# Dendritic cells licence regulatory B cells to produce IL-10 and mediate suppression of antigen-specific CD8 T cells

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**Abstract**

Regulatory B cells (Bregs) suppress and reduce autoimmune pathology. However, given the variety of Breg subsets their role is still unclear in the pathogenesis of type 1 diabetes. Here we dissect this fundamental mechanism. We show that natural protection from type 1 diabetes in non-obese diabetic (NOD) mice is associated with increased IL-10-producing B cells, while NOD mice that develop type 1 diabetes have compromised IL-10 production from B cells. However, B cells from diabetic mice regain IL-10 function if activated by innate immune receptor, TLR4, and can suppress insulin-specific CD8 T cells in a dendritic cell (DC) dependent IL-10-mediated fashion. Suppression of CD8 T cells was reliant on B cell contact with DCs. This cell contact results in deactivation of DCs, inducing a tolerogenic state, which in turn can regulate pathogenic CD8 T cells. Our findings emphasise the importance of DC: Breg interactions during the development of type 1 diabetes.

Regulatory B cells (Bregs) suppress immune responses and support immune tolerance. Breg cells mitigate inflammation via production of cytokines such as IL-10, TGFβ and IL-35[1-3], which modulate the response of pathogenic T cells and aid the generation of other regulatory lymphocyte populations. Bregs can suppress various autoimmune diseases, including models of experimental autoimmune encephalomyelitis (EAE) and arthritis[4-6]. However, in type 1 diabetes, a dual role for B cells is evident. B cells play a pathological role, as B cell depletion delayed and protected from diabetes[7-9]. Conversely Bregs can prevent the onset of disease in the NOD mouse model[2, 10]. B cells stimulated with lipopolysaccharide (LPS) upregulated FasL expression, increased TGFβ secretion and prevented the onset of diabetes upon adoptive transfer[2]. Furthermore, LPS-stimulated B cells induced CD8 T cell anergy in a membrane-bound TGFβ-dependent manner[11]. Activation of B cells through the B cell receptor (BCR) can also mediate protection from diabetes in NOD mice, via an IL-10-dependent mechanism[12]. In patients with type 1 diabetes, IL-10-producing B cells are diminished in peripheral blood, compared to healthy control individuals and autoantibody-positive relatives[13].

Similar to humans, NOD mice develop spontaneous type 1 diabetes. However, in most, if not all, NOD mouse colonies worldwide, approximately 20% (or more) of NOD mice remain normoglycemic and 'protected' from diabetes despite their genetic predisposition[14]. However, few studies have been done to uncover the mechanism of this natural protection. It is suggested that B cells, specifically anergic $CD40^{+}$IL-10-producing B cells found in the pancreatic islets of long term normoglycemic mice (protected)[13], may confer this natural protection. However, given the heterogeneity of regulatory B cells[15], which can be induced via stimulation with both TLR agonists or anti-CD40 we suggest that the mechanism by which B cells induce natural protection still remains unanswered.

Here we report that B cells from NOD mice, that have developed diabetes, have lost the capacity to produce IL-10, whereas B cells from naturally protected NOD animals have enhanced frequency of IL-10 producing B cells, regardless of the mode of B cell activation. However, if B cells from diabetic NOD mice receive signalling through innate immune receptor TLR4, they regain IL-10-producing function and are able to suppress insulin-specific CD8 T cells. For B cells to exert this suppression, contact with dendritic cells (DCs) is required and is mediated via IL-10. Direct B cell-DC interaction deactivates mature conventional DCs and promotes an IL-10 dependent tolerogenic DC population.

## Results

### Altered B cell function in NOD mice with type 1 diabetes

To determine the features of B cells in NOD mice that are "naturally protected" (not diabetic by 30 weeks of age and hereafter referred to as protected, as these mice rarely develop diabetes after 30 weeks), we compared B cell responses, from protected or diabetic NOD mice, to various stimuli. B cells were stimulated through adaptive immune co-stimulatory pathways (anti-CD40, $B_{CD40}$) and through innate immune pathways by microbial products (LPS - $B_{LPS}$ or CpG - $B_{CPG}$)[16-18]. Unstimulated B cells ($B_{US}$) were used as controls (Fig. 1). All stimuli up-regulated B cell activation markers, although the up-regulation of MHC I and II was not statistically significant, when compared to $B_{US}$. Key differences in surface markers, between B cells from protected and diabetic NOD mice, were that splenic B cells isolated from protected NOD mice had a lower expression of MHC I and a significantly lower frequency of CD86-expressing B cells, compared to B cells from diabetic mice (Fig. 1a). In both protected and diabetic NOD mice, the up-regulation of CD80 was evident, particularly after stimulation with anti-CD40 stimulation, compared to $B_{US}$ ($p<0.001$), whereas we observed that up-regulation of CD86 was greatest if stimulated by LPS

86 ($p<0.001$). Interestingly, the innate immune stimuli, LPS (TLR4) and CPG (TLR9) resulted
87 in higher up-regulation of PD-L1 expression on B cells ($p<0.05$ and $p<0.001$, respectively)
88 compared to $B_{US}$. We observed that after anti-CD40 stimulation ($B_{CD40}$), a classical B cell
89 stimulator of adaptive immunity, PD-L1 expression was similar to the unstimulated controls
90 (Fig. 1a). However, no difference in PD-L1 expression was found between protected and
91 diabetic NOD mice, corroborating previous literature [19].
92 Next, we determined cytokine-production from B cells, activated by the different types of
93 stimuli. Firstly, intracytoplasmic staining revealed significant induction of IL-10 in splenic B
94 cells from both protected and diabetic NOD mice following activation with the innate
95 immune stimuli, LPS and CPG ($p<0.05$), but not with anti-CD40, the adaptive immune
96 stimulator, compared to $B_{US}$ (Fig. 1b, c). However, a much higher proportion of B cells from
97 protected NOD mice expressed intracellular IL-10, in comparison to the diabetic NOD mice,
98 even without stimulation ($B_{US}$) *ex vivo* (Fig. 1c, top graph). In particular, there were
99 considerably lower proportions of IL-10-expressing B cells from diabetic NOD mice when
100 stimulated with anti-CD40 (Fig.1c; top graph). B cell secretion of IL-10 was only observed
101 with LPS and CPG stimulation, but not anti-CD40 (Fig. 1c; bottom graph), corroborating
102 previous literature[16]. Surprisingly, B cells from diabetic NOD mice secreted significantly
103 more IL-10, although only when stimulated with CPG, compared to B cells from protected
104 NOD animals (Fig. 1c, bottom graph; $p<0.01$). IL-10 secretion from LPS-stimulated B cells
105 was modestly increased in diabetic NOD mice, compared to equivalently stimulated
106 protected NOD mice, although this difference was not statistically significant (ns). To
107 address whether the greater proportion of IL-10-producing B cells in protected mice was age
108 related, but to circumvent the technical challenge that there are no diabetic NOD mice over
109 30 weeks of age, we investigated $B6^{g7}$ congenic mice, which have the same MHC haplotype

but are not prone to diabetes (Sup. Fig. 1). $B6^{g7}$ mice, aged >35-week-old, have little IL-10 production, indicating that potential for IL-10 production was not likely to be age associated. Only CPG stimulation promoted IL-6-producing B cells from both protected and diabetic mice (Fig. 1d), and no statistical difference between protected and diabetic NOD mice was observed in either intracytoplasmic or secreted IL-6 (Fig. 1d), indicating that diabetes protection was not related to a lack of pro-inflammatory cytokines in B cells.

These data demonstrated that B cells from diabetic NOD mice are more activated compared to mice protected from diabetes, particularly in CD86 expression (Fig. 1a, 4rd row). Furthermore, B cells from diabetic mice show a loss of overall intracellular IL-10 production, but can regain this function if stimulated via TLR4 or TLR9, highlighted by IL-10 secretion. Strikingly, when B cells are stimulated with anti-CD40, only protected mice have the potential to produce cytoplasmic IL-10.

**LPS-stimulated B cells suppress antigen-specific CD8 T cells in the presence of DCs**

We next investigated whether B cells from diabetic and protected NOD mice could regulate antigen-specific CD8 T cells via both innate and adaptive signalling. B cells were co-cultured with insulin-specific CD8 T cells from TCR transgenic G9C$\alpha^{-/-}$ mice[20] in the presence of BM-DCs from NOD.PI2tg mice, which express proinsulin (driven by the MHC class II promoter) in antigen-presenting cells (experimental scheme shown in Fig. 2a). This culture allowed intrinsic antigen-specific presentation, by proinsulin-expressing DCs, to insulin-specific CD8 T cells, without exogenous antigen. Considering that NOD.PI2tg DCs express proinsulin, they should be targeted by CD8 T cells from G9C$\alpha^{-/-}$ mice. Indeed, DC viability was significantly diminished in the control CD8 and DC culture (without B cells) (Fig. 2b). The addition of B cells prevented DCs from being killed by CD8 T cells (Fig. 2b) and importantly, stimulated B cells ($B_{LPS}$, $B_{CD40}$, $B_{CPG}$) promoted more DC survival compared to unstimulated B cells ($B_{US}$) (Fig. 2b). Next, we examined the proliferative function of CD8 T

cells in response to the intrinsic antigen presented by DCs in a CFSE dilution assay in the presence of B cells, unstimulated or stimulated, from protected and diabetic animals (Fig. 2c, d, e). CD8 T cell proliferation was significantly suppressed in the presence of LPS-stimulated B cells ($B_{LPS}$), compared to unstimulated B cell ($B_{US}$) cultures ($p<0.05$; Fig. 2c, d, e). Interestingly, no suppression by B cells, from either protected or diabetic NOD mice, was observed when activated by other stimuli (Fig. 2c, d, e). Control CD8 T cell proliferation (CD8+DC alone) is shown in supplementary figure 2a. Similarly, we observed less CD44 expression (Fig. 2f, g) and less MIP1β, the early chemokine produced by activated CD8 T cells, in the culture supernatant in the presence of $B_{LPS}$ (Fig. 2h). To confirm that CD8 T cell proliferation was impaired in $B_{LPS}$ cultures, and not due to increased CD8 T cell death, we assessed CD8 T cell viability and determined that there was no statistical difference between cultures (Sup. Fig. 2b).

To verify that the suppression by $B_{LPS}$ was TLR4 dependent, we used $B_{LPS}$ from $TLR4^{-/-}$ mice in the culture system and we observed no suppression of CD8 T cell proliferation or activation (Fig. 2i), confirming that LPS-mediated suppression was TLR4 dependent. To probe the direct effect of LPS-stimulated B cells on CD8 T cells, we stimulated $G9C\alpha^{-/-}$ CD8 T cells with plate-bound anti-CD3/28 and assessed CD8 T cell proliferation in the presence of B cells with or without stimulation. In contrast to the DC-stimulated T cells (Fig.2d), we did not observe suppression of the anti-CD3/28 stimulated CD8 T cells by LPS-stimulated B cells ($B_{LPS}$) (Fig. 2j). Taken together, our results suggest that the regulation of LPS-activated B cells on antigen-specific CD8 T cells requires the presence of DCs and importantly B cells from diabetic mice also have the ability to impair CD8 T cell responses, if stimulated via TLR4.

**IL-10 mediates CD8 T cell suppression**

To investigate the IL-10 contribution to CD8 T cell suppression by LPS-stimulated B cells, we measured the secreted IL-10 in culture supernatants of the experiments in Fig. 2. $B_{LPS,}$ from both protected and diabetic mice, had significantly increased IL-10 secretion compared to $B_{US}$ (Fig. 3a). Interestingly, when LPS-stimulated B cells from aged $B6^{g7}$ transgenic mice (>35 weeks old) were used in our *in vitro* culture system we neither observed significant CD8 T cell suppression (Sup. Fig. 3a), nor did we find significant IL-10 production (Sup. Fig. 3b), demonstrating that IL-10 mediates suppression. Furthermore, the addition of LPS-stimulated NOD B cells had no obvious effect on the secretion of IL-6 (Sup. Fig. 3c), suggesting that lower levels of IL-6 from protected NOD mice were not responsible for suppression of CD8 T cell proliferation.

