IL-1β produced in response to islet autoantigen presentation differentiates T-helper 17 cells at the expense of regulatory T cells: implications for the timing of tolerizing immunotherapy

Sebastien Bertin-Maghit¹, Dimeng Pang¹, Brendan O'Sullivan¹, Shannon Best¹, Emily Duggan¹, Sanjoy Paul², Helen Thomas⁴, Thomas W.H. Kay⁴, Leonard C. Harrison³, Raymond Steptoe¹* and Ranjeny Thomas¹*

¹The University of Queensland Diamantina Institute, Princess Alexandra Hospital, Brisbane, Queensland, 4102
²Queensland Clinical Trials & Biostatistics Centre, School of Population Health, The University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland, 4102
³Autoimmunity and Transplantation Division, Walter and Eliza Hall Institute, Melbourne
⁴St Vincent’s Institute, Melbourne, Australia.

* R. S. and R. T. contributed equally to the work

All correspondence should be addressed to: Professor Ranjeny Thomas
Email: Ranjeny.Thomas@uq.edu.au
OR
Dr Raymond Steptoe
Email: r.steptoe@uq.edu.au

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Objective: The effectiveness of tolerizing immunotherapeutic strategies, such as anti-CD40L or dendritic cells (DC), is greater when administered to young NOD mice than at peak insulitis. RelB\textsuperscript{lo} DC, generated in the presence of an NF-κB inhibitor, induce T regulatory (Treg) cells and suppress inflammation in a model of rheumatoid arthritis. IL-1β is over-expressed in humans and mice at risk of T1DM, dysregulates Treg cells, and accelerates diabetes in NOD mice. We investigated the relationship between IL-1β production and the response to RelB\textsuperscript{lo} DC in the pre-diabetic period.

Methods: We injected RelB\textsuperscript{lo} DC s.c. into 4 or 14 week-old NOD mice, and tracked the incidence of diabetes and effect on Treg cell function. We measured expression of proinflammatory cytokines by stimulated splenocytes and unstimulated islets from mice of different ages and strains, and proliferative and cytokine responses of T effectors to Treg in vitro.

Results: Tolerising RelB\textsuperscript{lo} DC significantly inhibited diabetes progression when administered to 4 week but not 14 week-old mice. IL-1β production by NOD splenocytes and mRNA expression by islets increased from 6-16 weeks of age, when MHC-restricted islet antigen presentation to autoreactive T cells occurred. IL-1 reduced the capacity of Treg cells to suppress effector cells and promoted their conversion to Th17 cells. RelB\textsuperscript{lo} DC exacerbated the IL-1-dependent decline in Treg function and promoted Th17 conversion.

Conclusion: IL-1β, generated by islet-autoreactive cells in MHC-susceptible mice, accelerates diabetes by differentiating Th17 at the expense of Treg. Tolerizing DC-therapies can regulate islet autoantigen priming and prevent diabetes, but progression past the IL-1β/IL-17 checkpoint signals the need for other strategies.
from at-risk relatives with islet autoantibodies but not healthy controls induced a gene expression signature characterized by upregulated expression of members of the IL-1 family and associated with IL-1 action, when incubated with healthy PBMC (15), and IL-1β was also over-expressed in the gene expression signature of PBMC from children with newly-diagnosed T1DM. IL-1 expression fell as hyperglycemia settled, suggesting at least some of the inflammatory drive is metabolic (22).

The effectiveness of tolerizing immunotherapies, such as anti-CD40L, IL-10, rapamycin or DC treated with NF-κB oligonucleotides, is greater when administered to young (4-6 weeks) NOD mice than mice with advanced insulitis (10-14 weeks) (23-26). The RelB subunit of NF-κB is a key determinant of DC antigen presenting cell (APC) function (27). Microbial, inflammatory or T cell-derived signals induce the nuclear translocation and transcriptional activity of RelB in DCs (28; 29). Induction of MHC class II and costimulatory molecules required for effective TCR signaling and T cell priming, is impaired in response to such activation of RelB−/− DC (28; 30; 31). T cell stimulatory function of RelB−/− DCs is deficient in vitro and in vivo, and transferred RelB−/− or RelBlo DC, generated in the presence of NF-κB inhibitors or RelB siRNA suppress primed T cell effector responses through induction of Tr1-type Treg, in an IL-10-dependent manner (31; 32). RelBlo DC suppress disease in a mouse model of rheumatoid arthritis (33). Here, we employed RelBlo tolerizing DC to prevent diabetes in NOD mice to define mechanisms that determine the age-dependent effectiveness of tolerizing therapies in T1DM.

