

Published in final edited form as:

*Eur J Immunol.* 2013 October ; 43(10): . doi:10.1002/eji.201343376.

## Insulinoma-released exosomes activate autoreactive marginal zone-like B cells that expand endogenously in prediabetic NOD mice

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### Summary

Exosomes (EXOs) are nano-sized secreted microvesicles that can function as potent endogenous carriers of adjuvant and antigens. To examine a possible role in autoimmunity for EXOs, we studied EXO-induced immune responses in non-obese diabetic (NOD) mice, an autoimmune-prone strain with tissue-specific targeting at insulin-secreting beta cells. EXOs released by insulinoma cells can activate various antigen-presenting cells to secrete several proinflammatory cytokines and chemokines. A subset of B cells responded to EXO stimulation in culture by proliferation, and expressed surface markers representing marginal zone (MZ) B cells, which was independent of T helper cells. Importantly, splenic B cells from prediabetic NOD mice, but not diabetic-resistant mice, exhibited increased reactivity to EXOs, which was correlated with a high level of serum EXOs. We found that MyD88-mediated innate TLR signals were essential for the B-cell response; transgenic B cells expressing surface immunoglobulin specific for insulin reacted to EXO stimulation, and addition of a calcineurin inhibitor FK506 abrogated the EXO-induced B-cell response, suggesting that both innate and antigen-specific signals may be involved. Thus, EXOs may contribute to the development of autoimmunity and type 1 diabetes (T1D) in NOD mice, partially via activating autoreactive MZ-like B cells.

### Keywords

Autoimmunity; autoreactive B cells; exosomes; NOD; T1D

### Introduction

Tissue-specific autoimmune disease such as type 1 diabetes (T1D) can be initiated following loss of tolerance to a single tissue-specific antigen and subsequent activation of its cognate T-cell clones [1, 2]. Several islet antigens including insulin, GAD65 and IA-2 are considered candidate autoantigens in T1D due to the high frequency of autoantibodies to these molecules in patients. Intriguingly, the expression of these autoantigens is not restricted to the pancreatic islets and can be expressed in thymus and peripheral lymphoid organs [3, 4]. It remains unclear whether these autoreactive T cells are activated in the periphery by the candidate islet antigens, or by unknown endogenous molecules or exogenous antigens. One plausible explanation is that processing and presentation [5–7] or modification [8] of the islet antigens in the pancreas may be different from that in thymus or lymphoid organs and

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

thus generate high affinity peptides or peptide-MHC complexes that can activate autoreactive T cells locally in the pancreas. But, such high affinity self peptide(s) have not been identified.

Non-obese diabetic (NOD) mice are highly genetically predisposed to T1D and serve as an excellent animal model of T1D [9]. In these mice, autoreactive T cells are activated very early in life (2–3 weeks of age) in the pancreas of almost all pups, suggesting that antigenic trigger(s) arise endogenously in the pancreas. It has been suggested that beta cell apoptosis occurs early during development and may act as the trigger of an autoimmune response [10], but the process of apoptosis commonly induces tolerance rather than a Th1-biased inflammatory response. Interestingly, type I IFN has been found upregulated only in the islets of 2 week-old NOD mouse strain [11], indicating unique endogenous inflammatory trigger(s) may be produced locally. Peri-islet Schwann-like glial cells have been suggested as the early autoimmune targets [12]; however, these cells do not express the candidate diabetes-causing autoantigens, and not all lymphocyte-infiltrated islets have peri-insulinitis. The initial triggering event remains unclear. Nevertheless, this event likely leads to activation of effector T cells specific for insulin, which appears the primary autoimmune target, at least in NOD mice [2].

Exosomes (EXOs) are small (30–100 nm) extracellular microvesicles that are produced by fusion of late endosomal compartments and/or multivesicular bodies with the plasma membrane. Under normal physiological conditions or in response to stress or tissue damage, a number of cell types secrete microvesicles containing RNA and/or proteins as a form of intercellular communication. Recent evidence suggests that some extracellular microvesicles, particularly EXOs, can stimulate immune responses [13]. Tumor cells actively release proinflammatory EXOs that can induce tumor-specific immunity [14, 15]; however, such responses are frequently immunosuppressive, possibly due to the induction of myeloid immune suppressor cells [16] and/or regulatory T cells [17]. Our group has pioneered the study of the immune response to EXOs in autoimmunity, a situation where pathogenic effectors, rather than regulatory T cells, are preferentially activated. We found that insulinoma-released EXOs contain innate stimuli and candidate islet antigens, and can activate autoreactive Th1 cells in prediabetic NOD mice [18]. In this continuing study, we found that EXOs can stimulate MZ-like B cells to proliferate, which does not require T-cell help, but requires MyD88-mediated innate signals and is partially dependent on antigen-specific signals. In agreement with the notion that EXOs may trigger autoimmunity, we demonstrate that prediabetic NOD mice have increased levels of endogenous serum EXOs and EXO-reactive B cells.

## Results

### EXOs activate a subset of splenic B cells independent of T-cell help

Both the MIN6 and NIT-1 mouse insulinoma cell lines release EXOs that were immunostimulatory to splenocytes of NOD mice. MIN6 is used for EXO production mainly because it has less spontaneous apoptosis in culture than NIT-1, and also because only MIN6 expresses GAD65 protein that can be released into the culture supernatant [18]. Previously, we found that EXOs can activate antigen-presenting cells (APCs) including B cells [18]. By tracking the EXO-induced cell division with CFSE dye, we noticed that not only CD4<sup>+</sup> T cells, but also many CD4<sup>-</sup> cells were proliferating when cultured with EXOs (Fig. 1A). These CD4<sup>-</sup> cells were found to be B220<sup>+</sup> B cells, which divided as early as 48 h of culture (T-cell proliferation starts at 72 h). We thus purified B220<sup>+</sup> cells (>95%) from NOD spleens using MACS beads, and stimulated them with EXOs. The purified splenic B220<sup>+</sup> cells responded vigorously to EXOs in culture. At the peak time of B-cell

proliferation (72 h), over one third of the cells are CFSE-low dividing cells (Fig. 1B). Thus, a subset of B cells can react to EXOs in the absence of T helper cells.