To identify the contribution of IL-10 from DCs in our culture system, we analysed secreted IL-10 from the cultures performed in the presence or absence of DCs (Fig. 3b). We found a significant increase in the amount of IL-10 secreted when B cells were added to DC+CD8 T cell cultures, regardless of whether the added B cells were unstimulated or stimulated, from both protected and diabetic mice. However, the greatest amount of secreted IL-10 was found in $B_{LPS}$ cultures. Cultures of CD8-DC alone were found to have minimal secreted IL-10 (average 124±25.4pg/ml), suggesting that upon the addition of B cells, either DCs or B cells, or both, contributed to the augmented IL-10 produced. To further address this finding, B cells ($B_{US}$, $B_{LPS}$ and $B_{CD40}$) from protected, diabetic or IL-10KO NOD mice were cultured with either BM-DCs from NOD.PI2tg or BM-DCs from IL-10KO NOD mice (Fig. 3c). IL-10 production in B cell+DC cultures was dependent on B cells capable of producing IL-10, as only a small amount IL-10 was observed when IL-10KO B cells were cultured with NOD.PI2tg BM-DCs (Fig.3c). Analysis of the reduced amount of IL-10 produced in IL-10KO cultures revealed no statistically significant difference between NOD.PI2tg BM-DCs

cultured without B cells, and IL-10KO B cell cultures (Sup. Fig 4a). Furthermore, equivalent amounts of IL-10 were produced when B cells from protected and diabetic NOD mice were cultured with IL-10KO BM-DCs (Fig. 3c), indicating that the IL-10 augmentation observed upon the addition of B cells, is independent of IL-10 from BM-DCs.

As the secreted IL-10 contributed to the suppression of CD8 T cell proliferation, we evaluated the effect of recombinant IL-10. Surprisingly, increasing concentrations of recombinant IL-10 alone had little effect on G9C$\alpha^{-/-}$ CD8 T cell proliferation when cultured with NOD.PI2tg DCs (Sup. Fig. 4b). Moreover, blocking the IL-10 receptor (anti-IL-10R) also had little effect on both CD8 T cell proliferation (Sup. Fig.4c, left) and CD44 expression on CD8 T cells (Sup. Fig. 4c, right). Yet, when $B_{LPS}$ cells were present in the CD8-DC culture system, adding anti-IL-10R not only reduced suppression of CD8 T cell proliferation (Fig. 3d), compared to $B_{US}$, but also promoted a significant increase of CD44 surface expression on CD8 T cells (Fig. 3e), compared to control. This was confirmed using B cells from IL-10KO mice (Fig. 3d, e). In keeping with published reports[21, 22], IL-10 reduced the expression of co-stimulatory molecules on DCs to exert immunosuppression (Sup. Fig. 4d). We also found that the significant reduction of CD80 on mature DCs in $B_{LPS}$ cultures, was reversed by the addition of anti-IL-10R or using B cells from IL-10KO mice (Fig. 3f). However, CD86 expression on DCs was less affected by the addition of anti-IL-10R or the use of IL-10KO B cells (Sup. Fig. 4e). Our results, thus far, indicated that IL-10 produced by LPS stimulated B cells down-regulated CD80 expression on DCs and suppressed antigen-specific CD8 T cells.

**Insulin-specific CD8 T cell suppression is DC-B cell contact dependent**

To determine if cell contact is also required for $B_{LPS}$ -mediated CD8 T cell suppression, we used a transwell culture system. CD8-DC cultures were either in cell contact (cont) or separated (trans) from B cells, which were unstimulated, or stimulated with LPS ($B_{LPS}$) or anti-CD40 ($B_{CD40}$) (Fig. 4a). Our results revealed that G9C$\alpha^{-/-}$ CD8 T cell proliferation and the expression of $CD44^{+}$ on the CD8 T cells were significantly suppressed when $B_{LPS}$ from both protected and diabetic NOD mice were in contact with CD8:DCs. This suppression was significantly reduced when the B cells were separated from CD8:DC cultures (Trans) (Fig. 4a, b).

**B cell: DC cell contact synergises cytokine production**

Since we observed that CD8 T cell suppression was mediated via both IL-10 and contact dependent mechanisms, we sought to determine whether cytokine production was B cell: DC contact dependent. We measured cytokine secretion when B cells were either in contact with mature NOD.PI2tg BM-DCs, or cultured in transwells, in the presence (Fig. 4c, e) or absence of T cells (Fig. 4d, f). Firstly, we found that IFNγ production was unchanged by the addition of B cells from either protected and diabetic NOD mice, when not in direct contact (Trans) with CD8 T cells and DCs, compared to contact cultures (Cont) (Fig. 4c). However, in the absence of CD8 T cells, B cells from diabetic NOD mice, in direct contact with DCs, induced more IFNγ compared to B cells from protected NOD mice ($p<0.01$), regardless of the type of stimulus (Fig.4d). Furthermore, IFNγ production was significantly increased when B cells were stimulated by anti-CD40 ($B_{CD40}$) (protected; $p<0.05$, diabetic; $p<0.001$), which was also contact dependent (Fig. 4d).

Secondly, we found increased IL-10 in both $B_{LPS}$ protected and diabetic cultures, in the presence or absence of CD8 T cells, whereas we observed considerably less IL-10 in $B_{LPS}$

transwell cultures (Fig. 4e, f). Interestingly, we observed IL-10 production in $B_{LPS}$ cultures was greater in diabetic NOD mice, compared to protected NOD mice, in the presence of CD8 T cells (Fig. 4e). In contrast, in the absence of CD8 T cells, $B_{LPS}$ cultures in protected NOD mice had the greatest levels of IL-10 (Fig. 4f). Of note, we observed no differences in IL-12p70 or IL-6 production comparing B cell contact or transwell cultures, indicating they were not affected by direct B cell contact (data not shown). Taken together, our data suggest that CD8 T cell suppression is mediated via IL-10 secretion, which is dependent on direct contact between LPS-stimulated B cells and mature BM-DCs.

**$B_{LPS}$ condition mature BM-DCs**

We next investigated if B cells induced tolerance in mature BM-DC. Firstly, we co-cultured B cells with BM-DCs from NOD.PI2tg mice for 3 days. The B cells were then removed, and the conditioned BM-DCs were washed and co-cultured with G9C$\alpha^{-/-}$ CD8 T cells for 3 days (scheme shown in Fig. 5a), before being assessed for proliferation (Fig. 5b) and activation of CD8 T cells (Fig. 5c), as well as cytokine production from DC:CD8 T cell cultures (Fig. 5d). Overall, CD8 T cell proliferation (CFSE dilution) and activation (assessed by CD44 surface expression) was reduced when mature NOD.PI2tg BM-DCs had been exposed to B cells, regardless of stimulation; however only $B_{LPS,}$ from both protected and diabetic NOD mice, significantly suppressed insulin-specific CD8 T cells, compared to control (no B cell exposure).(Fig. 5b). Following pre-exposure to B cells, stimulated with LPS or anti-CD40, the BM-DCs from DC:CD8 cultures secreted IL-10 (Fig. 5d, left). However, only LPS-stimulated B cells reduced pro-inflammatory cytokine secretion, IL-12p70 (Fig. 5d, middle) and IL-6 (Fig. 5d, right), by mature BM-DCs.

**B cells modulate activation of mature conventional DCs**

We have shown, thus far, that LPS B cells suppress CD8 T cell function, mediated via IL-10, which is dependent on DC-B cell contact. Considering the importance of B cell: DC contact, we determined how B cells affect the activation of mature NOD.PI2tg BM-DCs, in the absence of T cells. We found that all B cells significantly increased survival of DCs in co-cultures (Sup. Fig. 5a); however, B cells from protected NOD mice were more effective at enhancing DC survival, compared to B cells from diabetic NOD mice (Sup. Fig. 5a). In protected NOD mice, B cell contact was required for complete DC deactivation, as we found that MHC I, CD80, CD86 and CD69 were all significantly downregulated on matured BM-DCs when cultured in contact (Cont) with LPS-stimulated B cells, compared with unstimulated B cells (Fig. 6a, b). B cells from protected NOD mice, when not in direct contact with DCs (Trans), were less effective in deactivating BM-DCs, with reduced down-regulation of CD86 and CD69 (Fig. 6a, b). Our data suggest that B cells from protected mice suppress MHC I and CD80 expression on BM-DCs via soluble factors, whereas the suppression of CD86 and CD69 is mediated via contact mechanisms.

When investigating B cells from diabetic NOD mice, $B_{LPS}$ also downregulated all measured activation markers on DCs if in direct contact (Cont) (Fig. 6a, b). In contrast, when the same B cells were separated (Trans) from DCs, we observed minimal effects on mature BM-DCs. Interestingly, supernatants taken from B cells, which had been stimulated in culture with the various stimuli (Sup), had no effect on activation of BM-DCs, compared to the DC controls (dotted line, Fig. 6a, b). However, in $B_{LPS}$ transwell cultures from protected mice, MHC I, CD80 and CD69 on BM-DCs were still significantly reduced compared to $B_{LPS}$ supernatants but this effect was not observed in the diabetic B cell transwell cultures (Fig. 6a, b). This suggests that B cells from protected mice can respond to BM-DCs, in turn deactivating BM-DCs. Conversely, in B cells from diabetic mice this function is absent. Therefore, B cells

from diabetic mice are less effective in mediating deactivation of mature BM-DCs, but can regain some of this function if stimulated via TLR4 and have direct contact with BM-DCs. Of note, B cells from protected and diabetic NOD mice, stimulated via TLR4 or CD40 interaction also significantly downregulated MHC II (Sup. Fig. 5b, c), independent of cell contact. Collectively, these results reveal that 'deactivation' of mature conventional BM-DCs by B cells requires both direct contact and soluble mediators; with MHC I, II and CD80 more affected by soluble factors (most likely, cytokines) whereas CD86 and CD69 are more dependent on direct cell contact. Furthermore, B cells that received signalling via TLR4 are more efficient in BM-DC 'deactivation' and B cells from protected NOD mice are most effective in this process.

**$B_{LPS}$ induce CD45RB$^{+}$CD11c$^{low}$ tolerogenic BM-DCs**

To determine if LPS stimulated B cells not only deactivated mature BM-DCs but induced a tolerogenic DC population, we cultured B cells and NOD.PI2tg BM-DCs and evaluated different tolerogenic surface markers (Fig. 7). We demonstrated that $B_{LPS}$ cells, from both protected and diabetic NOD mice, significantly induced a subset of CD45RB$^{+}$ DCs compared with DCs either cultured alone or with $B_{US}$ (Fig. 7a). However, $B_{LPS}$ from protected NOD mice induced significantly more CD45RB$^{+}$ DCs compared to $B_{LPS}$ from diabetic NOD mice ($p<0.05$). Interestingly, B cells from diabetic mice stimulated with anti-CD40 ($B_{CD40}$), also induced a CD45RB$^{+}$ population. Furthermore, the induction of CD45RB$^{+}$ DCs was dependent on B cell production of IL-10, as neither $B_{LPS}$ and $B_{CD40}$ from IL-10KO mice induced CD45RB on BM-DCs (Fig. 7a). Similarly, the addition of B cells from B6$^{G7}$ mice, which have fewer IL-10 producing B cells, had no effect on the induction of CD45RB on BM-DCs (data not shown).

This population of CD45RB$^{+}$ BM-DCs had a lower expression of CD11c compared to the CD45RB$^{-}$ counterpart (Fig. 7b, c), a phenotype associated with regulatory DCs[23, 24].

300 Furthermore, significantly fewer CD45RB$^{+}$ BM-DCs had CD80 or CD86 expression (Fig. 7b,
301 c), compared to CD45RB$^{-}$ BM-DCs. No difference in CD45RB$^{+}$ phenotype was observed
302 between protected and diabetic cultures, therefore overall phenotype is shown (Fig. 7b, c).
303 We also investigated the expression of PD-L1 or ILT3 on BM-DCs with or without B cell
304 contact and did not find obvious differences (data not shown), indicating that tolerogenic
305 DCs induced by B cells in this study do not function through the inhibitory markers PD-L1 or
306 ILT3 [25, 26].

