 5 g l⁻¹ gelatin, 7 mg l⁻¹ MgSO₄·7H₂O, 21 mg l⁻¹

MgCl₂·6H₂O, 17 mg l⁻¹ CaCl₂·2H₂O, 113 mg l⁻¹ NaHCO₃). The cell suspension was then centrifuged at 350 × g to pellet the cells. Isolated cells were re-suspended in FACS buffer (0.1 M PBS, 3% fetal calf serum, 0.01% sodium azide) and stained in 96 well U-bottom plate. The plate was centrifuged up to 730 × g to sediment cells and supernatant was discarded. The cells were incubated on ice for 20 min with unlabeled anti-CD16/32 (Fc Block), washed and stained with an antibody mixture containing pre-determined optimized dilutions of IgM^b-FITC, CD23-PE, IgE-biotin, TCR-APC and CD19-PE-Cy7, on ice for 20 min and washed. To visualize biotin stained cells, Streptavidin-Alexa750 was added to each well and incubated for 20 min on ice and washed. The stained cells were re-suspended in FACS buffer. At least 50 000 events were collected on LSR II FACS machine and analyzed using Diva software (BD Biosciences, San Jose, CA).

Adoptive transfers

B cell hybridomas producing murine IgE anti-TNP (clone IGELb4) [25] were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) with 5% FCS. Supernatant of the cell culture was collected and ran through a Sepharose column (GE Healthcare Lifesciences, Buckinghamshire, UK) conjugated with monoclonal rat anti-mouse κ Ab (clone 187.1.10) [26]. IgE anti-TNP in the supernatant bound to the column was then eluted with 0.1 M glycine-HCl elution buffer (pH 2.8) and dialyzed against PBS. Purified IgE anti-TNP was sterile filtered and its concentration was determined by measuring absorbance at 280 nm ($1.5 A_{280 \text{ nm}} = 1 \text{ mg/ml}$). Antibodies were stored at -20 °C before use. OVA-TNP conjugation was made by adding 1 ml picrylsulfonic acid (5%; Sigma-Aldrich) to 100 mg OVA dissolved in 10 ml 0.28 M cacodylate buffer (pH 6.9). The mixture was incubated at 22.5 °C for 170 min in the dark and the reaction was stopped by adding 100 mg glycyl-glycine (Merck, White House Station, NJ). The solution was dialyzed against 1 l PBS for 8 h and the dialysis was repeated thrice. OVA-TNP was then sterile filtered and the absorbance at 280 and 340 nm was determined. The concentration of OVA and TNP was calculated according to the following equations:

$$\text{OVA (mol/l)} = \frac{A_{280} - 0.337 \times A_{340}}{45 \times 10^3 \times 0.74};$$

$$\text{TNP (mol/l)} = \frac{A_{340} - 0.257 \times A_{280}}{1.14 \times 10^4}.$$

Adoptive transfers in CD23^{-/-} KO mice were performed as described in Henningsson et al. [27]. Briefly, T cells were isolated from DO11.10 spleens using the CD4⁺ T cell isolation kit and B cells from NOD and B6 spleens were isolated using the B cell isolation kit with MACS columns as per manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Eight weeks old CD23^{-/-} KO recipients were adoptively transferred i.v. with 5×10^6 T cells from DO11.10 mice on day one and with 17×10^6 B cells from NOD or B6 mice 24 h later. Twenty micrograms of OVA-TNP and 50 µg of IgE anti-TNP were mixed at RT (left for maximum 1 h at RT) and injected into the recipients 3 h post-B cells transfer. Spleens from the recipients were analyzed 72 h after the last injection.