RelBlo DC, which express reduced MHC class II and CD40, suppress effector function both by reduced capacity to signal Teff and through induction of Treg (31). The induction of Treg by tolerizing immunotherapies depends on the presence of FoxP3+ natural Treg (34; 35). Thus, factors which dysregulate Treg function are candidates for interference with effective suppression of autoimmune disease by RelBlo DC. Since IL-1 plays an important role in the pathogenesis of T1DM in mice and humans, and its production in NOD mice dysregulates Treg cell function, we hypothesized that IL-1 impairs the response to RelBlo DC.

**MATERIALS & METHODS**

**Mice.** NOD.Lt, C57BL/6, NOD.I-Ak, NOD.CD45.2 (36), NOD.IR1−/− (21) and pINS.NOD (37) mice were obtained from the Animal Research Centre (Perth, Australia) James Cook University or bred at Walter and Eliza Hall Institute (WEHI, Melbourne, Australia) or the University of Queensland. Mice were housed at Princess Alexandra Hospital (Brisbane, Australia) or WEHI.

**Cytokine assay.** Cytokines were assayed in supernatants by ELISA for IL-1 (eBioscience, San Diego, USA) and IL-17 (Biolegend, San Diego, USA), and bead array (BD Bioscience, Franklin Lakes, USA). Supernatants in triplicates were pooled and cytokine assays were made in duplicate. Data represent mean ±SD.

**Intracellular staining.** All surface antibodies were purchased from Biolegend. Cells were stained fresh or after culture with anti-CD3ε mAb for 72h, or PMA, with addition of Brefeldin A (Sigma, St Louis, USA) for 4 h. After surface mAb staining, cells were fixed and permeabilized, then incubated on ice with anti-IL-1 (eBioscience) or isotype control. When biotinylated antibody was used, cells were washed and incubated for 15 minutes with labelled streptavidin (Biolegend). Cells were suspended in 10% formalin, read on a FACS Calibur (BD Bioscience) and analysed using FlowJo software (Tree Star, Inc.).

**Assessment of diabetes and insulitis.** Mice were classified diabetic and euthanized following two consecutive weekly blood
glucose readings >12mM. For analysis of insulitis, pancreata were collected from 4 mice per group at 12 weeks. Insulitis was graded in hematoxylin and eosin stained sections as described (38).

**Islet purification and qPCR.** Islets of Langerhans were isolated by collagenase P (dissolved in HBSS containing 2mM Ca²⁺ and 20 mM HEPES) digestion and density gradient centrifugation as described previously (39). RNA was extracted (RNeasy Mini Kit, Qiagen) and cDNA synthesized; qPCR used Taqman primers on a AB 7900 (Applied Biosystems) machine.

**Proliferation and suppression assay.** CD11c⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from spleen and lymph nodes using immunomagnetic beads and AutoMacs separator (Miltenyi Biotec, Bergisch Gladbach, Germany). One hundred thousand CD11c⁺, 10⁵ CD4⁺CD25⁻ and 7.5 x 10⁴ CD4⁺CD25⁺ cells were incubated for 72 hours in presence of anti-CD3ε antibody. Supernatants were collected for cytokine assays and 1µ Cu of tritiated thymidine (Perkin Elmer, Waltham, USA) was added for 18h before counting. Data represent mean ±SD of triplicates performed twice.

**In vivo administration of RelBlo DC and transfer of Treg.** Bone marrow was collected from long bones of NOD mice aged 8±2 weeks. After Ficoll-Histopaque gradient separation, cells were cultured in RPMI + 10% FCS in presence of murine IL-4 and GM-CSF (Peprotech, Rocky Hill, USA) and 2.5µM BAY-11-7082 (Calbiochem, San Diego, USA) for 12 days, media being refreshed every 3 days. RelBlo DC (5x10⁵ per animal) were injected s.c. to the tail base. For Treg transfer, CD4⁺CD25⁺ were isolated from spleen and lymph nodes of 4 week NOD.Lt or NOD.IL-1R1⁻/⁻ mice using immunomagnetic beads. 2 x 10⁶ cells were transferred i.v. at the same time as DC.