### **EXO-reactive B cells express the surface markers of MZ B cells**

To identify the B-cell subset(s) that contain the EXO-reactive B cells, we examined the expression of several B-cell markers on the CFSE-low, EXO-reactive B cells (Fig. 1C). These cells were IgM<sup>+</sup>/CD23<sup>-</sup>, excluding the subset of follicular B cells; they were IgD negative, which could be downregulated during the culture, and intermediate for CD1d. Thus, the majority of them could be defined as MZ B cells. Most of the dividing cells are CD21<sup>-high</sup>, but about one third of the dividing cells are CD21-low-to-intermediate (CD21<sup>-lo/int</sup>). In contrast, LPS stimulated only CD21<sup>-high</sup> MZ B cells to proliferate. About 20% of EXO-reactive cells were CD27<sup>+</sup> (Fig. 1C), which is a marker for activated/memory B cells in humans [19]; whereas LPS activated far fewer (<3%) CD27<sup>+</sup> cells. Therefore, the in vitro EXO-reactive B cells may include previously activated or memory B cells in addition to MZ B cells.

### **Increased EXO-reactive B cells and serum EXOs in NOD mice**

To examine a possible correlation between the number of MZ B cells and the in vitro B-cell response to EXOs, we compared NOD and B6 mouse strains since adult NOD mice exhibit an abnormal increase in MZ B-cell numbers in comparison with B6 mice [20]. We confirmed that MZ B cells are increased in 8–12 week-old NOD mice (Fig. 2A); at the same age, B6 mice contain, on average, twofold fewer MZ B cells than NOD mice. When their splenocytes were stimulated in vitro by EXOs, the average number of EXO-reactive B cells in NOD was more than two-fold higher than that in B6 mice (Fig. 2B). In fact, serum EXO levels were found to be three-fold higher in adult (8–12 week-old) NOD mice, as measured by CD81 total protein in purified serum EXOs (Fig. 2C). These results indicate that the high levels of serum EXOs in NOD mice at the age of 8–12 week-old may cause expansion of memory-type autoreactive B cells, which can be recalled by in vitro stimulation with EXOs.

### **Increased numbers of EXO-reactive MZ-like B cells in prediabetic NOD female mice**

To further examine whether EXO-reactive B cells are associated with T1D development, we compared NOD female with NOD male mice, since only females are highly susceptible to T1D. The number of MZ B cells in 8–12 week-old NOD females is slightly higher, average 6.52% in females vs. 5.94% in males (Fig. 3A). However, the percentage of EXO-reactive B cells in the females was two-fold higher than that in age-matched males, 10.67% vs. 5.45% (Fig. 3B), suggesting an increased frequency of EXO-reactive, memory-type B cells in female mice. Among individual mice, the percentage of EXO-reactive B cells varies largely for both genders, from 2 to 16% of total splenic B cells, but the variation of MZ B-cell number in these mice was lower (from 4 to 9%). Thus, development of EXO-reactive B cells in NOD mice could not be predicted solely by the percentages of MZ B cells; additional marker(s) are required for their identification. Interestingly, 10 week-old females exhibited the strongest B-cell response to EXOs as compared with 7 or 14 week-old females (Fig. 3C), indicating a role of these EXO-reactive B cells in the early stage of the disease. The lack of response to EXOs in the older mice might be a result of the persistent high level of serum EXOs, which may lead to B-cell anergy or tolerance.

### **EXO immunization expands EXO-reactive B cells in young NOD mice**

MZ B cells in young NOD mice (< 5 week-old) are not expanded, similar to the percentages in B6 mice. In agreement, serum level of EXOs was also undetectable in both mouse strains at this young age. As expected, the number of EXO-reactive B cells in both strains at this young age is low, less than 5%. To examine whether EXO immunization can induce specific

B-cell expansion in these young mice, we injected NOD pups (one week-old) with the EXOs and examined the mice at 4 weeks of age. Fig. 4A showed that EXO-reactive B cells increased to 14.9% in the injected mice from 4.7% in control mice; however, the percentages of MZ B cells were comparable between the control and EXO-injected mice (Fig. 4B), suggesting that the early EXO immunization may induce EXO-reactive memory B cells, which may not completely exhibit MZ B-cell phenotype.

### EXO-induced B-cell activation requires innate TLR signals

EXO-induced innate immune responses require the MyD88-mediated signaling pathway. In the absence of MyD88, NOD splenocytes failed to produce inflammation cytokines after EXO stimulation (Fig. 5A). Also, there was no B-cell proliferation in the NOD.MyD88<sup>-/-</sup> splenocytes. To address which TLR(s) are involved, we first examined Unc93b1 mutant mice. Unc93b1 is an adaptor molecule required for endosomal TLRs (TLR3, 7/8 and 9), and a single mutation *3d* completely destroys Unc93a1 function and innate responses by these TLRs [21, 22]. We found that EXO-induced secretion of inflammatory cytokines and lymphocyte proliferation was intact in the *3d* mutant mice (Fig. 5B&5C). This suggests that cell surface TLRs may be crucial for the EXO-induced innate response. We thus examined each of the TLR2, 3 and 4 deficient mice for their responses to EXO stimulation in 48 h cytokine secretion, 72 h CFSE-labeled B-cell division and 96 h <sup>3</sup>H-thymidine incorporation assays. We found that both TLR2 and TLR4 partially contributed to the EXO-induced responses (Table I).