Immunohistochemistry and immunofluorescence

Spleens were flash frozen with Tissue-Tek OCT (Sakura, The Netherlands) in liquid nitrogen and pancreata were first fixed in 4% paraformaldehyde for 2 h and incubated in 30% sucrose solution before flash frozen with OCT in liquid nitrogen. Cryostat sections of 8 μ m thickness were used for stainings. Sections from the adoptive transfer experiment were stained using immunofluorescence with anti-DO11.10 TCR biotin with Streptavidin-APC for OVA specific T cells and B220-FITC for B cells and viewed using Nikon confocal microscope (Nikon Instruments Europe, Amsterdam, Netherlands). The images were analyzed in the NIS elements software. The spleens and pancreata were stained for IgE using immunohistochemical method with anti-IgE-biotin or, GL7-biotin or anti-B220-biotin or anti-CD4-biotin which were revealed using Streptavidin HRP and 3,3'-diaminobenzidine substrate and counter stained with methyl green. Microscopy was done using Nikon fluorescence microscope and images were analyzed with NIS elements software.

ELISA

Sera from mice were analyzed for IgE using ELISA. Nunclon ELISA plates were coated with IgE diluted in coating buffer (sodium carbonate buffer pH 9.6) and incubated overnight at 4 °C. The ELISA plate was blocked using 1% bovine serum albumin (BSA) in PBST (PBS with 0.05% Tween20). The plate was incubated with serum, overnight at 4 °C. IgE was detected using goat polyclonal anti-IgE, incubated overnight at 4 °C. Streptavidin alkaline phosphatase was added to the wells and incubated at room temperature for 2 h. The substrate (pNPP in dietanolamine buffer 1 mg/ml) was added and the resultant color change was read at 405 nm in Varioskan plate reader (Thermo Fisher Scientific, Waltham, MA).

Anti-IgE treatment and diabetes incidence

Anti-IgE antibodies were purified from 23G3 clone of hybridoma (kind gift from Prof. D.H. Conrad, Virginia University) using HiTrap Protein-L column (GE Healthcare Lifesciences) and diluted in PBS. NOD mice were given either a total of 1 mg anti-IgE in 10 injections containing 100 μ g each, starting at 3 weeks of age for 5 weeks, or 100 μ l PBS per injection via intraperitoneal route. The mice were monitored for diabetes for 45 weeks by measuring urine glucose levels weekly using Clinistix (Bayer Pharmaceuticals, Leverkusen, Germany). Mice that showed high glucose levels 2 weeks in a row were considered diabetic.

Statistical analysis

The data were analyzed using Mann–Whitney test for unpaired samples or Student's *t*-test where applicable and a two-tailed *p* value was calculated. A *p* value ≤ 0.05 was considered significant. Chi-square test was performed for the diabetes incidence after anti-IgE treatment.

Results

IgE capture on NOD B cells

We have previously observed that NOD B cells aberrantly capture IgG and IgM on the cell surface [20]. To determine if NOD B cells also captured other isotypes such as IgE, we

stained NOD B cells with anti-IgE antibodies. We noticed significantly increased surface IgE level on NOD B cells in spleen, lymph nodes and blood (Figure 1A, B and C and Supplemental Figure S1). To elucidate if this capture was restricted to a certain subset of B cells, the presence of IgE on splenic sections from NOD and B6 were analyzed. As shown in Figure 1(D), increased level of IgE was detected in all areas of the NOD spleen including the marginal zone. This observation was further supported by flow cytometry analyses, distinguishing follicular (CD23⁺) and marginal zone (CD23[−]) B cells, where similar binding was detected (data not shown). Marginal zone B cells lack the low affinity receptor for IgE (CD23/Fc ϵ R1I), which implies that the binding may be mediated by an alternative ligand. An increased level of IgE was also detected in the red pulp, in accordance with the fact that CD23 is present on other cell types including macrophages and eosinophils [28].

Capture of IgE is increased across all ages in NOD mice and does not correlate with IgE levels in serum

Next we analyzed IgE levels on B cells across different ages of NOD and B6 mice. IgE levels on B cells were consistently increased in NOD mice, even in as young as two-week-old mice (Figure 2A and Supplemental Figure S2). One possibility for the increased level could be that serum IgE is increased in NOD mice. Thus, we analyzed the levels of IgE in the serum and as displayed in Figure 2(B), levels of serum IgE fluctuated throughout the age span. No correlation was found between the IgE level in the serum and IgE on the splenic B cells in the same mice.