## Discussion

We have several novel findings in this study. Firstly, we report that B cells from NOD mice, that are naturally protected from diabetes, have increased IL-10-expressing B cells, while B cells from non-protected diabetic NOD mice show an altered IL-10 profile (Fig. 8 parts 1, 2). Secondly, we find that TLR4-activated B cells amplify their IL-10 production in response to contact with mature BM-DC, in turn inducing a DC tolerogenic state, which can suppress pathogenic CD8 T cells (Fig. 8, part 3). Thirdly, B cells from diabetic NOD mice have a diminished response to BM-DCs (Fig 8, part 4); however, they regain IL-10 production and the function of suppressing insulin-specific CD8 T cells, when in the presence of proinflammatory cytokines and stimulated via TLR4 (Fig. 8, part 5). Lastly, we show that B cells deactivate mature BM-DCs, via both soluble mediators and cell contact mechanisms. IL-10-producing B cells (B10) restrain inflammatory responses and it has been widely documented that B10 cells can negatively regulate autoimmune disease. Furthermore, it is implied that IL-10-producing B cells are lost in patients with type 1 diabetes, compared to healthy control individuals[13]. Notably, B10 cells can be induced through both adaptive (anti-CD40) and innate TLR signalling[16], regulated via different mechanisms[2, 27, 28] under various inflammatory conditions. Our results reveal a loss in cytoplasmic IL-10 in non-protected diabetic NOD mice, specifically when stimulated via CD40. In line with this, patients with systemic lupus erythematosus (SLE) have impaired $CD19^{+}CD24^{hi}CD38^{hi}$ Breg populations that are refractory to CD40 stimulation compared to healthy controls[29]. This was associated with a lack of STAT-3 phosphorylation after CD40 engagement and not altered expression of CD40[29]. There was no difference in CD40 expression on splenic B cells from either protected or diabetic mice in our study (data not shown) as well as in the studies of others[13]. The lack of IL-10 response with anti-CD40 stimulation could be due to other factors. These other factors include retention of IL-10 by B cells from protected mice,

or B cells that respond to CD40 stimulation have trafficked out of the spleen, or the B cells have been destroyed *in vitro* after stimulation (however no difference was observed in B cell viability) or *in vivo* by other cell types such as $FasL^{+}CD5^{+}$ B cells[30]. Altogether, our current results add to the concept that there is a strong association between CD40 stimulation and the immune regulation of autoimmune disease[4, 31].

Importantly, our study shows, for the first time, that the impaired IL-10 response by B cells in diabetic NOD mice can be restored when activated via TLR4 or TLR9 signalling. Under these circumstances, secreted IL-10 from B cells in diabetic NOD mice was increased compared to B cells from protected mice stimulated with CPG, and to a lesser extent LPS. Considering that we find B cells from diabetic mice are more activated, it is possible these cells are primed to secrete cytokines more rapidly. Although others have reported that LPS-stimulated B cells from very young NOD mice exert regulatory effects in type 1 diabetes via secreted TGFβ[2]; however, we did not detect any TGFβ secretion in our assays (data not shown). This disparity may be due to the differences in the age of mice studied.

We demonstrate, in this study, that NOD B cells can deactivate mature BM-DCs, via both soluble mediators and cell-contact mechanisms. TLR4-stimulated B cells, which produce more IL-10, exhibit a stronger capacity to deactivate BM-DCs; specifically, the expression of MHC I, MHC II and CD80 on BM-DCs is clearly reduced. Furthermore, the expression of CD86 and CD69 are down-regulated by direct B cell: DC contact. Importantly, B cells from diabetic NOD mice, stimulated via anti-CD40, are not as effective in deactivation of DCs as B cells from diabetes-protected mice. This could be, in part, due to the increased secretion of IFNγ upon B cell-DC contact in diabetic NOD mice. In addition, we found that BM-DCs exposed to LPS-stimulated B cells produced less IL-12p70 and IL-6, but increased IL-10, in line with B cell–DC interactions noted previously[32]. B cells, activated to produce IL-10 by *Leishmania major* infection, induced suppression of IL-12 production by DCs[33]. Similarly,

CpG-activated neonatal B cells were able to suppress IL-12 production by neonatal dendritic cells[34]. Direct B cell-DC interaction has been shown using B cell deficient ($\mu MT^{-/-}$) mice, which produce higher levels of IL-12p70 from DCs compared to wild-type animals[35]. Furthermore, it is known that DCs cultured with IL-10 can shift from a Th1 pathway by reducing IL-12 secretion[21] and IL-10 can also affect DC antigen presentation[36]. It is conceivable that the reduction of MHC II expression on BM-DC by IL-10 producing B cells in our study could impact antigen presentation from DCs to CD4 T cells, leading to suboptimal CD4 T cell activation.

It is clear that TLR4-activated NOD B cells operate directly on BM-DCs to inhibit CD8 T cell activation. We find that B cell-DC contact also amplifies B cell secretion of IL-10, which is exaggerated in the presence of IFNγ-producing CD8 T cells. Our finding is consonant with a previous study suggesting that inflammatory cytokines can increase IL-10 production from Breg cells[37]. However, we also find IL-10 alone is not sufficient to inhibit BM-DC induced CD8 T cell proliferation, suggesting a contact-dependent change in BM-DCs upon initial engagement with B cells. Furthermore, if this initial contact-dependent change is reciprocal, or if $CD45RB^{hi}CD11c^{low}$ DCs have any reverse effect on B cells is not yet understood.

In this study, we also demonstrate an IL-10 dependent induction of $CD45RB^{+}CD11c^{lo}$ BM-DCs, a distinct subset of tolerogenic $CD45RB^{hi}CD11c^{low}$ DCs[38], which were induced with LPS-stimulated B cells more efficiently from protected NOD mice. A previous study suggests that a similar tolerogenic DC population produces IL-27 and promotes T cell tolerance mediated via IL-10[24]. Interestingly, this population can be induced with galectin-1[24] which has recently been described to be required for regulatory B cell function[39]. Whether this mechanism is involved in the induction of $CD45RB^{+}CD11c^{lo}$ tolerogenic DC population by B cells in our study is for future investigation.

Our results are in line with the findings in human B cell-DC interactions, where human B cells influence the differentiation of DCs[40-42]. B cells activated by CD40 and TLR9 can also restrict monocytes from developing into mature DCs and reduce the expression of activation molecules and cytokine production by DCs[40]. Similarly, B cells activated via BCR signalling can induce DC maturation, which then drives differentiation of CD4 T cells to Th2 cells[42]. Again, this maturation is dependent on B cell–DC cell contact and reliant on B cell factors such as BAFFR (B cell activating factor receptor), TACI (transmembrane and calcium-modulating cyclophilin ligand interactor) and CD69[42]. It is clear that there is important cross-talk between B cells and DCs, and this is dependent on which signals B cells receive[41]. Our results suggest that the cross-talk between B cells and DCs is mutually modulated and both cell contact dependent and independent.

In summary, we have found that B cells play a novel role in the natural protection of diabetes in NOD mice. B cells from protected NOD mice are high IL-10 producers, and suppress the activation status of BM-DCs, which in turn control pathogenic CD8 T cells. In contrast, the B cells from the non-protected diabetic NOD mice have reduced IL-10 expression, especially when activated via CD40, and weak suppressive function. Interestingly and importantly, if B cells from the non-protected diabetic mice are stimulated through innate immune signalling pathways, in particular TLR4 (LPS), these B cells have the capacity to produce IL-10 and immune suppressive function is restored. This alteration of suppressive B cell function under innate immune activation or inflammatory conditions may contribute to the dysregulation or abnormalities in DC populations found in individuals with type 1 diabetes[43]. Our study, thus, may point to a possible therapeutic target for future investigation.

403 **Methods**

404 **Mice.**

405 NOD/Caj mice, originally from Yale University, were bred in-house at Cardiff University.
406 The G9Cα$^{-/-}$NOD mice were bred in-house at Cardiff University as previously described[20].
407 NOD TLR4$^{-/-}$ mice were bred in-house at Yale University. NOD.PI2$^{tg}$ mice, with transgenic
408 overexpression of PI2 on the MHC class II promotor, were kindly provided by Prof. L
409 Harrison and Dr. A. Lew. B6$^{g7}$ were bred in-house at Cardiff University. NOD.129P2(Cg)-
410 *Il10tm1Cgn*/DvsJ (IL-10KO) were bred in house at Yale University. Mice were maintained at
411 Cardiff or Yale Universities in specific pathogen-free isolators or scantainers. All animals
412 received water and food *ad libitum,* and were housed in a 12h dark/light cycle. The animal
413 experiments were conducted in accordance with United Kingdom Animals (Scientific
414 Procedures) Act, 1986 and associated guidelines.

415 **Diabetes Incidence.** Mice were monitored weekly for glycosuria (Bayer Diastix) from 12
416 weeks of age and when blood glucose levels were greater than 13.9mmol/L were diagnosed
417 as diabetic. NOD mice that were 35 weeks of age or older and had never tested positive for
418 glycosuria, and had blood glucose less than 13.9mmol/L were considered to be protected
419 from diabetes, as the incidence of diabetes after this age is very low.

420 **Reagents.**

421 *Invivo*Mab anti-mouse IL-10R (CD210) and *Invivo*Mab rat IgG1 isotype control, anti-CD3
422 (clone 2C11) and anti-CD28 (clone 37.51) were all purchased from Bioexcell. Recombinant
423 IL-10 was purchased from Miltenyi Biotec.

424 **Cell preparation**

425 Bone marrow (BM-DCs) cells were flushed out from the hind legs (femur and tibia) and
426 cultured with granulocyte macrophage colony stimulating factor (GM-CSF) at 1.5ng/ml and
427 stimulated overnight with LPS (Sigma) at 1μg/ml before co-culture set up. Whole

splenocytes or freshly isolated splenic B cells, selected using B cell isolation kit (Miltenyi), were either seeded at $1x10^6$/ml in a 24-well plate (for phenotyping) or $5x10^6$ cells/ml in a 6-well plate (for co-cultures), and left either unstimulated or stimulated with 5μg/ml lipopolysaccharide (LPS), 5μg/ml anti-CD40 (Bioexcell) or 0.5μg/ml CPG (Eurofins MWG) for 24hrs before harvesting for further assays. CD8 T cells were negatively selected with CD8 T cell isolation kit (Miltenyi). Purity for all cell sorting was >95%.

**B cell: DC co-cultures**

NOD.PI2$^{tg}$ BM-DCs that were stimulated with LPS, were cultured either alone or with B cells, unstimulated or stimulated with various stimuli at a ratio of 1:3 (DC: B cell), and cultured in a 24-well plate for 3 days. For transwell experiments, B cells and DCs were separated by a 0.4μM membrane (Corning), with DCs placed in the bottom chamber, and unstimulated/stimulated NOD B cells placed in the top chamber. For DCs cultured with various NOD B cell supernatants, supernatants were centrifuged to ensure cell removal before the addition to $4x10^5$ DCs (1ml per well). After 3 days, assays were analyzed by flow cytometry.

**B cell: DC: CD8 co-cultures**

NOD.PI2$^{tg}$ BM-DCs stimulated with LPS were cultured with freshly isolated G9C$\alpha^{-/-}$ CD8 T cells, with or without unstimulated or stimulated NOD or TLR4$^{-/-}$ B cells, at a ratio of 1:2:7 (CD8: DC: B cell) for 3 days. G9C$\alpha^{-/-}$ CD8 T cells were labelled with 0.5μmol/L CFDA-SE (CFSE) (Invitrogen), before culture set up. For transwell experiments (Corning), 0.4μM membranes separated B cells from BM-DCs and CD8 T cells with unstimulated/stimulated B cells placed in the top chamber and BM-DCs and CD8 T cell mixed culture in the bottom. For anti-IL-10R blocking experiments 10μg/ml of anti-IL-10R or isotype was added for the 3-day culture. $4x10^5$ NOD.PI2$^{tg}$ BM-DCs were also cultured with recombinant IL-10 (Miltenyi Biotech) alone at increasing concentrations. Plate-bound anti-CD3/CD28 (0.1μg/ml

anti-CD3, 1ug/ml anti-CD28) was coated on a 48 well plate overnight at 4°C, washed with PBS, before plating G9C$\alpha^{-/-}$ CD8 T cells labelled with CFDA-SE and cultured with or without NOD B cells from different stimulations. After 3 days, the assays were analysed by flow cytometry.

**Flow cytometry**

Single cell suspensions were incubated with TruStain (anti-mouse CD16/32 [Biolegend]) for 10min at 4°C, followed by fluorochrome-conjugated mAbs against cell surface markers for 30min at 4°C. Multi-parameter flow cytometry was carried out using mAbs: CD8 PE594 (53-6.7), CD19 AF700 (6D5) CD11b APC or BV421 (M1/70), CD11c Pe-Cy7 (N418), MHC I PE (SF1-1.1), MHC II FITC (10-3-6), CD80 BV650 (16-10A1), CD86 Pe-Cy7 or AF700 (PO3), CD44 BV711 (IM7), PD-L1 APC (10F-962), OX-40L PeCy7 (RM134), CD69 BV510 (H1-2F3), CD45RB PerCPCy5.5 (C363-16.7) all from Biolegend. APC-Cy7 (1D3) and CD11c PerCPCy5.5 (N418) were purchased from eBioscience and BAFFR BV786 (7H22-E16) was purchased from BD Biosciences. Cells were also stained with live/dead exclusion 605 (Invitrogen) or 780 (eBioscience). For intracellular cytokine analysis, splenocytes were either unstimulated or stimulated for 24hrs. Three hrs before antibody staining, PMA (50ng/ml) and ionomycin (500ng/ml) and monensin (3µg/ml) (all from Sigma-Aldrich) were added to the cells. Fc receptors were blocked using TruStain and after extracellular staining, cells were fixed using fixation/permeabilisation kit according to the manufacturer's instructions (BD Biosciences), and subsequently stained for intracellular cytokines or with appropriate isotype controls. Cells were acquired on LSRFortessa (FACS Diva software) and analysis was performed using Flowjo software (Treestar).