### Insulin-specific transgenic B cells can react to EXOs

To examine whether EXOs can stimulate antigen-specific autoreactive B cells, we tested a BCR-transgenic mouse strain (Tg125), which expresses both H- and L-chains of an insulin-specific Ig [23]. A control mouse strain (VH125) that expresses only the H-chain of the insulin-specific Ig was used. Tg125 has two-fold more MZ B cells than that in VH125 mice (Fig. 6A), whereas the number of CFSE-low/CD21<sup>+</sup> dividing B cells was five-fold higher in Tg125 than that in VH125 (Fig. 6B). B cells from the Tg125 mice were not hyper-responsive in culture and produced a similar background as VH125 cells. MIN6-derived EXOs contain insulin protein as found in previous mass spectrometry analysis [18], which may contribute to the response of Tg125 B cells to EXOs.

### FK506 inhibits the EXO-induced B-cell response

FK506 is a calcineurin inhibitor that blocks calcium flux, and can inhibit anti-IgM-induced B-cell response [24]. As shown in Fig. 7A, FK506 can inhibit the EXO-induced proliferation response. In contrast, it has no effect on LPS-induced B-cell proliferation at the same dose range (1–10 μM). At a lower dose of FK506 (200 nM), both anti-IgM and EXOs, but not LPS –induced B-cell proliferation remained sensitive to the inhibition (Fig. 7B), confirming this calcineurin-dependent activation of B cells by EXO stimulation. However, we found that phosphorylation of Syk was only induced by anti-IgM, but not by EXO stimulation (Fig. 7C), although a weak phosphorylation of ERK was detected after EXO stimulation. Alternative signaling pathway(s), independent of Syk phosphorylation, may be activated by EXOs.

## Discussion

The reason behind the expansion of MZ B cells in the NOD mouse strain, particularly in adult females, is unknown. In this study, we found that EXOs can stimulate autoreactive MZ-like B cells. Since natural autoantibodies recognize a large array of different autoantigens [25], and are also abundant in NOD mouse strain [26], it is possible that EXOs may contain some of the autoantigens recognized by the natural autoantibodies. The

interaction between exosomal antigens and the autoreactive B cells is one of low affinity since there was no phosphorylation of Syk tyrosine kinase induced; instead, it requires MyD88-mediated TLR signals. Whether this strong dependence on the innate signals is also true *in vivo* remains to be addressed. Nevertheless, the weak antigen-specific signals are sufficient to allow us to distinguish the NOD females from males, or diabetes-susceptible from resistant mice in their responses to EXOs, confirming an autoreactive nature of the EXO-reactive B cells that are expanded in prediabetic mice. Although it is not clear how this natural autoimmune response to a widely expressed antigen source, EXOs, leads to a highly tissue-specific autoimmune response in the pancreatic islets; with their unique adjuvant activity and expression of candidate autoantigens, EXOs appear to be an excellent tool for studying tissue-specific autoimmunity and endogenous pathways priming effector autoreactive T cells.

MZ B cells are responsible for primary immune response to T-independent antigens. They express germ-line-encoded, partially autoreactive antigen receptors and may play an important role in natural immunity and/or autoimmunity to stress-induced self antigens [27]. It has been shown that adult NOD mice have increased expansion of MZ B cells in their spleens as compared with B6 mice [28]. However, the expansion of MZ B cells in NOD mouse strain occurs only in the adult stage [20], and the percentage of MZ B cells is comparable with B6 mouse strain at a younger age (<5 week-old). This suggests that the expansion of MZ B cells is not due to dysfunction of B-cell development; instead, it is more likely that a continuous stimulation by some endogenous antigens may expand MZ B cells. It is unclear why increased MZ B cells associate with autoimmunity. One possibility is that MZ B cells may function as APCs to activate T helper cells [29]; but the antigens that stimulate MZ B cells are mostly T-independent ones, and more problematically, T cells rarely appear in the MZ area of the spleen. One related observation by Bachmann and colleagues [30] is that natural IgM antibodies that are mostly produced by MZ B cells preferentially bind to particulate, but not soluble antigens to facilitate antigen-capture by follicular dendritic cells. It might be helpful to examine the percentage of plasma B cells and the levels of serum Ig in prediabetic NOD mice if immune complex-mediated antigen-capture or APC activation is the key function attributed by the expanded MZ B cells.

Cross-linking BCR with TLRs induces functional properties of B cells that cannot be induced by engaging either receptors alone [31], suggesting a role for innate signals in breaking B-cell tolerance. TLR stimulation of B cells upregulates the expression of MHC and costimulatory molecules, and increased secretion of proinflammatory cytokines [32]. In fact, IL-6-producing B cells are the key mediators for the pathogenesis in experimental autoimmune encephalomyelitis [33], demonstrating that at least a subset of autoreactive B cells can be activated in the presence of innate stimuli. Early autoreactive B cells are believed to be low affinity and have broad antigen specificity [34, 35], which could be induced by EXOs that carry both innate and antigen specific stimuli.

With respect to the differences between female and male mice in terms of autoimmune responses, it has been shown that NOD females (>5 week-old) have increased anti-insulin titer [36], but this titer drops in older female mice [37]. Although NOD splenocytes responded to LPS three-fold stronger than B6 cells, the difference between female and male NOD mice in response to LPS stimulation was insignificant (data not shown). However, the gender difference became obvious (two-three-fold higher in the females) when EXOs were used to stimulate the splenocytes. The different response to EXOs could not be solely explained by the amount of MZ B cells since the females contain slightly higher percentages with an average of 6.52% and 5.93% for the males. More likely, the autoreactive B cell precursors or memory cells accumulated or expanded in the prediabetic females accounted for the gender difference in their response to EXOs. Interestingly, the EXO-reactive MZ B

cells decreased in older female mice (14 week-old), possibly due to anergy or tolerance imposed on these B cells at the late prediabetic stage. This decrease in the B-cell response fits with the drop of anti-insulin titer in these mice [37], and also agrees with an observation by a non-invasive imaging method that islet inflammation peaks at 10 week-old, not at the onset of diabetes [38]. Likely, in 8–10 week-old female mice, the battle between effectors and regulatory cells might be accelerated, at which immune interventions would be the most effective.