IgE capture is partly but not exclusively mediated by CD23 and CD23 dependent enhancement of immune response is not affected

As mentioned above CD23 is expressed on follicular B cells among other cell types. To elucidate the role of CD23 in the increased IgE capture of NOD B cells, we used a protocol by Rogosch et al. [29] where a short incubation in a low pH buffer leads to the dissociation of CD23 and IgE. As shown in Figure 3(A), IgE levels decreased after acid treatment, but the difference remained significant between NOD and B6 B cells, indicating that increased CD23 mediated IgE binding in the NOD cannot be the sole explanation for this feature.

Furthermore, to test whether CD23 mediated enhancement of IgE-dependent immune response was increased in the NOD mouse, we performed an adoptive transfer experiment in a CD23^{−/−} system, as described by Henningsson et al. [27]. We hypothesized that NOD B cells would promote an increased OVA-specific T cell response, compared to B6 B cells, in the context of IgE-OVA immune complexes. OVA-specific T cells within the T cell zones of the splenic follicles were similarly recruited (Figure 3B). This indicated that both NOD and B6 B cells were able to rescue the IgE-mediated immune response.

Anti-IgE treatment of pre-diabetic NOD mice delays diabetes development

In the NOD mouse, early depletion of B cells using anti-IgM antibodies has been shown to prevent diabetes