**Statistical analysis.** Student’s t-tests or one-way ANOVA with Bonferroni post-test compared means of two or multiple groups respectively, and Kaplan-Mayer based on log-rank analyses test for comparisons of survival time. We made 5 comparisons, with ‘PBS-treated’ as the standard comparator. The Bonferroni-corrected threshold was 0.01 based on family-wise significance-level at 0.05. Two-way ANOVA compared time-dependent changes in Treg function.

**RESULTS**

**RelBlo DC inhibit diabetes when administered to young, but not insulitic NOD mice.** To investigate the consequences of administration of RelBlo DC to young or insulitic NOD mice, we generated RelBlo DC from NOD mice by incubation of bone marrow cells in the presence of GM-CSF, IL-4 and Bay11-7082 (an irreversible inhibitor of NF-κB and inflammasome, but not p38 MAP kinase (40; 41)) as previously described (33; 35). CD40 and MHC class II expression were reduced by addition of Bay11-7082 to NOD DC cultures compared to control DC without Bay11-7082, as expected (Supplementary Figure 1 in the online appendix available at http://diabetes.diabetesjournals.org). The concentration of inhibitor required to inhibit expression of CD40 and MHC class II in NOD bone marrow cell cultures was generally 50% of that required for C57Bl/6 RelBlo DC cultures, consistent with the constitutively higher levels of NF-κB expression in NOD cells (11). To determine the potential of RelBlo DC to suppress disease generation in an antigen-specific manner, we also generated RelBlo DC from NOD mice where proinsulin is driven as a transgene by the MHC class II promoter (pINS.NOD) (42). Mean survival without diabetes was increased from 161 (95%CI 132-191) days if mice were untreated, to 232 (215-248) days in mice administered 5 x 10⁵ RelBlo DC s.c., generated from NOD.Lt mice (p=0.002). The smaller increases in survival time in mice administered pINS.NOD DC, or control DC,
at 28 days of age were not statistically significant (Figure 1). There was a trend for reduction in insulitis at 85 days in mice treated with NOD.Lt or pINS.NOD RelB\textsuperscript{lo} DC compared to PBS, but because islets from only 4 mice per group were examined, it was not meaningful to analyze the data statistically (Figure 1E). In contrast, diabetes incidence was not reduced relative to saline treated controls by transfer of RelB\textsuperscript{lo} or control NOD or pINS.NOD DC at 100 days of age and if anything, NOD RelB\textsuperscript{lo} DC reduced the survival time without diabetes at this age (Figure 1C, D). Thus, as expected, administration of RelB\textsuperscript{lo} DC prevented development of diabetes when administered to 28 but not 100 day-old NOD mice. This was not dependent on transgenic expression of proinsulin autoantigen by the DC.

**IL-1β is over-expressed as islet inflammation develops, in response to self-antigen presentation.** We showed previously that IL-1β over-production in NOD mice is systemic and can be measured by stimulation of splenocytes with anti-CD3; IL-1 production in humans is also systemic and measurable in PBMC (22). IL-1 dysregulates Treg cell function, and RelB\textsuperscript{lo} DC suppress inflammation through Treg cells. We therefore determined the timing and mechanism of systemic IL-1β over-production prior to the onset of diabetes relative to the timing of administration of RelB\textsuperscript{lo} DC. We first analyzed supernatants from splenocytes stimulated with anti-CD3 every 4 weeks from weaning till diabetes. IL-1β secretion by NOD splenocytes was increased relative to C57BL/6 controls, between weeks 6 and 16, peaking around 12 weeks of age (Figure 2A), as insulitis develops (43). IL-1β was not over-expressed by splenocytes from NOD.I-A\textsuperscript{k} congenic mice, which do not express the MHC class II proinsulin antigen restriction element, I-A\textsuperscript{g7}, or from pINS.NOD transgenic mice. These mice express mouse proinsulin II under control of the I-E\textsubscript{α} \textsuperscript{k} MHC class II promoter, are tolerant to pro-insulin, and do not develop diabetes (but remain susceptible to other autoimmune diseases) (44; 45). When anti-CD3-stimulated splenocytes were analyzed with intracellular staining and flow cytometry, we found that T cells including both CD4\textsuperscript{+} and CD8\textsuperscript{+} subsets, and APC including CD11c\textsuperscript{+} DC and F4/80\textsuperscript{+} macrophages produced IL-1β (Figure 2B). Together, the data indicate that IL-1β is over-expressed by innate immune cells and T cells, and unexpectedly, that IL-1β over-production during the development of islet inflammation is driven or accelerated by islet autoantigen presentation in the context of the NOD genetic background.