We found that the serum level of EXOs is dramatically increased and persist in adult NOD but not in B6 mice, which may be responsible for expanding the MZ B cells in NOD mice. The source of serum EXOs in NOD mice is unknown, but likely do not originate from the islets; in addition to endo/epithelial cells, both T and B cells can also release EXOs to regulate immune responses [39, 40]. It remains to be examined whether the increase of serum EXOs is triggered by the initial islet autoimmune response, but it cannot be excluded that abnormal EXO production may occur systemically with or without the islet-specific autoimmune responses, possibly due to a dysregulation of EXO secretion in this autoimmune-prone mouse strain. Interestingly, Epstein-Barr virus (EBV) -transformed B lymphoma cells can release EXOs carrying the viral gp350 protein, which targets B cells specifically via binding to CD21 [41]. Thus, not only genetic abnormalities but also environmental triggers may also affect EXO secretion and the immune responses. The consequence of the increased serum EXOs to the islet-specific autoimmune response remains to be studied. It could cause expansion of autoreactive MZ B cells as we found in the spleen of prediabetic NOD mice, but a persistently high level of serum EXOs might also induce tolerance to both B and T antigens expressed by the EXOs. Additional antigenic and/or costimulatory signals may have to be provided locally within the islets in order to activate effector functions of the EXO-reactive B and T cells.

The antigens inducing the innate and the B-cell response are unknown. We found that MIN6 cells express insulin, GAD65 and several Heat shock proteins (HSP) [18]. Likely, the insulin-specific Tg125 transgenic B cells recognize insulin carried by the EXOs. HSP can stimulate TLRs to induce innate response [42]. The difficulty separating EXOs from other microvesicles such as apoptotic bodies hinders the studies specifically addressing innate or antigen-specific responses to EXOs. Considering the large number of EXO-reactive B cells in NOD female mice (over 10% in some mice), it is unlikely that all of these B cells recognize one islet antigen such as insulin; rather some common cross-reactive exosomal antigens may also attribute to the low affinity B cell recognition. It is noteworthy that phospholipid content in EXOs is unique -- EXOs contain more phosphatidylserine (PS) but less phosphatidylcholine (PC) as compared with that in the cell membrane, and a higher ratio of saturated PC [43]. Also, EXO formation or budding was reduced after inhibiting sphingomyelinase [43].

Interestingly, anti-PC antibodies are known to contribute to natural immune defense, and are also present in autoimmune responses [44]; more interestingly some HIV neutralization antibodies can also cross-react to lipid antigens [45]. Future research will aim at understanding how this low affinity autoantibodies and/or autoreactive B cells participate in T-cell-mediated autoimmunity, which has much higher tissue and/or antigen specificity and is difficult to achieve tolerance.

## Materials and methods

### Mice

NOD/ShiLtJ and C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as inbred strains at the animal facility of the Torrey Pines

Institute for Molecular Studies (TPIMS). Splenocytes from NOD.MyD88<sup>-/-</sup>, NOD.TLR2<sup>-/-</sup>, NOD.TLR3<sup>-/-</sup> and NOD.TLR4<sup>-/-</sup> mouse strains were provided by Li Wen at Yale University (New Haven, CT). Splenocytes from B6.*Ipr*<sup>-/-</sup>.Unc93b1 *3d* mutant and B6.*Ipr*<sup>-/-</sup> mouse strains were provided by Dwight Kono at the Scripps Research Institute (La Jolla, CA). Splenocytes from NOD mice expressing transgenes of anti-insulin Ig H+L-double-chains (Tg125) or H-single-chain (VH125) were provided by James Thomas at Vanderbilt University (Nashville, TN). Experimental protocols were conducted with approvals from the Ethical Review Committee of TPIMS.

### EXO Preparation

MIN6 insulinoma cell line [46] maintained in high glucose DMEM with 10% fetal calf serum (FCS) was used for EXO preparation following a previously described method [18]. Briefly, FCS was pre-centrifuged at 100,000 g × 90 min to remove serum EXOs and other microparticles. Culture supernatants were harvested every 2–3 days. When the cells reached confluence, trypsin–EDTA treatment was applied and one-third of the cells were inoculated for subculture. The supernatants were centrifuged at 3000 rpm × 15 min, followed by membrane filtration (0.22 μm). EXOs were collected by spinning the filtered supernatant in an ultracentrifuge (Sorvall Discovery 90SE; Hitachi) at 100,000 g × 90 min. After washing once with PBS, protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). One liter of MIN6 culture supernatant yields 0.5–1.0 mg of EXOs. EXOs were examined by electromicroscope and mass spectrometry as previously described [18], and expression of exosomal signature proteins was further confirmed by flow cytometry analysis using anti-CD63/CD81-coated beads and detected by fluorescence-labeled anti-CD81 (see supplementary Fig.s1).

### Flow cytometry

Flow cytometry was performed on FACSCalibur flow cytometer (BD Biosciences). Fluorescent antibodies against mouse B-cell surface markers, CD21, CD23, CD27 and IgD antibodies were purchased from BioLegend (San Diego, CA), IgM, B220/CD45R, CD4, and CD1d antibodies from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA).

### CBA Cytokine Assay

A cytometric bead array (CBA)-based flow cytometry method (BD Biosciences) was used to analyze six different inflammatory cytokines or chemokines, IL-6, IL-10, MCP-1, IFN- $\gamma$ , TNF- $\alpha$ , and IL-12p70, according to the manufacturer's protocol with modification as previously described [18]. The concentration of each cytokines was extrapolated from the standard curves by testing with the respective recombinant proteins of the cytokines or chemokines.

### Carboxyfluorescein Succinimidyl Ester (CFSE) Cell Proliferation Assay

Total splenocytes ( $10^7$  cells/ml PBS) were labeled with 5 μM of CFSE (Sigma-Aldrich) at 37°C for 10 minutes, and then cultured in a 96-well flat bottom plate at  $8 \times 10^5$  cells per well in 200 μl of RPMI complete medium containing antigens. After 72 hours, the cells were harvested and stained with fluorescence-labeled B220 and CD4 antibodies to identify CFSE-low, proliferating B220<sup>+</sup> B cells and CD4<sup>+</sup> T cells by flow cytometry.