**Cytokine assays**

Supernatants were taken from cell culture assays at the 3-day endpoint to analyze IL-10, IL-12p70, MIP1β, IFNγ and IL-6. Cytokines IL-10, IL-12p70, IFNγ and IL-6 were measured by

the Meso Scale Discovery (MSD) system and MIP1β measured by ELISA (R&D systems). MSD was performed according to the manufacturer's (Meso Scale Diagnostics, LLC.) instructions and detected using MSD Sector Imager 6000. MIP1β ELISA was run according to the manufacturer's instructions (R&D systems).

**Statistical analysis.**

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Significance was determined by One-way ANOVA followed by a Dunn's multiple comparison or a Two-way ANOVA followed by a Bonferroni post-test for more than two variables, and a Mann-Whitney U test was performed for only two variables. Data were considered significant at $p<0.05$.

## Data Availability

The datasets generated or analysed during the current study are available on reasonable request.


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## Author contributions

J. Boldison, L. Wen and F.S. Wong designed the experiments and wrote the manuscript. J. Boldison performed the experiments and analyzed the data. L. Carmargo da Rosa and J. Davies contributed to experimental procedures. All authors reviewed the manuscript. FSW conceived the project and is the guarantor of this work.

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**Figure 1.** *Phenotypic analysis of B cells.* Splenic B cells from protected (>35weeks old) and diabetic NOD mice were left unstimulated ($B_{US}$; white circle) or stimulated with LPS ($B_{LPS}$; black square), anti-CD40 ($B_{aCD40}$; dark grey triangle) or CPG ($B_{CPG}$; light grey diamond) for 24hrs before analysis for surface markers and cytokine production. (a) Isolated B cells were analysed for various surface markers. Representative flow cytometric plots (left) and graphical summary (right). (b) Representative flow cytometric plots for intracytoplasmic IL-6 and IL-10 staining in total splenic B cells. Representative flow cytometric plots (top), graphical summary (bottom) (c) Graphical summary of intracytoplasmic (top) and secreted IL-10 (bottom) (d) Graphical summary of intracytoplasmic (top) and secreted IL-6 (bottom). Cytokines were measured by MSD. Black line represents the median value. B cells were gated on live $CD19^+$ cells. Data represent at least 4 independent experiments. ns; non-significant, +P<0.05, ++P<0.01, +++P<0.001, versus $B_{US}$ (two-way ANOVA). $^*$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001, protected versus diabetic (two-way ANOVA).

**Figure 2.** *LPS-stimulated B cells suppress insulin-specific CD8 T cells.* NOD.PI2$^{tg}$ BM-DCs, CFSE-labelled G9C$\alpha^{-/-}$ CD8 T cells and splenic B cells that were unstimulated ($B_{US;}$ white bar) or stimulated with one of LPS ($B_{LPS;}$ black bar), anti-CD40 ($B_{aCD40}$; dark grey bar) or CPG ($B_{CPG}$; light grey bar), from protected or diabetic NOD mice, were co-cultured for 3 days before being examined by flow cytometry. CD8 T cells were gated on $CD11c^-CD11b^-CD19^-CD8^+$ cells. (a) Co-culture set up and experimental design. (b) Live DC percentages ($CD11c^+CD11b^+$), **P<0.01, ***P<0.001, versus control (DC+CD8 alone, patterned bar) (one-way ANOVA) (c, d, e) CD8 T cell proliferation from B cell cultures; (c) CFSE representative flow plots and (d) graphical representation and (e) proliferation index. (f, g) CD44 surface staining on CD8 T cells (f) Representative flow plots and (g) graphical summary (h) ELISA of MIP1β cytokine levels in supernatants of cultures. (i) NOD.PI2$^{tg}$ BM-DCs, G9C$\alpha^{-/-}$ CD8 T cells co-cultured with splenic B cells from TLR4KO mice, showing

(left) CD8 T cell proliferation by CFSE dilution, and (right) CD44 surface staining on CD8 T cells. In d-h, data were normalised to control (DC+CD8 alone). (j) G9C$\alpha^{-/-}$ CD8 T cell proliferation from plate-bound anti-CD3/anti-CD28 (0.1μg/ml, 1μg/ml respectively), cultured with unstimulated or stimulated B cells from protected or diabetic NOD mice. Data shown are mean ± SEM and represent at least 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001 versus $B_{US}$ (two-way ANOVA).

**Figure 3.** *IL-10 partially mediates insulin-specific CD8 T cell suppression.* (a, b) NOD.PI2$^{tg}$ BM-DCs, G9C$\alpha^{-/-}$ CD8 T cells and unstimulated ($B_{US}$) or B cells stimulated with LPS ($B_{LPS}$) or anti-CD40 ($B_{aCD40}$) from protected or diabetic NOD mice, were co-cultured for 3 days. (a) cytokine levels in B cell cultures; IL-10; dotted line represents baseline from CD8+DC control culture (124±25.4pg/ml); **P<0.01, ***P<0.001 (two-way ANOVA) (b) IL-10 cytokine levels from protected or diabetic NOD B cell cultures, either plated alone or with NOD.PI2$^{tg}$ DCs and G9C$\alpha^{-/-}$ CD8 T cells (B+DC+CD8); *P<0.05, **P<0.01, ***P<0.001 (Mann-Whitney U test); horizontal line represents the median value. (c) Unstimulated ($B_{US}$) or B cells stimulated with LPS ($B_{LPS}$) or anti-CD40 ($B_{aCD40}$) from protected, diabetic or IL-10KO NOD mice, were co-cultured with BM-DCs from either NOD.PI2$^{tg}$ or IL-10KO mice for 3 days before IL-10 was measured. Dotted line (NOD.PI2tg) and dashed line (IL-10KO) represents baseline from DC alone cultures (347±34.6pg/ml; 218.2±69.2 respectively). (d, e, f) Unstimulated ($B_{US}$) or stimulated B cells either with LPS ($B_{LPS}$) or anti-CD40 ($B_{aCD40}$) from protected and diabetic NOD mice, treated with either isotype control (control), or anti-IL-10 receptor (anti-IL-10R) or IL-10KO mice were cultured with NOD.PI2$^{tg}$ BM-DCs, G9C$\alpha^{-/-}$ CD8 T cells and investigated for (c) CD8 T cell proliferation (d) CD44 expression on CD8 T cells (e) CD80 expression on NOD.PI2$^{tg}$ DCs. Data were normalised to control (DC+CD8 alone, dotted line) *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA). Data shown are mean ± SEM. Data shown represent at least 3 independent experiments.

**Figure 4.** *B cell: DC cell contact suppresses CD8 T cell proliferation and activation.* Activated NOD.PI2$^{tg}$ BM-DCs and unstimulated ($B_{US}$) or B cells stimulated with either LPS ($B_{LPS}$) and anti-CD40 ($B_{aCD40}$) from protected or diabetic NOD mice were cultured with G9C$\alpha^{-/-}$ CD8 T cells (a-d) or without G9C$\alpha^{-/-}$ CD8 T cells (e, f) for 3 days before analyses. B cells were cultured either in contact (Cont) or separated from BM-DCs +/- CD8 T cells in transwells (Trans). (a, b) flow cytometric analyses on CD8 T cells. Representative plots (left) and summary graphs (right) on (a) CFSE dilution illustrating proliferation (b) CD44 surface expression. Data were normalised to DC+CD8 alone control cultures (dotted line). (c, e) Supernatants from protected and diabetic NOD DC-B cell-CD8 T cell co-cultures (c) IFNγ (e) IL-10. (d, f) Supernatants from protected and diabetic NOD DC-B cell co-cultures (d) IFNγ (f) IL-10. Data shown are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, versus $B_{US}$ (two-way ANOVA); $^{+}$P<0.05, $^{++}$P<0.01, Cont vs Trans (two-way ANOVA). Data represent at least 3 independent experiments.

**Figure 5.** *$B_{LPS}$ have lasting tolerogenic effects on mature BM-DCs.* Activated NOD.PI2$^{tg}$ BM-DCs and unstimulated ($B_{US}$) or B cells stimulated with either LPS ($B_{LPS}$) and anti-CD40, ($B_{aCD40}$) from protected or diabetic NOD mice, were co-cultured for 3 days before being removed, and remaining NOD.PI2$^{tg}$ BM-DCs were cultured with G9C$\alpha^{-/-}$ CD8 T cells for 3 days, before being assessed by flow cytometry. (a) Experimental set up; (b) CFSE dilution illustrating CD8 T cell proliferation; (c) CD44 surface expression on CD8 T cells. CD8 T cells were gated on live CD11b$^{-}$CD11c$^{-}$CD19$^{-}$CD8$^{+}$ cells. (d) Cytokine analysis of supernatants for IL-10 (left), IL-12p70 (middle), IL-6 (right). Control (chequered bar) represents DCs not cultured with B cells, prior to the addition of CD8 T cells. Data shown are mean ± SEM. *P<0.05, **P<0.01, two way ANOVA. Data represent 3 independent experiments.

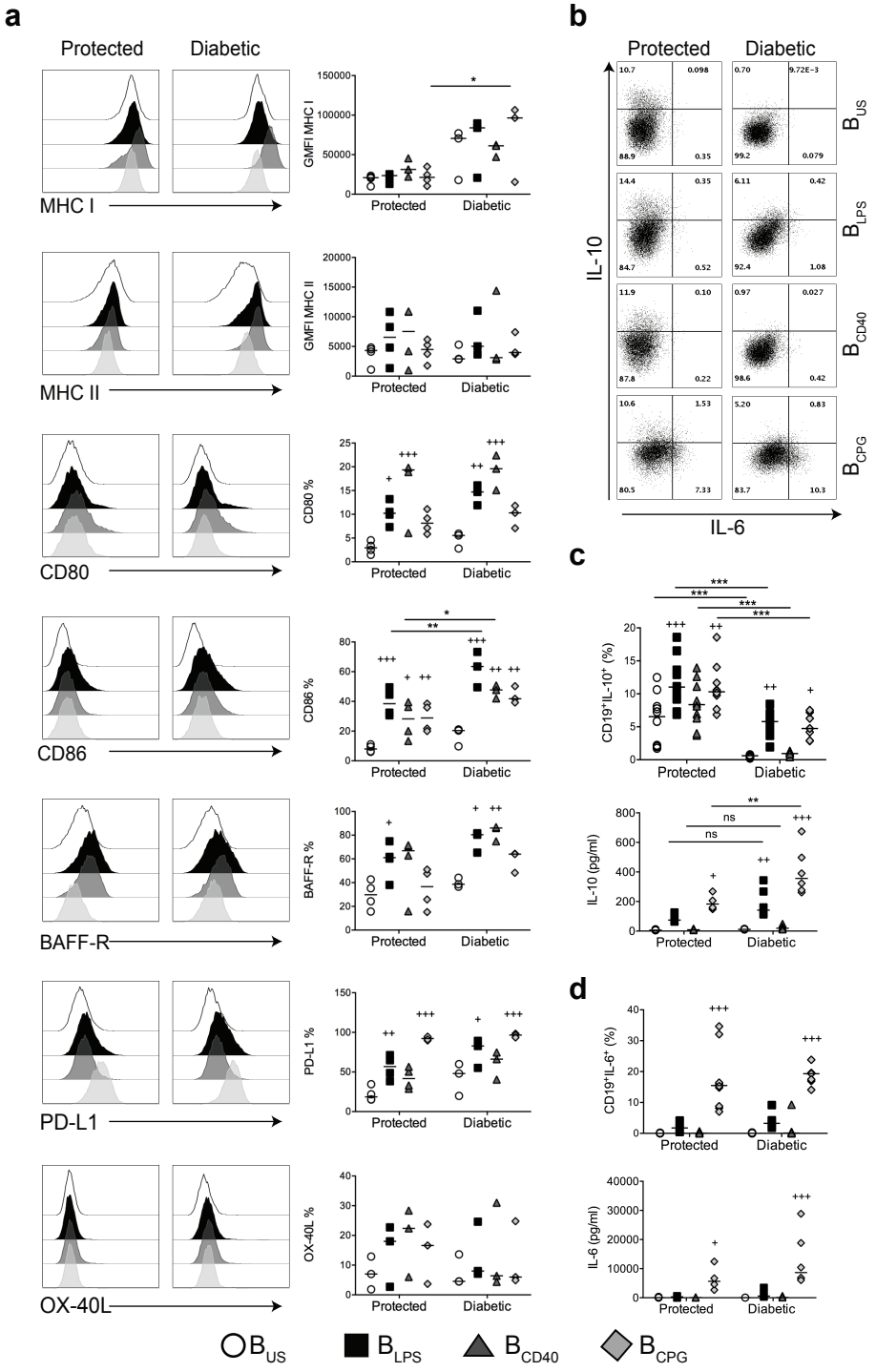
**Figure 6.** *B cells modulate mature DC activation.* Mature NOD.PI2$^{tg}$ BM-DCs and unstimulated ($B_{US}$) or B cells stimulated with either LPS ($B_{LPS}$) and anti-CD40 ($B_{aCD40}$) from protected or diabetic NOD mice were co-cultured for 3 days before analysis (a, b) DC-B cell co-cultures were either cultured together in contact (Cont) or in a transwell plate (Trans) or BM-DCs were treated with supernatants from B cells either unstimulated or different stimuli (24hrs prior to co-culture) (Sup), and mature NOD.PI2$^{tg}$ DCs analysed for various surface markers. (a) Representative plots and (b) combined graphical data. Dotted line represents DC control (no B cells). All data were normalized to DC control cultures. *P<0.05, **P<0.01, ***P<0.001, cont vs trans vs sup (two-way ANOVA); $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001, versus $B_{US}$ (two-way ANOVA). Data shown are mean ± SEM. Data represent at least 3 independent experiments.