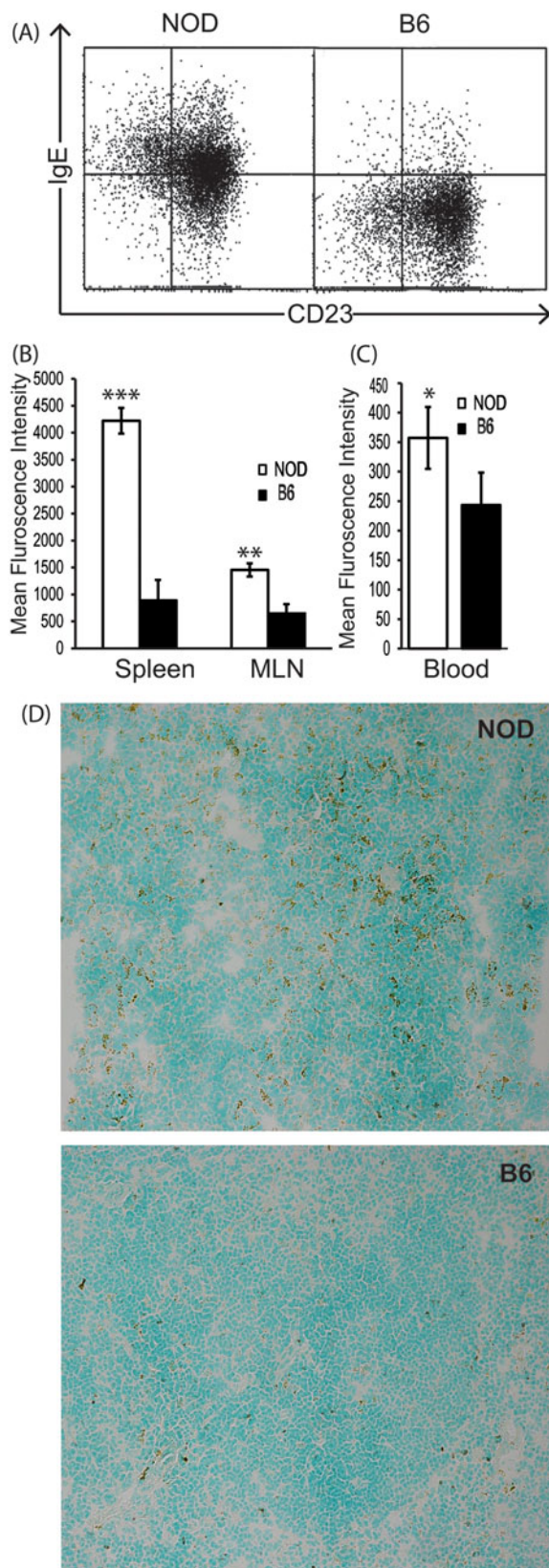


Figure 1. Increased IgE levels on NOD B cells compared to B6 B cells. (A) Single cell suspensions from spleen, mesenteric lymph nodes and peripheral blood were stained for CD19, CD23 and IgE and analyzed by flow cytometry. Representative FACS plot showing a distinct population shift of the splenic follicular B cells (CD19⁺) in NOD when stained with anti-IgE. (B and C) Observed MFI levels of IgE staining in the specified populations in 8-week-old NOD and B6 ($n=3$ for both strains, plot representative of eight experiments, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.0001$). (D) Immunohistochemical staining of 8 μm splenic cryosections from NOD and B6 detecting IgE (brown staining).

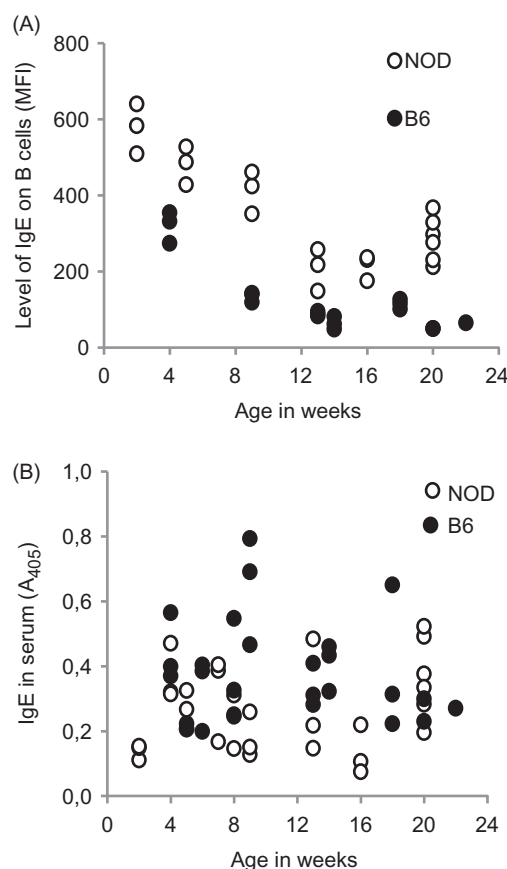


Figure 2. IgE levels are higher in NOD mice across all ages but have no correlation with levels of IgE in the serum. Serum and cells were collected from mice of different ages (2–23 weeks) and analyzed by ELISA and FACS, respectively. (A) NOD and B6 mice of different ages ($n=21$ for each strain) were sacrificed and the splenocytes were stained for CD19 and IgE. NOD mice were observed to have significantly higher IgE compared to B6 mice across all ages ($p < 0.01$). (B) Sera from the NOD and B6 mice were analyzed by ELISA and no significant difference was observed in IgE levels across the ages between the strains.

development [30]. We investigated whether targeted depletion of IgE in particular would also have an effect on diabetes development. Depletion of IgE by anti-IgE injection during 5 weeks, starting in 3-week-old NOD mice, delayed diabetes development (Figure 4, $p=0.06$). No decrease in B cell numbers in the anti-IgE treated group compared to the PBS treated group was observed (data not shown).

To elucidate the potential specificity of the IgE antibodies, we stained pancreata sections from NOD and B6 mice at 9–20 weeks ($n=18$ for each group) and studied the occurrence of IgE in these sections. We observed distinct IgE staining in one NOD mouse (13 weeks; Figure 5). This implies that localized IgE production may occur. The presence of tertiary lymphoid structures in and around the infiltrated pancreatic islets as observed by us and others [31] suggests that class switching could occur at the site of inflammation and that the IgE might be specific to islet antigens.