### Measuring serum EXOs by Enzyme-linked Immuno-sorbent Assay (ELISA)

Serum EXO concentration was measured using an ExoQuick kit (System Biosciences, Mountain View, CA). Briefly, 250 μl of serum was incubated with 63 μl of ExoQuick polymer solution for 30 min to precipitate EXOs. EXO pellets were lysed by 200 μl EXO-binding buffer, followed by centrifugation at 1500 g for 5 min. Then, 50 μl of the lysed

supernatant were coated on Microtiter plates for overnight at 37°C. After three washes, plates were incubated with anti-CD81 for 1 h, and then HRP-conjugated secondary antibody. Substrate 3,3',5,5'-tetramethylbenzidine was used for color reaction. The optical density (OD) was read at 450 nm. The levels of CD81 protein in serum EXOs are shown as OD values.

### **<sup>3</sup>H-Thymidine Incorporation Assay**

Total splenocytes ( $6 \times 10^5$  cells/200  $\mu$ l/ well) were cultured in RPMI complete medium with or without antigens in 96-well flat bottom plates for 78 h, followed by pulsing with 0.5  $\mu$ Ci/ well <sup>3</sup>H-thymidine (Moravek Biochemicals, Brea, CA) for 18 h. Cells were harvested with a Micro Cell Harvester (Skatron Instruments, Sterling, VA), and incorporation of [<sup>3</sup>H]-thymidine was measured on a Wallac MicroBeta Trilux counter (Perkin Elmer, Boston, MA). Stimulation indexes (SI) were calculated as counts per minute (cpm) of stimulated cells / cpm of the medium control.

### **SDS-PAGE and western blotting analysis**

Cell lysates of stimulated splenocytes were similarly prepared and separated on SDS-PAGE as previously described [18]. After transferring the separated proteins onto a nitrocellulose membrane (Amersham, GE Healthcare Life Sciences, Piscataway, NJ), immuno-blotting was performed using 1  $\mu$ g/ml of primary antibodies, followed by a respective secondary HRP-labeled anti-IgG (Amersham). The protein bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham). Primary antibodies specific for phosphorylated Syk (Tyr323), ERK (Erk1/2) or total ERK were purchased from Cell Signaling Technology (Danvers, MA).

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **Acknowledgments**

This work was supported by grants to Y.D.D. from the National Institutes of Health (R01DK091663) and the Diabetes National Research Group (DNRG). We thank Dr. Linda Wicker at the University of Cambridge UK and Dr. Linda Sherman at the Scripps Research Institute (San Diego, CA) for their guidance. We thank Dr. James Thomas at the Vanderbilt University, Dr. Li Wen at the Yale University (New Haven, CT) and Dr. Dwight Kono at the Scripps Research Institute (San Diego, CA) for providing mouse strains and discussion.

### **Abbreviations**

<b>T1D</b>	type 1 diabetes
<b>NOD</b>	non-obese diabetic
<b>MZ</b>	marginal zone
<b>EXO</b>	exosome
<b>TLR</b>	Toll-like receptor

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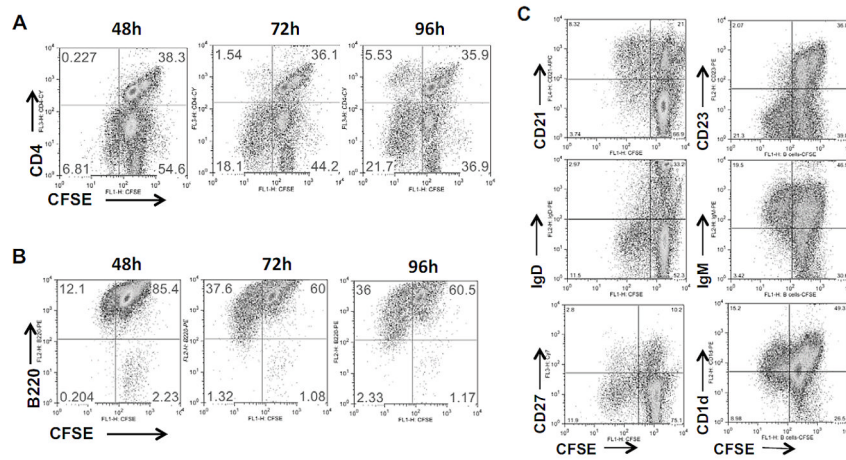
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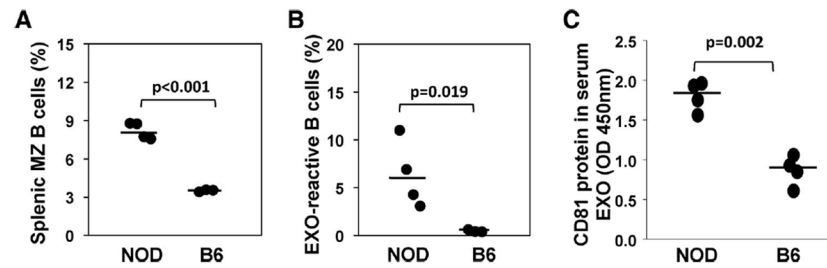
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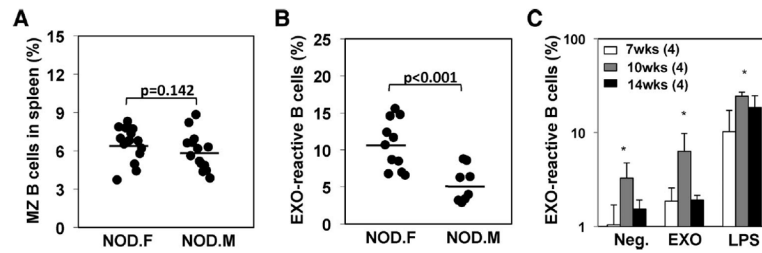
**Fig. 1.**

EXOs activate MZ B cells in the absence of T-cell help. (A) CFSE-labeled splenocytes ( $8 \times 10^5/200 \mu\text{l/well}$ ) of NOD females (8–10 weeks old) were stimulated with  $20 \mu\text{g/ml}$  of EXOs in a flat-bottom 96-well plate. Cultured cells were harvested at different times and stained with CD4 or B220 antibodies. (B) B cells were purified from the CFSE-labeled NOD splenocytes with MACS beads coated with anti-B220, and then stimulated with EXOs. (C) CFSE-labeled splenocytes (left) or purified B cells (right) were stimulated with EXOs for 72 h, and stained with antibodies shown. Data shown are from one experiment representative of three performed.