**Figure 7.** *$B_{LPS}$ induces a CD45RB$^{+}$CD11c$^{lo}$ tolerogenic DC population.* Activated NOD.PI2$^{tg}$ BM-DCs and unstimulated ($B_{US}$; white circle) or B cells stimulated with either LPS ($B_{LPS}$; black square) and anti-CD40 ($B_{aCD40}$; grey triangle) from protected or diabetic or IL-10KO NOD mice were co-cultured for 3 days before analysis. (a) representative plots (left) and summary graph (right) of CD45RB$^{+}$ DC (gated on Live CD11c$^{+}$CD11b$^{+}$) populations, dotted line represents DC alone (control DC). Horizontal line represents median value b) representative flow plots for CD11c, CD80 and CD86 expression on CD45RB$^{+}$ (solid line) and CD45RB$^{-}$ (dashed line) populations. (c) CD11c, CD80 and CD86 summary graphs on CD45RB$^{+}$ and CD45RB$^{-}$ populations from pooled protected and diabetic NOD mice. Data shown are mean ± SEM. Data shown represent 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, two-way ANOVA.

**Figure 8.** *Schematic summary of B cell-directed CD8 T cell suppression in the context of type 1 diabetes.* 1. B cells from protected NOD mice stimulated via TLR4 or anti-CD40 express substantial intracellular IL-10 but only secrete IL-10 with TLR4 stimulation. 2. B cells from

diabetic NOD mice express reduced intracellular IL-10 when stimulated via TLR4 and lack IL-10 expression with anti-CD40 stimulation, but can secrete IL-10 with TLR4 stimulation. 3. (a) BM-DCs engage with TLR4 activated B cells, from protected NOD mice (b) On initial engagement a possible reciprocal contact-mediated process, inducing an altered BM-DC in order to generate an IL-10 feedback loop and tolBM-DC induction by IL-10 (dotted arrows) (c) B cell: BM-DC contact augments IL-10 production from B cells, creating a feedback loop as shown by solid black arrows. (d) B cell: BM-DC contact and IL-10 secretion induces a deactivated tolerogenic BM-DC population (tolBM-DCs). (e) tolerogenic deactivated BM-DCs suppress pathogenic CD8 T cells (f) Effect of tolBM-DCs on B cells, after induction, is still unknown (dashed arrow). 4. B cells from diabetic NOD mice, have a diminished response to contact with BM-DCs, which results in fewer deactivated tolBM-DCs. 5. In the presence of CD8 T cells and proinflammatory cytokines i.e. IFNγ, B cells, stimulated with LPS, from diabetic NOD mice, in contact with BM-DCs, amplify their IL-10 response and induce CD8 T cell suppression as effectively as B cells from protected NOD mice.

# Figure 1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Protected
Diabetic
MHC I
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MHC II
GMFI MHC II
CD80
CD80 %
CD86
CD86 %
BAFF-R
BAFF-R %
PD-L1
PD-L1 %
OX-40L
OX-40L %
Protected
Diabetic
B_US
B_LPS
B_CD40
B_CPG
b
Protected
Diabetic
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c
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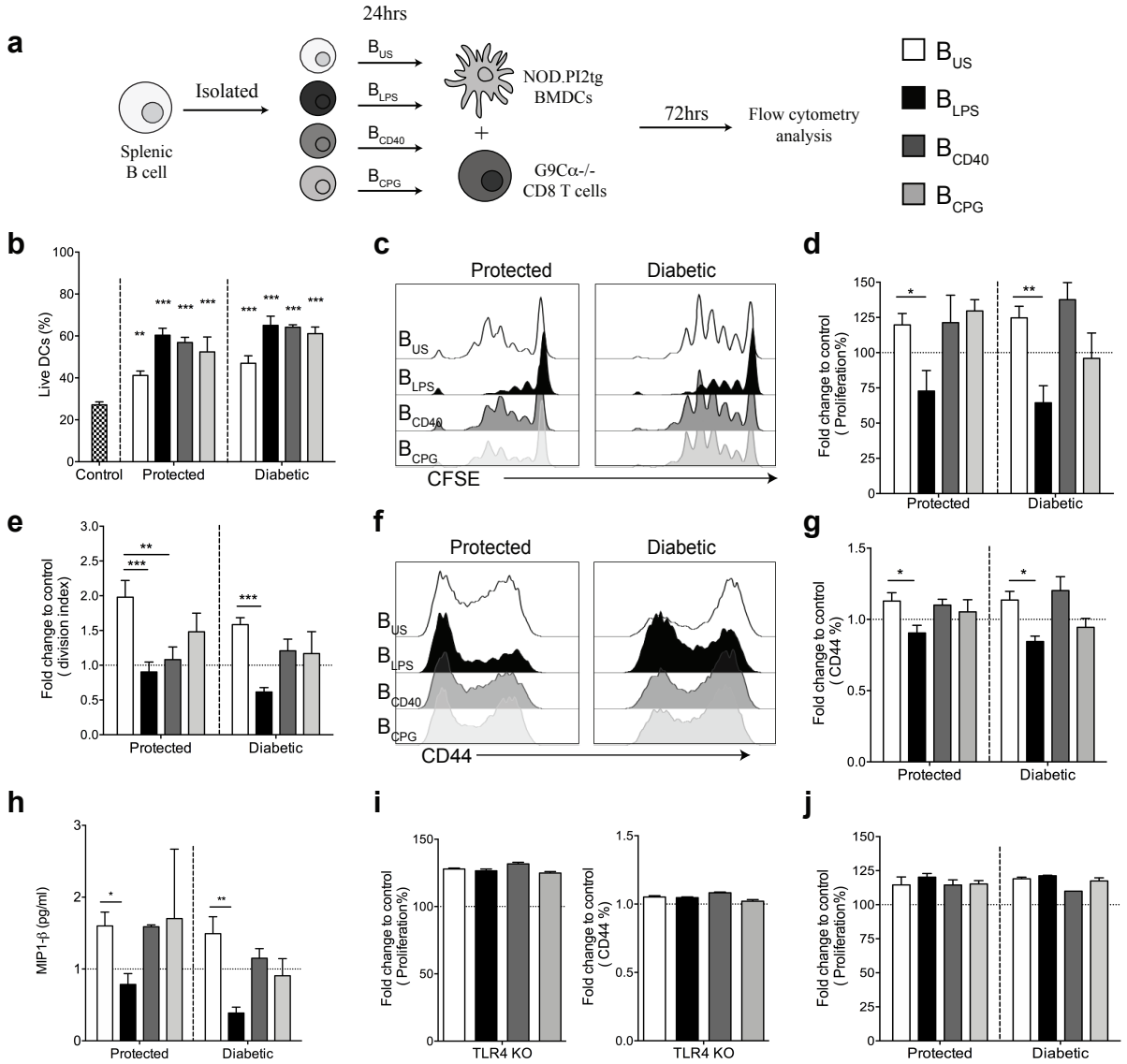
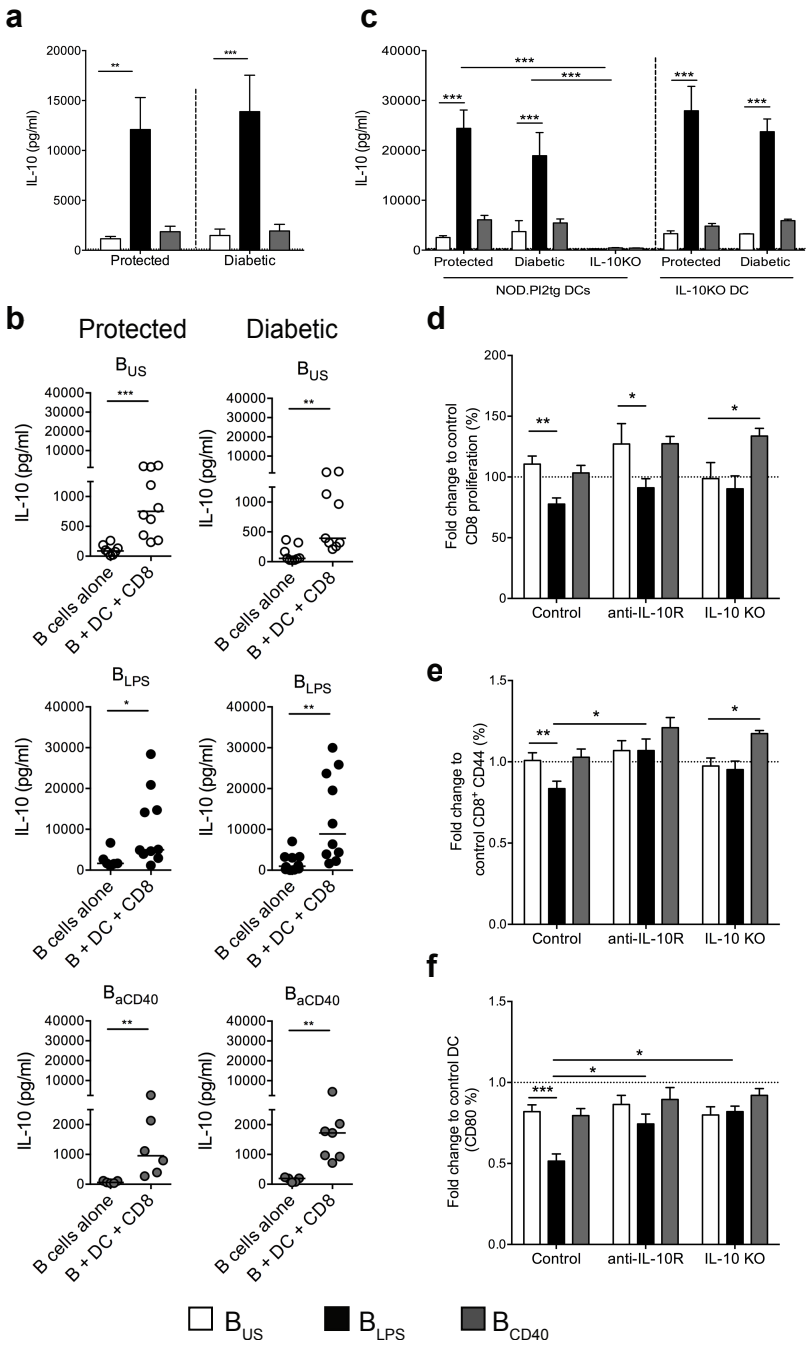
IL-10 (pg/ml)
ns
d
CD19+IL-6+ (%)
IL-6 (pg/ml)