Discussion

Although the presence of IgE in different autoimmune disease, including T1D, has been reported [2–7,32,33] the potential contribution to the pathogenesis in the different diseases is still to be clarified. In this study, we have explored

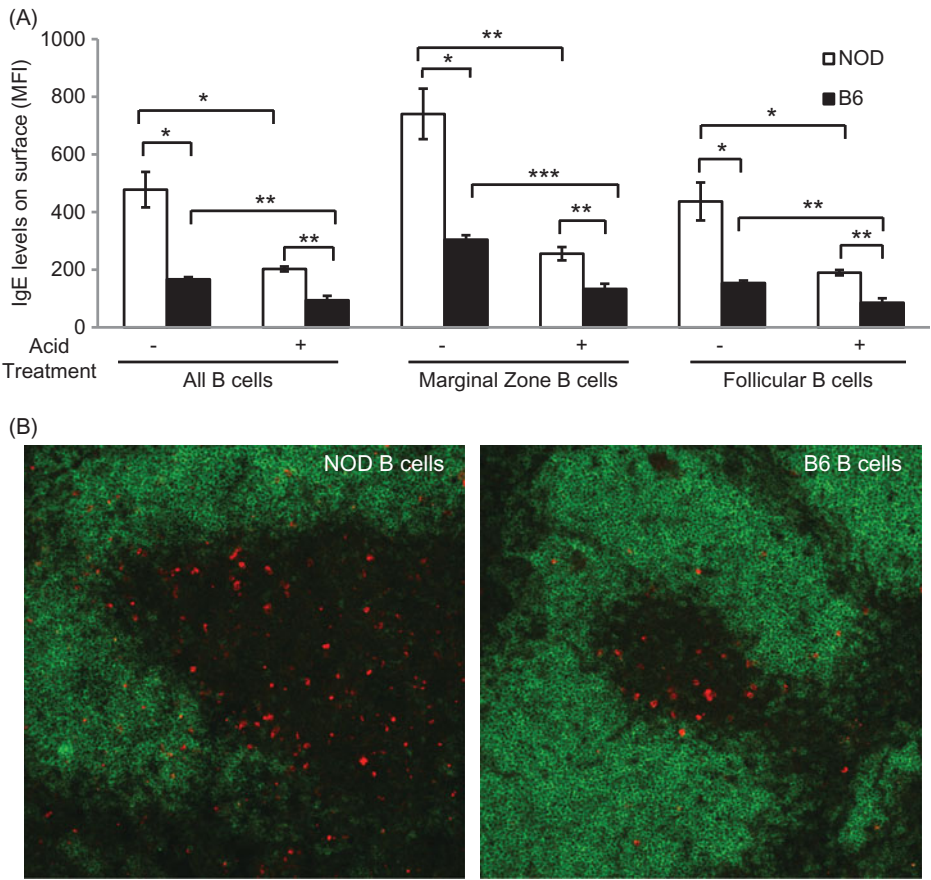


Figure 3. Acid treatment of NOD and B6 B cells does not eliminate the difference in IgE binding between both strains. The functionality of CD23 also remained similar as both NOD and B6 B cells were able to recruit OVA specific T cells to the follicle when adoptively transferred into CD23^{-/-} mice. (A) Graphical representation of FACS analyses (MFI) before and after acid treatment ($n=3$, representative of five experiments, $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.0001$). (B) Immunofluorescence staining of 8 μm spleen sections from adoptively transferred recipient CD23^{-/-} mice, with either NOD B cells or B6 B cells, reveal recruitment of DO11.10 T cells that are specific to OVA to the T cell region of the splenic follicles in mice transferred. The difference in recruitment capacity was not significant. Both NOD and B6 B cells were able to rescue the IgE-immune complex mediated response.