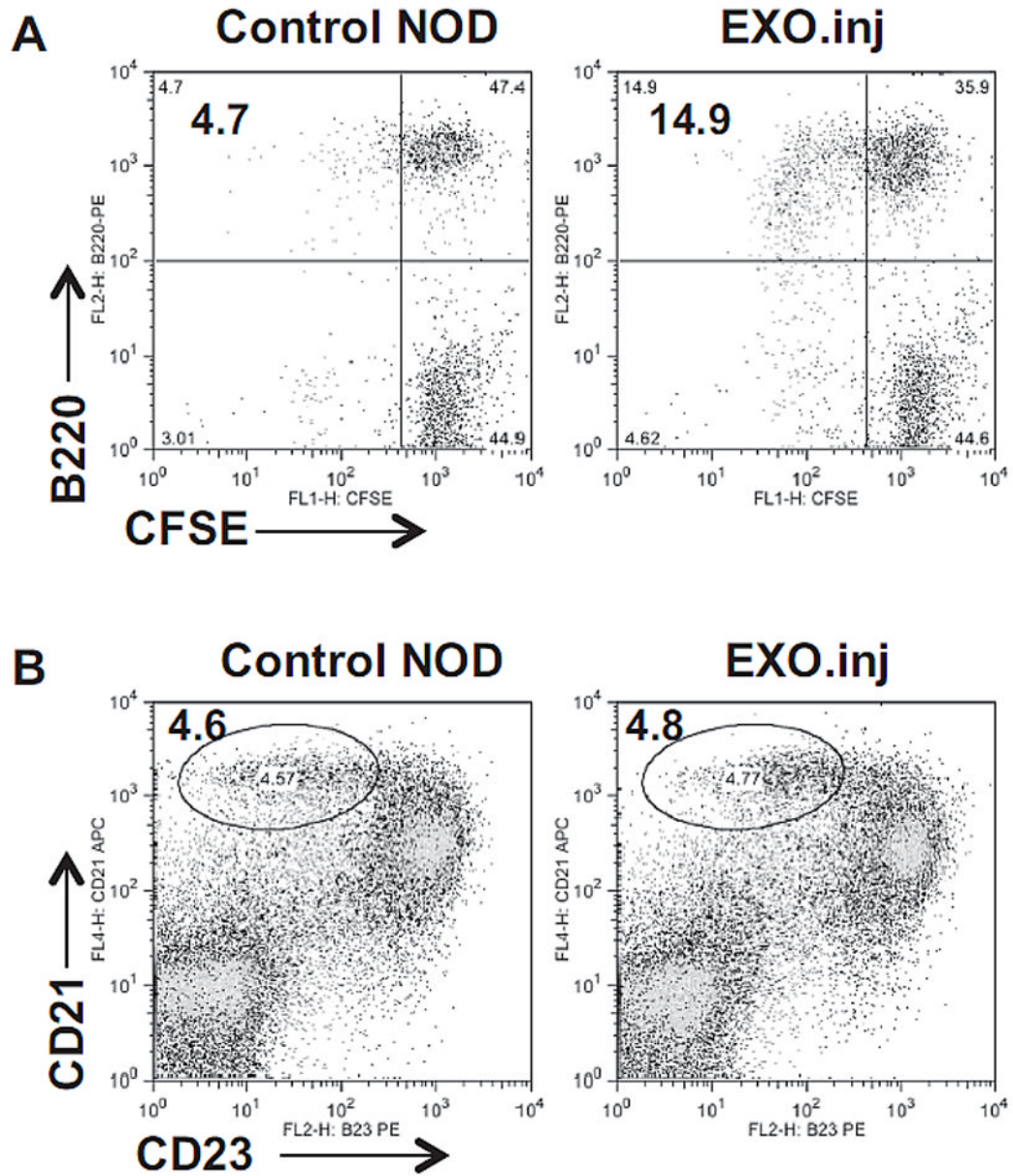
**Fig. 2.**

Comparison of NOD and B6 B-cell responses to EXOs. (A) Splenic MZ B cells in NOD or B6 mice (8–12 week-old) were identified as  $CD21^{-hi}/CD23^{-lo}$ . (B) CFSE-labeled splenocytes of NOD or B6 mice were stimulated with EXOs (20  $\mu\text{g/ml}$ ). After 72 h, proliferating B cells were identified as  $B220^{+}/CFSE^{-low}$ . Data shown are from one experiment representative of two performed. (C) Serum samples were collected from 8–12 week-old NOD or B6 mice. EXOs were precipitated using ExoQuick and EXO concentration was measured by ExoELISA and total CD81 protein in the EXO samples was presented as OD values. Each symbol represents a single mouse, bar represents the mean. Data shown are from one experiment representative of three performed. Statistical significance determined by student T-test.