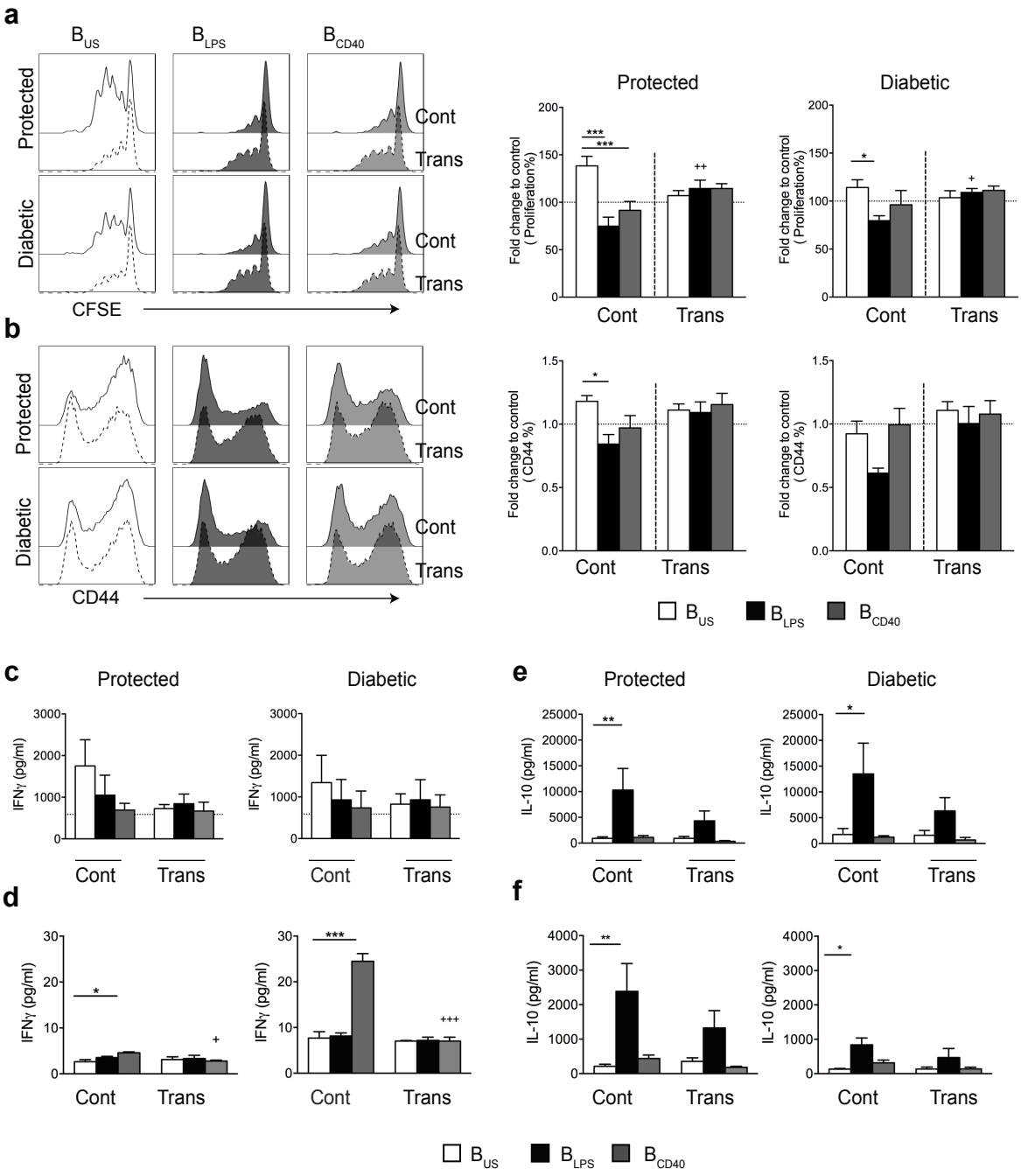
## Figure 2


a
24hrs
Isolated
Splenic B cell
B_US
B_LPS
B_CD40
B_CPG
NOD.PI2tg BMDCs
+
G9Cα-/- CD8 T cells
72hrs
Flow cytometry analysis
B_US
B_LPS
B_CD40
B_CPG
b
Live DCs (%)
Control
Protected
Diabetic
c
Protected
Diabetic
B_US
B_LPS
B_CD40
B_CPG
CFSE
d
Fold change to control ( Proliferation%)
Protected
Diabetic
e
Fold change to control ( division index)
Protected
Diabetic
f
Protected
Diabetic
B_US
B_LPS
B_CD40
B_CPG
CD44
g
Fold change to control ( CD44 %)
Protected
Diabetic
h
MIP1-β (pg/ml)
Protected
Diabetic
i
Fold change to control ( Proliferation%)
TLR4 KO
Fold change to control ( CD44 %)
TLR4 KO
j
Fold change to control ( Proliferation%)
Protected
Diabetic

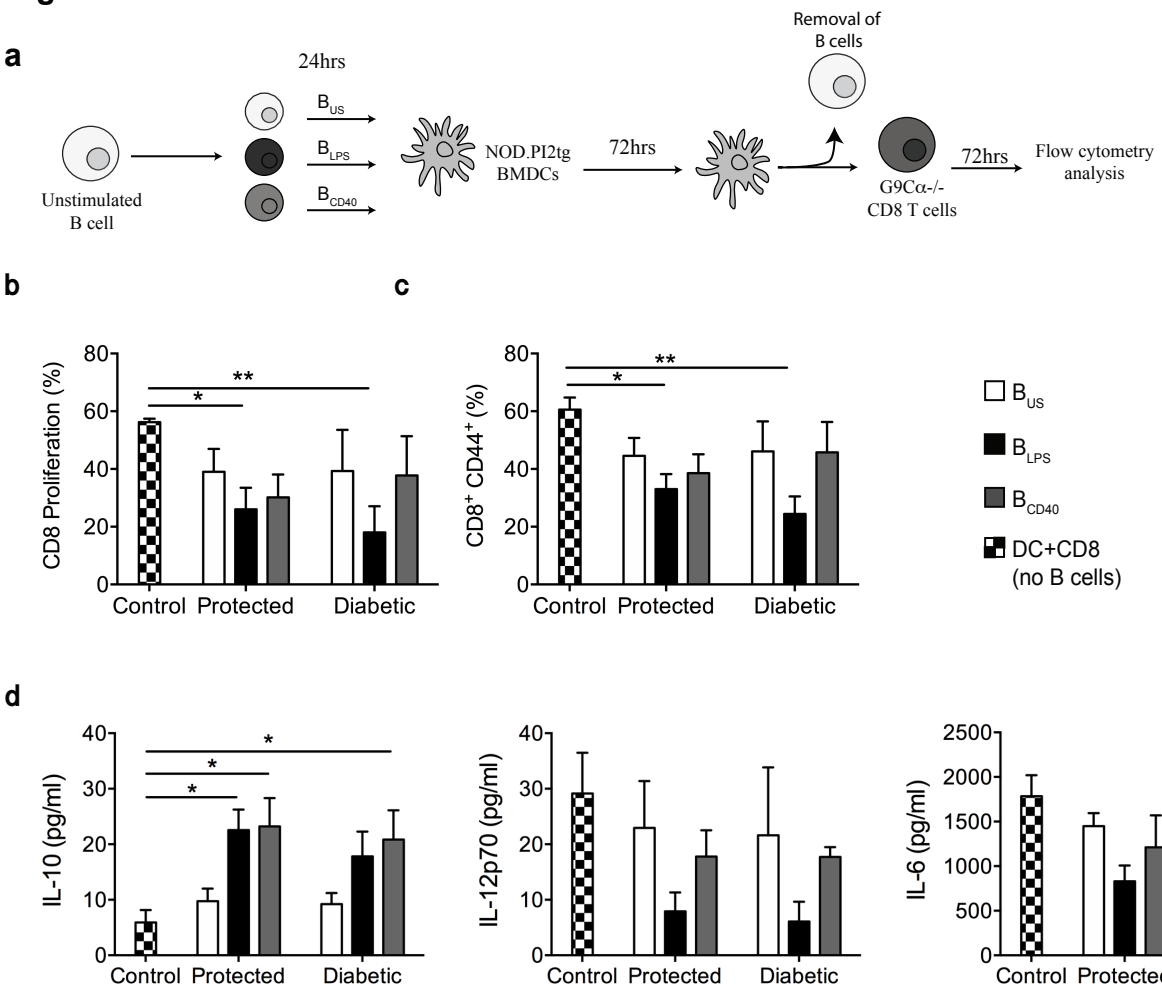
# Figure 3


a
IL-10 (pg/ml)
Protected
Diabetic
c
IL-10 (pg/ml)
Protected
Diabetic
IL-10KO
Protected
Diabetic
NOD.PI2tg DCs
IL-10KO DC
b
Protected
Diabetic
B_US
B_LPS
B_aCD40
IL-10 (pg/ml)
B cells alone
B + DC + CD8
d
Fold change to control CD8 proliferation (%)
Control
anti-IL-10R
IL-10 KO
e
Fold change to control CD8+ CD44 (%)
Control
anti-IL-10R
IL-10 KO
f
Fold change to control DC (CD80 %)
Control
anti-IL-10R
IL-10 KO
B_US
B_LPS
B_CD40

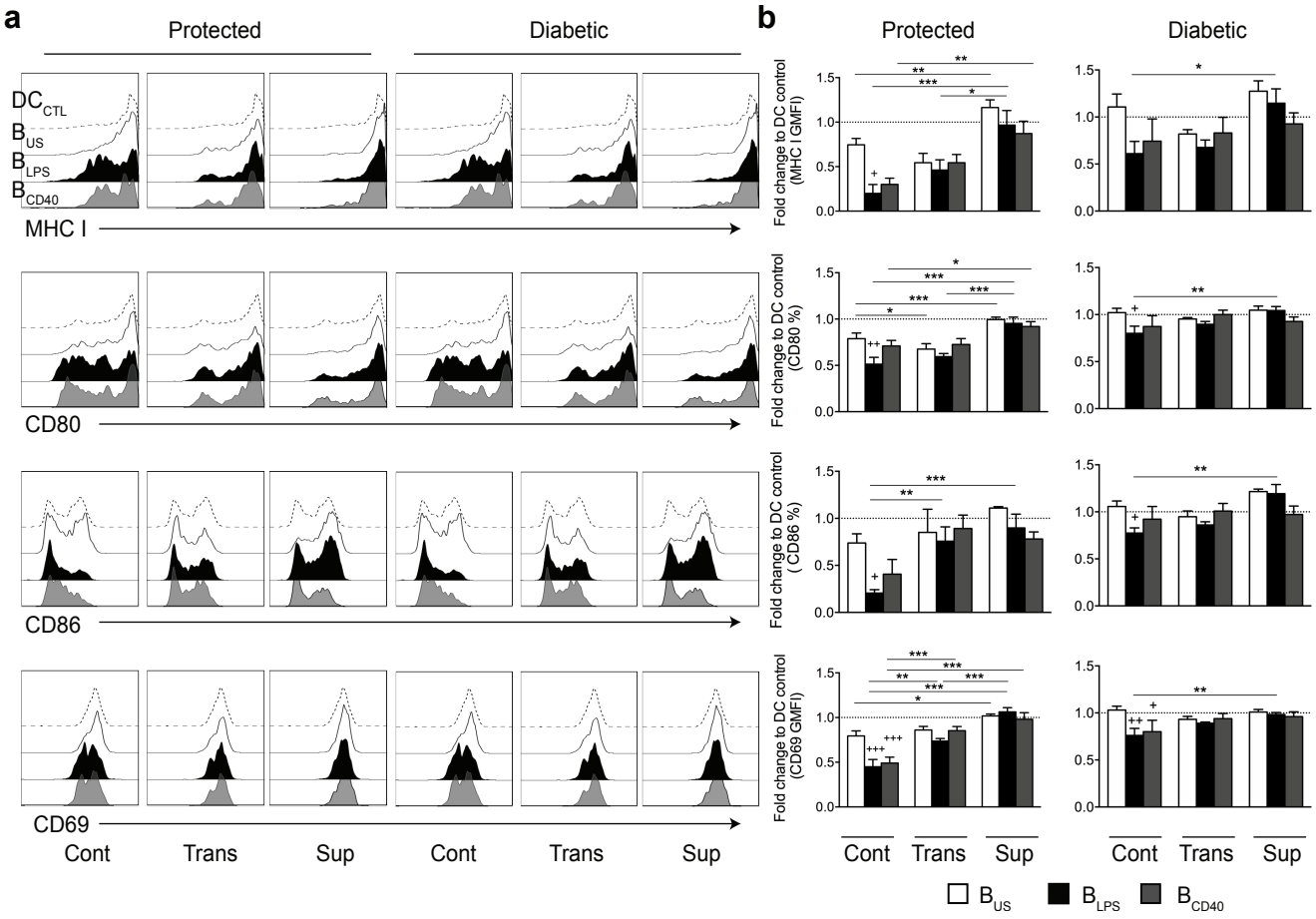
# Figure 4


a
B_US
B_LPS
B_CD40
Protected
Diabetic
Cont
Trans
CFSE
b
CD44
Protected
Diabetic
Fold change to control (Proliferation%)
Fold change to control (CD44 %)
Cont
Trans
B_US
B_LPS
B_CD40
c
IFNγ (pg/ml)
d
e
IL-10 (pg/ml)
f