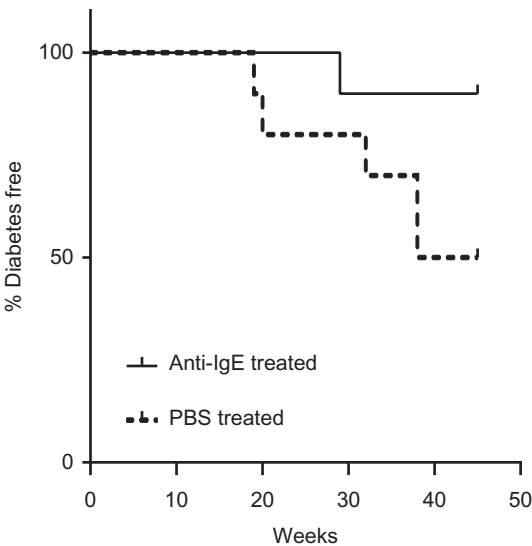


Figure 4. Anti-IgE treatment of NOD mice delays diabetes incidence. Mice ($n=10$ in both groups) were injected 10 times in total from 3 weeks of age for 5 weeks with either 100 μg anti-IgE (full line) or with PBS (hatched line) only. Diabetes was monitored weekly.

the role of IgE in a T1D model, i.e. the NOD mouse. Interestingly, we found that NOD B cells display increased levels of IgE on the surface using flow cytometry. Immunohistochemical analyses confirmed this phenotype to

be present throughout the spleen including the red pulp. One possible explanation for this phenotype could be increased serum levels of IgE. Indeed, it has been reported previously that IgE level is increased in NOD serum compared to B6 ($n=2$ for each group) [34]. However, we could not confirm this difference ($n=27$ for each group), possibly because the number of mice analyzed in the previous studies has been limited. In addition, no correlation between individual serum IgE levels and the detected surface IgE levels was found, supporting the notion that this is not the cause.

One plausible ligand for the IgE binding is CD23. This molecule is expressed on B cells and additional cell types in the red pulp of the spleen [28]. However, as we observed similar levels of IgE on marginal zone B cells that do not express, or at least display low levels of CD23 on the surface, in contrast to the follicular B cells, we consider an alternative ligand to be more likely. In fact, we have previously shown that NOD B cells capture IgM and IgG immunoglobulins aberrantly on their surface [20]. It is plausible that the same ligand that is responsible for binding IgM and IgG is also binding IgE. The existence of an alternative ligand was further supported by the fact that the IgE binding was of significant strength to withstand an acid treatment, which previously has been shown to disrupt CD23-IgE binding [29]. We acknowledge the possibility that the increased IgE level could be solely due to class switching although we believe