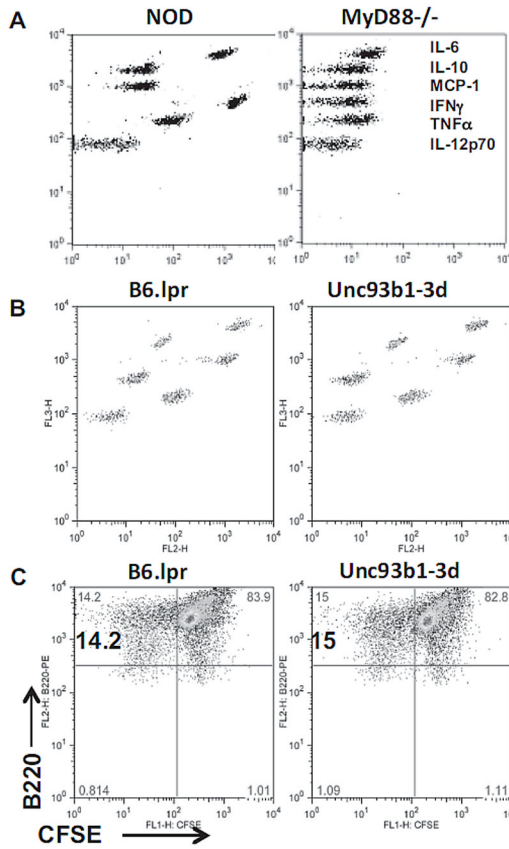


**Fig. 3.**

Increased EXO-reactive MZ B cells in prediabetic NOD female mice. (A) The percentages of MZ B cells in the spleens of 8–12 week-old NOD females or males were compared. (B) CFSE-labeled splenocytes of NOD females (NOD.F) or males (NOD.M) were stimulated with EXOs for 72 h, and the percentage of B220<sup>+</sup>/CFSE<sup>low</sup> cells were shown. (A, B) Each symbol represents one mouse, bar represents mean. Data shown are pooled from three or four experiments performed. Statistical significance determined by T-test. (C) CFSE-labeled splenocytes from different ages of NOD females (4 mice per group) were stimulated with EXOs or 2 µg/ml of LPS for 72 h. The percentage of B220<sup>+</sup>/CFSE<sup>low</sup> dividing cells are shown as mean + SD from one experiment representative of two performed. \*p<0.05, T-test.

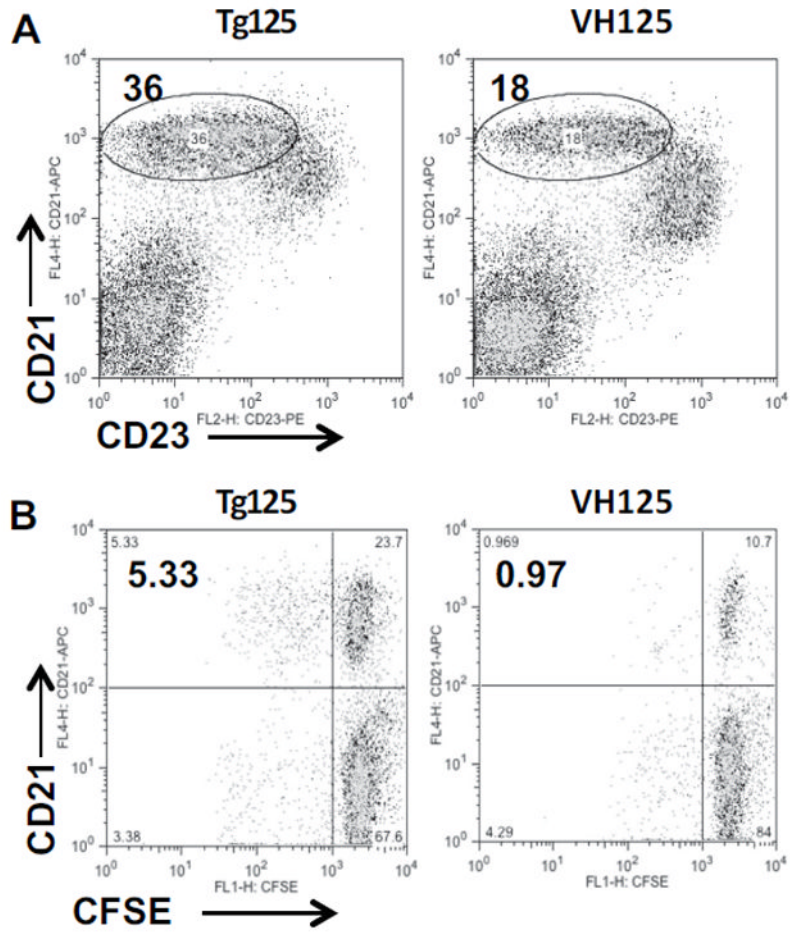


**Fig. 4.** EXO immunization expands EXO-reactive B cells in young NOD mice. NOD pups (1 week-old) were injected intraperitoneally with 3  $\mu$ g of EXOs in 50 $\mu$ l of PBS, and boosted once with 10  $\mu$ g of EXOs one week after. The mice were examined at 4 weeks of age. (A) CFSE-labeled splenocytes from the injected or control mice were stimulated by EXOs. EXO-reactive B cells are shown as CFSE<sup>low</sup>. (B) Percentages of MZ B cells in the freshly isolated splenocytes are shown. Data shown are from one experiment representative of three performed.