## Figure 5

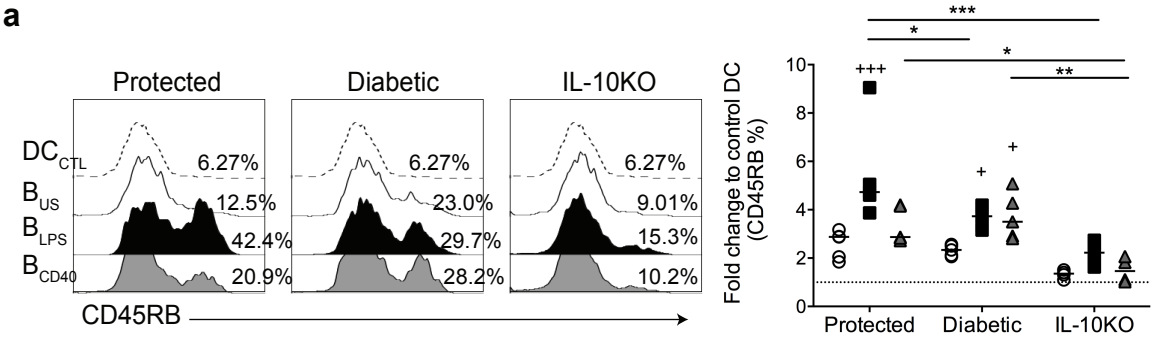

a
24hrs
Removal of
B cells
Unstimulated
B cell
B_US
B_LPS
B_CD40
NOD.PI2tg
BMDCs
72hrs
72hrs
Flow cytometry
analysis
G9Cα-/-
CD8 T cells
b
CD8 Proliferation (%)
Control
Protected
Diabetic
c
CD8+ CD44+ (%)
Control
Protected
Diabetic
B_US
B_LPS
B_CD40
DC+CD8
(no B cells)
d
IL-10 (pg/ml)
Control
Protected
Diabetic
IL-12p70 (pg/ml)
Control
Protected
Diabetic
IL-6 (pg/ml)
Control
Protected
Diabetic

## Figure 6

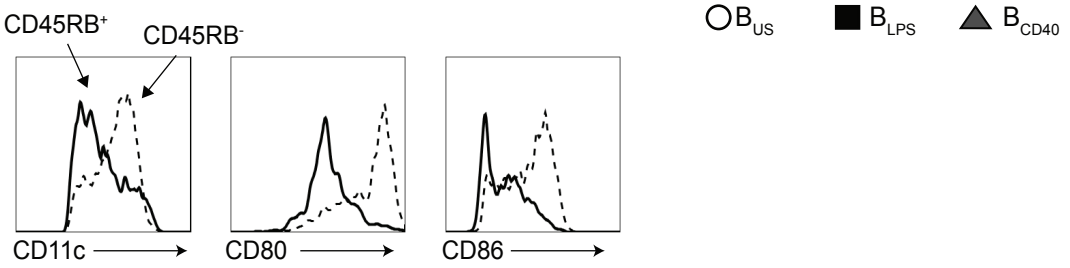

a
Protected
Diabetic
DC_CTL
B_US
B_LPS
B_CD40
MHC I
CD80
CD86
CD69
Cont
Trans
Sup
b
Protected
Diabetic
Fold change to DC control (MHC I GMFI)
Fold change to DC control (CD80 %)
Fold change to DC control ( CD86 %)
Fold change to DC control (CD69 GMFI)
B_US
B_LPS
B_CD40

## Figure 7

**a**


Protected
Diabetic
IL-10KO
DC_CTL
B_US
B_LPS
B_CD40
6.27%
12.5%
42.4%
20.9%
6.27%
23.0%
29.7%
28.2%
6.27%
9.01%
15.3%
10.2%
CD45RB
Fold change to control DC (CD45RB %)
Protected
Diabetic
IL-10KO

**b**


CD45RB+
CD45RB-
B_US
B_LPS
B_CD40
CD11c
CD80
CD86

**c**

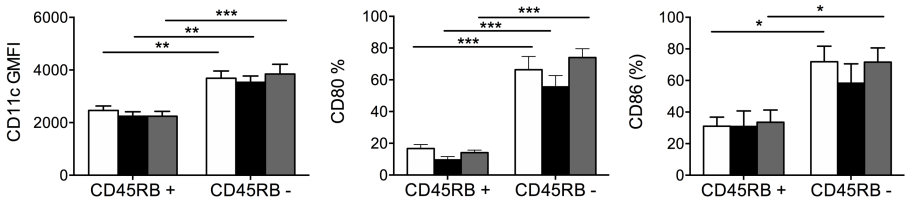
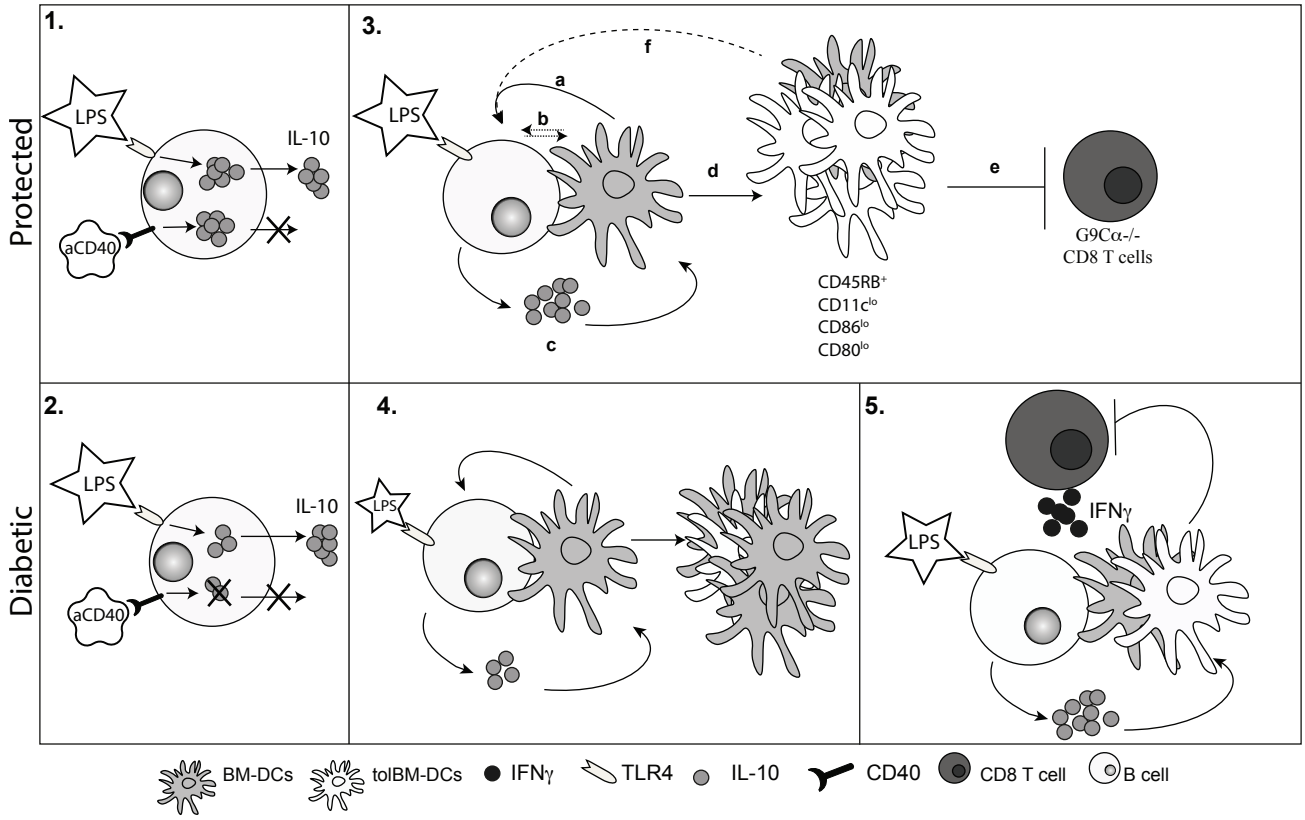
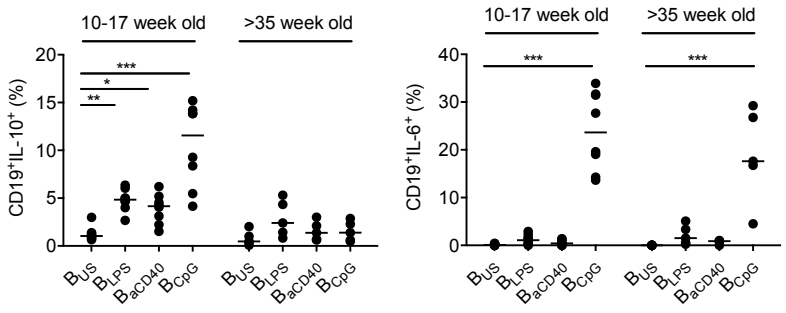
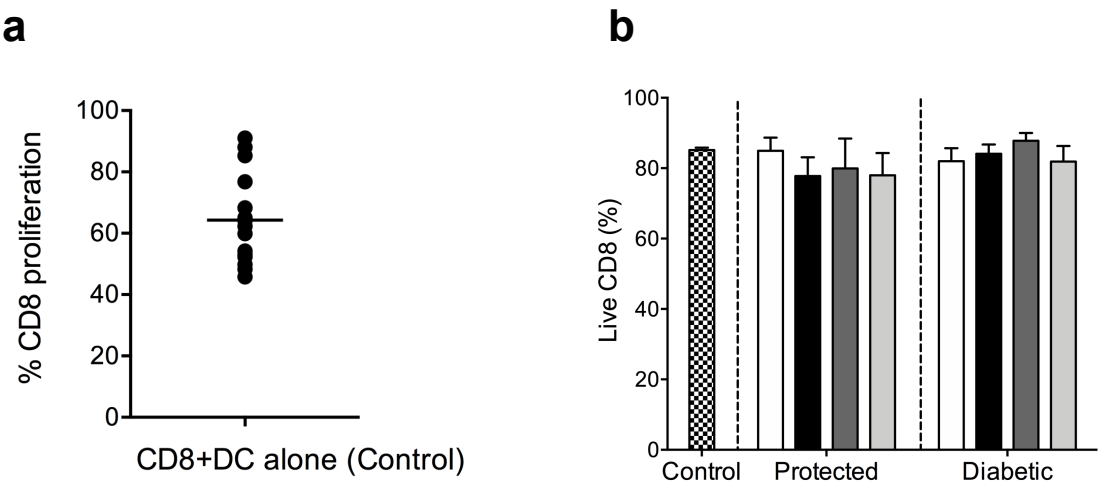

CD11c GMFI
CD80 %
CD86 (%)
CD45RB +
CD45RB -

**Figure 8**

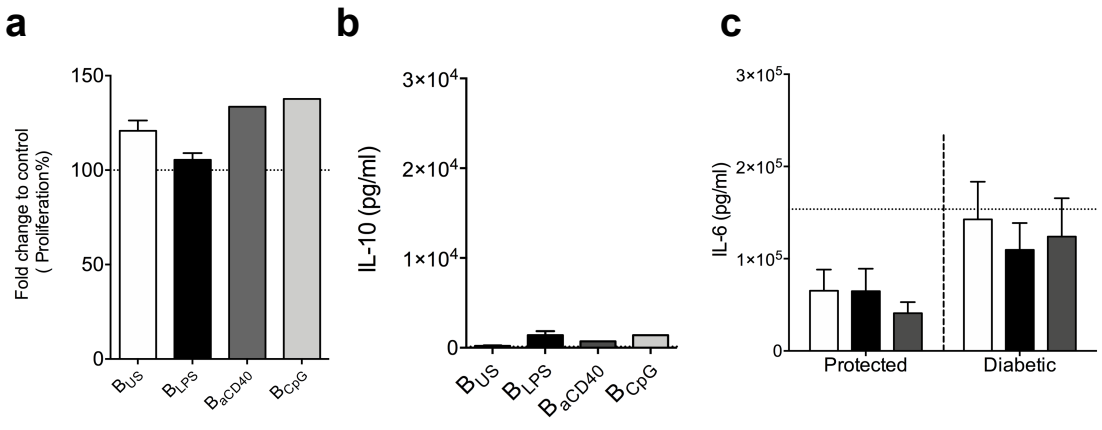

Protected
Diabetic
1.
LPS
IL-10
aCD40
3.
LPS
a
b
c
d
e
f
CD45RB+
CD11clo
CD86lo
CD80lo
G9Cα-/-
CD8 T cells
2.
LPS
IL-10
aCD40
4.
LPS
5.
IFNγ
LPS
BM-DCs
tolBM-DCs
IFNγ
TLR4
IL-10
CD40
CD8 T cell
B cell