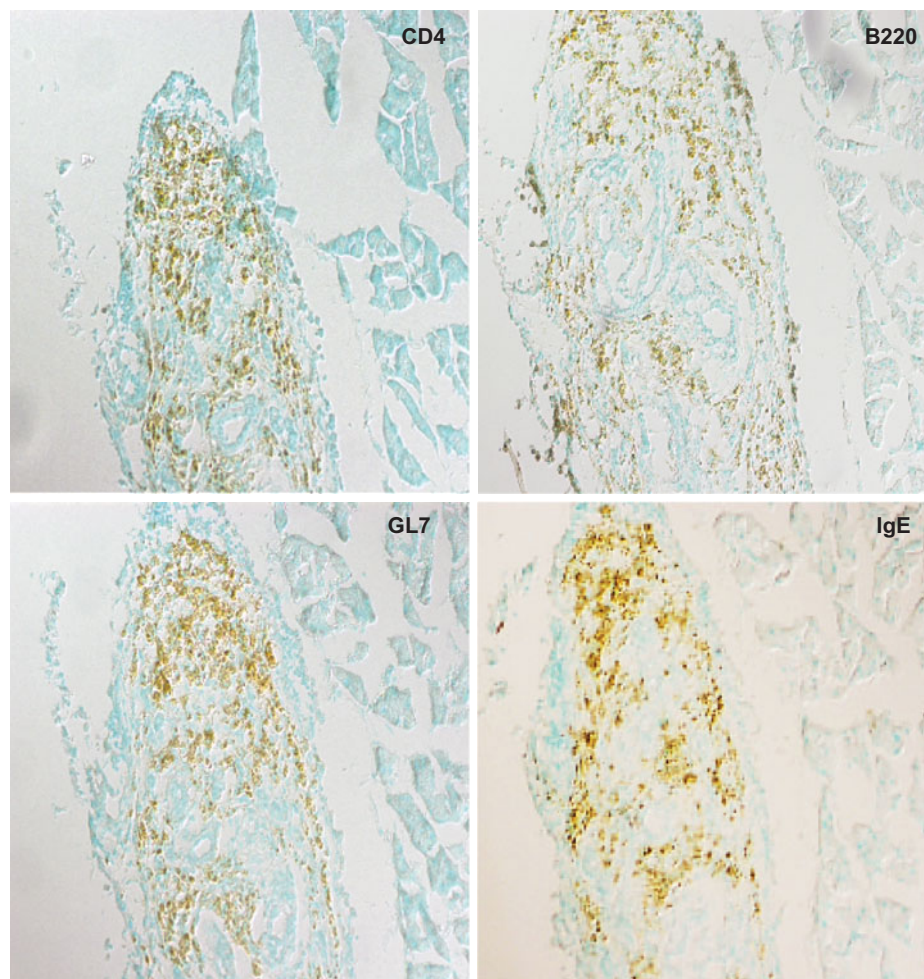


Figure 5. Pancreata sections from NOD mice revealed the presence of tertiary lymphoid structures with staining for T cells (CD4), B cells (B220), germinal centers (GL7) and IgE (possible class switching). Images are from one NOD mouse 13 weeks of age.

that a stronger staining pattern of IgE would have been observed if this had been the case.

To test if this excessive binding made NOD B cells more efficient in rescuing IgE-mediated immune responses, we performed the adoptive transfer experiment described by Henningsson et al. [27]. The adoptive transfer experiment revealed no difference between the ability of NOD B cells and B6 B cells to rescue IgE-mediated immune response. One possibility for this outcome is that this experimental system is exclusively dependent on the CD23 molecule and this is identical between NOD and B6 as verified by the SNPs from the MGI database (<http://www.informatics.jax.org>). Moreover, we did not detect any difference in the expression level of CD23 by flow cytometry (Figure 1A). Another explanation could be that since NOD mice have more CD23^{low} MZ B cells [35,36], and this was not adjusted for in the transfer of a specific number of B cells, the recruitment of similar number of transgenic T cells to the follicles still reflects an increased ability of the NOD B cells to promote an IgE dependent immune response. Finally, it is possible that additional capture of the IgE–OVA–TNP complex may also be prevented due to the saturation of NOD B cells with natural IgE.

In conclusion, we suggest that IgE autoantibodies contribute to the pathogenesis of Type 1 diabetes in the

NOD mouse. It has been suggested that IgE mediates feedback on the immune system, by increasing antigen presentation and thus, enhancing the immune response [24,37–40]. This may lead to sustained inflammation due to the IgE feedback mechanism or faster destruction of the β cells mediated by IgE opsonization. In addition, it is plausible that as a consequence excessive class-switching in germinal centers due to sustained inflammation in tertiary follicles, could initiate class switching to IgE. This supports the concept of autoallergy and demonstrates that IgE is also important in the development of autoimmune disease.

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Declaration of interest

The authors declare no conflict of interest. This study was supported by grants from the Swedish Diabetes Foundation, The Swedish Children's Diabetes Foundation, Stiftelsen Lars Hiertas Minne, Insamlingsstiftelsen vid Umeå universitet.

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Supplementary material available online.
Supplemental Figures S1 and S2