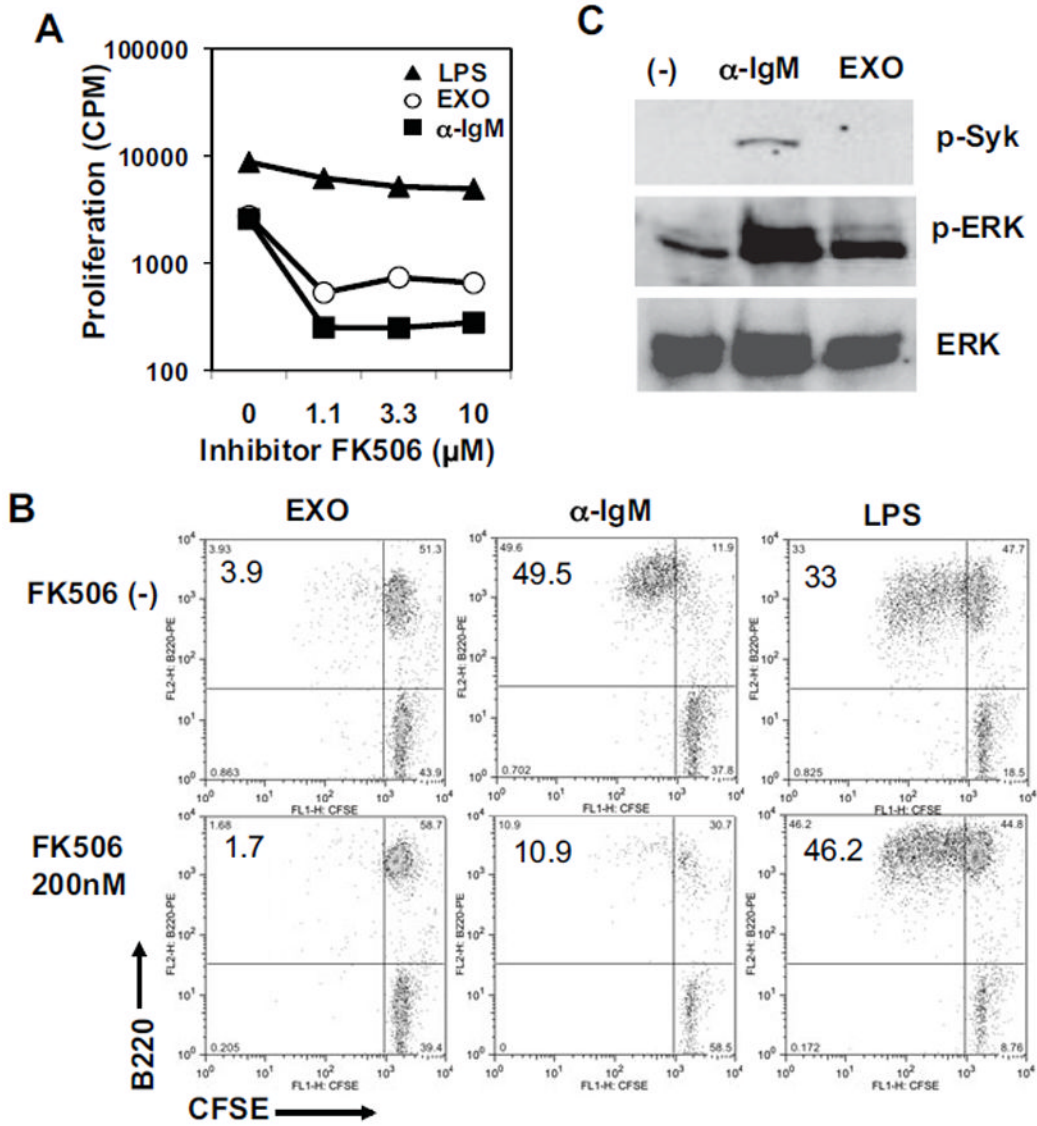


**Fig. 5.** EXO-induced B-cell activation depends on MyD88 but not Unc93b1-mediated innate signaling pathways. (A) Splenocytes ( $8 \times 10^5/200 \mu\text{l/well}$ ) from 8–10 week-old female NOD or NOD.MyD88<sup>-/-</sup> mice were stimulated with EXOs. Cytokines in the 48 h culture supernatants were measured by CBA assay. (B, C) Splenocytes were collected from B6.lpr<sup>-/-</sup>/Unc93b1.WT (B6.lpr) or B6.lpr<sup>-/-</sup>/Unc93b1-3d mutant (Unc93b1-3d). (B) Cytokine release at 48 h and (C) B-cell proliferation at 72 h were examined by CBA or CFSE assays respectively. Data shown are from one experiment representative of three performed.





**Fig. 6.** Activation of insulin-specific BCR transgenic B cells by EXOs. (A) Increased MZ B cells in transgenic mice expressing both H- and L- chains of Insulin-specific Ig (Tg125) as compared with single H-chain transgenic mice (VH125). (B) CFSE-labeled splenocytes from the Tg125 or VH125 mice were stimulated by EXOs for 72 h, followed by staining with anti-CD21 and analyzed by flow cytometry. Data shown are from one experiment representative of two performed.



**Fig. 7.** FK506 inhibits EXO-induced B-cell response. (A, B) Splenocytes from NOD females were stimulated with 20 μg/ml of EXOs, 3 μg/ml of anti-IgM, or 2 μg/ml of LPS, in the presence of FK506 in (A) a proliferation assay or (B) a CFSE-dilution assay. Similar results were observed in three experiments. (C) Splenocytes (10<sup>7</sup> cells) were stimulated by 4 μg/ml of anti-IgM for 2 minutes, or 50 μg/ml of EXOs for 30 minutes at 37°C, followed by western blotting assay using antibodies for phosphorylated Sky (p-Syk), phosphorylated ERK (p-ERK), or total ERK kinases. Data shown are from one experiment representative of two performed.

Table 1

TLR requirements by EXO-induced immune responses

Immune responses	TLR2 <sup>-/-</sup>	TLR3 <sup>-/-</sup>	TLR4 <sup>-/-</sup>	MyD88 <sup>-/-</sup>	
EXO (20µl/well)	IL-6	21.6	185.5	52.2	9.2
	IFN	12.7	122.3	31.8	7.0
	TNF	12.0	34.5	22.1	5.3
<b>Proliferation</b>	(SI)	4.8	7.9	4.0	1.6
<b>CFSE-low<sup>**</sup></b>	(%)	4.31	8.86	7.64	0.34

Splenocytes from TLR or MyD88 deficient NOD mice were stimulated with EXOs. Cytokines in the supernatants of 48h culture were measured by CBA assay.

\* Proliferation of total splenocytes was monitored by incorporation of <sup>3</sup>H-thymidine at the last 18h of total 96h culture.

\*\* CFSE-low, proliferating cells within total CFSE-labeled splenocytes were calculated after 72h of stimulation by EXOs.