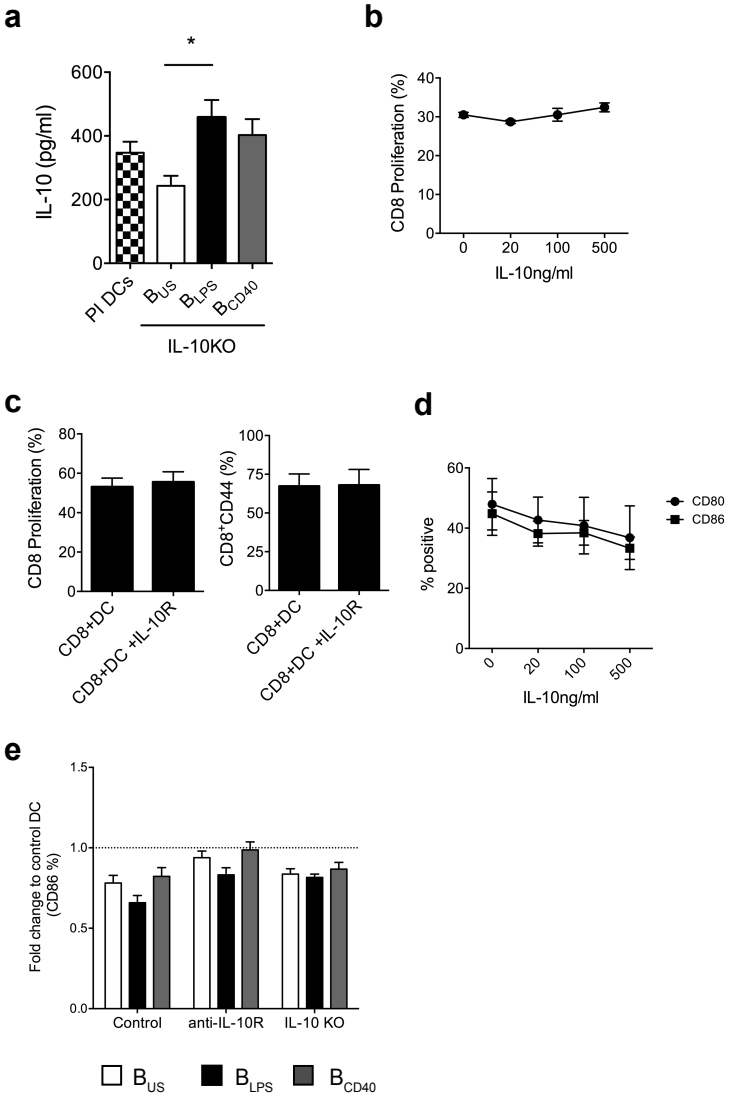

**Supplementary figure 1.** *Age matched B6G7 mice have little IL-10 potential.* Splenic B cells from $B6^{G7}$ mice, aged either 10-17 weeks or more than 35 weeks, were left unstimulated ($B_{US}$) or stimulated with LPS ($B_{LPS}$), anti-CD40 ($B_{aCD40}$) or CPG ($B_{CPG}$) for 24hrs before (a) being analysed for IL-10 (left) and IL-6 (right) potential by intracytoplasmic staining. B cells were gated on live $CD19^+$ cells. The horizontal line represents the median value. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; two-way ANOVA. Data represent at least two independent experiments.



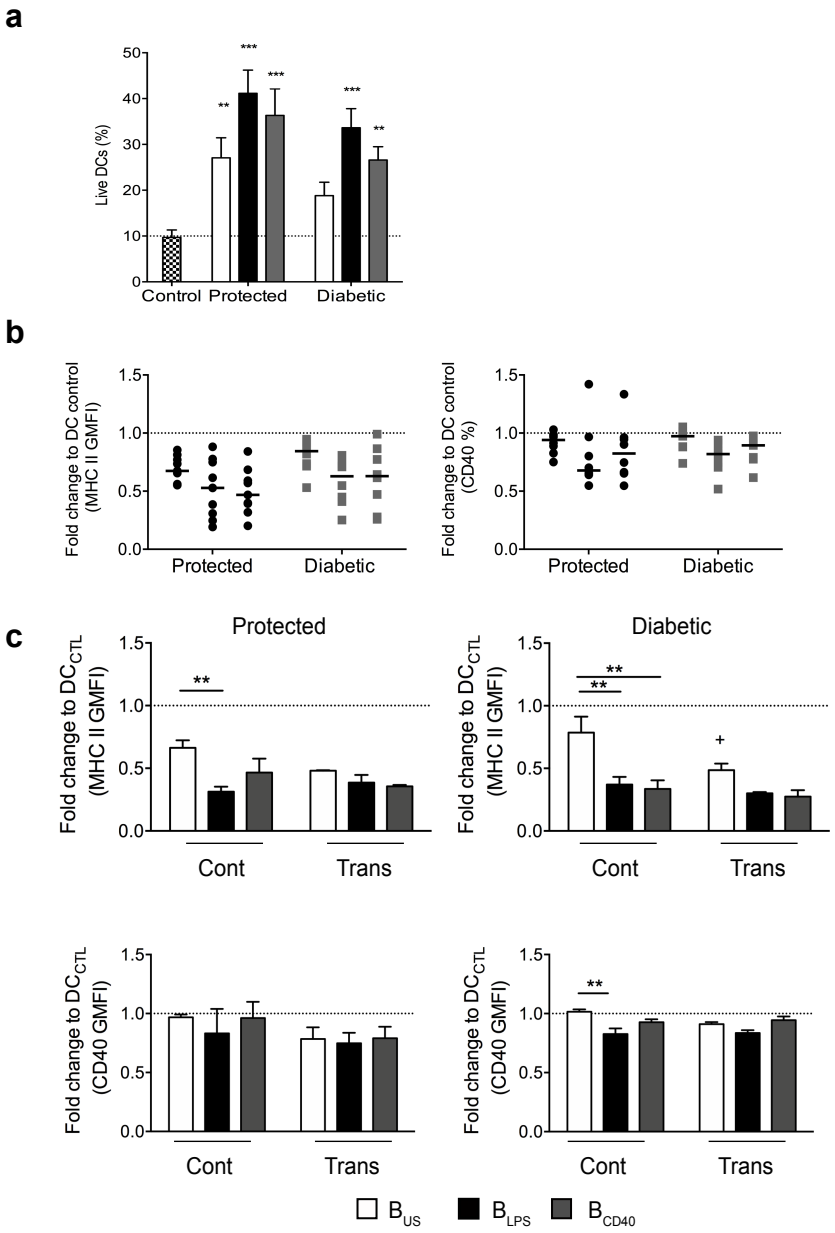

**Supplementary figure 2.** *CD8 T cell suppression is not due to increased CD8 T cell death.* NOD.PI2$^{tg}$ BM-DCs, CFSE-labelled G9C$\alpha^{-/-}$ CD8 T cells and splenic B cells that were unstimulated ($B_{US;}$ white bar) or stimulated with one of LPS ($B_{LPS;}$ black bar), anti-CD40 ($B_{aCD40}$; dark grey bar) or CPG ($B_{CPG}$; light grey bar), from protected or diabetic NOD mice, were co-cultured for 3 days before being examined by flow cytometry. CD8 T cells were gated on CD11c$^-$CD11b$^-$CD19$^-$CD8$^+$ cells. (a) CD8 T cell proliferation in controls (DC+CD8 T cells alone) is shown for all assays. Line represents the median value. (b) Graph shows viable live CD8 T Cells. The control was DC+CD8 alone, shown as the patterned bar. Data shown are mean ± SEM (Data represent at least 3 independent experiments).




**Supplementary figure 3.** *B cells from B6$^{g7}$do not suppress insulin-specific CD8 T cells* NOD.PI2$^{tg}$ BM-DCs, CFSE-labelled G9Cα$^{-/-}$ CD8 T cells and splenic B cells that were unstimulated ($B_{US;}$ white bar) or stimulated with one of LPS ($B_{LPS;}$ black bar), anti-CD40 ($B_{aCD40}$; dark grey bar) or CPG ($B_{CPG}$; light grey bar), from *B6$^{g7}$* (a, b) or protected or diabetic NOD mice (c), were co-cultured for 3 days. (a) CD8 T cell proliferation, normalised to control (DC+CD8 alone; dotted line). CD8 T cells were gated on CD11c$^{-}$CD11b$^{-}$CD19$^{-}$CD8$^{+}$ cells. (b) Supernatant detection of IL-10 cytokine in culture medium. (c) Supernatant detection of IL-6 cytokine in culture medium, dotted line represents DC+CD8 alone. Data shown in (a, b) for $B_{aCD40}$ and $B_{CPG}$ cultures represent one experiment. Data shown in (a, b) for $B_{US}$ and $B_{LPS}$ and (c) represent 3 independent experiments.




**Supplementary figure 4.** *IL-10 affects co-stimulatory markers on mature BM-DCs.* (a) NOD.PI2$^{tg}$ BM-DCs were cultured either alone (chequered bar) or with splenic B cells that were unstimulated ($B_{US;}$ white bar) or stimulated with LPS ($B_{LPS;}$ black bar) or anti-CD40 ($B_{aCD40}$; dark grey bar) from IL-10KO B cells, for 3 days before measurement of IL-10, *P<0.05, one-way ANOVA (b, c, d) NOD.PI2$^{tg}$ BM-DCs and CFSE-labelled G9Cα-/- CD8 T cells were cultured for 3 days and CD8 T cells were gated on live $CD11c^-CD11b^-CD8^+$ cells; (b) DC and CD8 T cells cultured with or without increasing concentrations of recombinant IL-10 and the graph shows CD8 T cell proliferation; (c) DC and CD8 T cells cultured with 10μg/ml anti-IL-10R and the graph shows CD8 T cell proliferation (left) and $CD8^+CD44^+$ surface expression (right); (d) DC and CD8 T cells cultured with increasing concentrations of recombinant IL-10 and the graph shows CD86 and CD80 expression on BMDCs, gated on live $CD8^-CD11c^+CD11b^+$ cells. (e) NOD.PI2$^{tg}$ BM-DCs, G9Cα-/- CD8 T cells and unstimulated ($B_{US}$) or B cells, stimulated with either LPS ($B_{LPS}$) or αCD40 ($B_{aCD40}$) from protected/diabetic NOD mice, were either treated with isotype control (control) or 10μg/ml anti-IL-10R (anti-IL10R), or IL-10KO B cells, and co-cultured for 3 days. The cultures were analyzed for CD86 on $CD8^-CD19^-CD11c^+CD11b^+$ BM-DCs. Data were normalised to control (DC+CD8 alone, dotted line). Data shown are mean ± SEM. Data represent at least 3 independent experiments.




**Supplementary figure 5.** *Stimulated B cells can regulate MHC II expression on mature BM-DCs independent of cell contact.* Activated NOD.PI2$^{tg}$ BM-DCs and unstimulated ($B_{US}$) or B cells stimulated with either LPS ($B_{LPS}$) and αCD40 ($B_{aCD40}$) from protected or diabetic NOD mice were co-cultured for 3 days before analysis. (a) Live DC percentages from DC-B cell cultures; (b) MHC II (left) and CD40 (right) expression on live CD19$^{-}$CD11b$^{+}$CD11c$^{+}$ BMDCs; (c) DC-B cell co-cultures were either cultured together in contact (Cont) or in a transwell plate (Trans) and analysed for MHC II and CD40 expression on live CD19$^{-}$ CD11b$^{+}$CD11c$^{+}$ BMDCs. **P<0.01, versus $B_{US}$ (two-way ANOVA), $^{+}$P<0.05, versus cont vs trans (two-way ANOVA). Dotted line represents DC control (no B cells) ($DC_{CTL}$). Data were normalized to DC control. Data shown are mean ± SEM. Data represent at least 3 independent experiments.