Since sera from patients with or at risk of T1DM, when incubated with healthy PBMC, promote a microarray signature dominated by IL-1β (15), we assessed whether a factor in mouse serum similarly promotes IL-1β mRNA expression when incubated with splenocytes from either C57BL/6 or 6 week-old NOD mice. While serum from diabetic NOD mice induced expression of IL-1β by healthy splenocytes, serum derived from NOD mice during the insulitis phase did not (Supplementary Figure 2). Glucose at concentrations similar to those in diabetic mice or humans promote IL-1 secretion by monocytes in vitro (46). Thus, our data are consistent with the conclusion that islet autoreactivity and not metabolic factors, such as hyperglycemia, drive IL-1β over-expression during the insulitis phase in NOD mice.

**IL-1β blocks Treg function during the insulitis phase in NOD mice.** We next determined the effects of IL-1β in NOD mice. We first tracked the capacity of NOD effector T cells (Teff) to be suppressed by regulatory (Treg) T cells, in parallel to IL-1β production, at different ages in NOD.Lt mice. We incubated syngeneic splenic DC, CD4\textsuperscript{+}CD25\textsuperscript{−} Teff, CD4\textsuperscript{+}CD25\textsuperscript{+} Treg and anti-CD3, and
plotted the percent suppression of Teff by Treg over time (Figure 3). Splenic Treg suppressed proliferation of Teff in vitro before 6 weeks of age to 40% maximal. However, Treg were unable to suppress Teff by 8 weeks of age and this function only returned by 18 weeks, associated with the fall in splenocyte IL-1 secretion (Figure 2A). The same experiments were carried out using cells from NOD mice lacking the IL-1R1 receptor (NOD.IL-1R1−/−). The capacity of NOD.Lt Treg to suppress NOD.Lt Teff was significantly different from the capacity of NOD.IL-1R1−/− Treg to suppress NOD.IL-1R1−/− Teff over the same time period (p<0.001 by 2-way ANOVA). Thus, the reduced capacity of NOD.Lt splenic CD4+CD25+ T cells to suppress Teff is IL-1β and time dependent. The results are consistent with reduction in Treg function in NOD mice in vivo after 4-6 weeks of age (7).

**An inflammatory profile associated with IL-1β.** Given the marked change in pro-inflammatory IL-1β secretion associated with altered Treg function during the insulitis phase in NOD mice, we extended our analysis to determine whether other pro-inflammatory cytokines were produced simultaneously, and whether these could be suppressed by Treg cells. IL-6, TNF, IL-17, IFN-γ and IL-10 were over-expressed at the same time as IL-1β (8-12 weeks). Treg did not suppress these cytokines when added to Teff (Figure 4A). We did not observe similar increases in IL-6, IL-17, IFN-γ or IL-10 when analyzing cells from NOD.IL-1R1−/− mice (Figure 4B). TNF was over-expressed in NOD.IL-1R1−/− mice between weeks 5 and 10, but was suppressed by addition of Treg. IL-23 was undetectable in these supernatants (data not shown). IL-17 production increased again in supernatants from NOD mice around 25 weeks, and in IL-1R1−/−.NOD mice at 18 weeks, concomitant with reduction in Treg function (Figure 4), and heralding the onset of diabetes. Levels of the IL-17-regulatory cytokine, IFN-γ, dropped after 12 weeks, while IL-17 levels increased. The data suggest that in the presence of high levels of IL-1β, Treg are unable to contain the expression of a set of pro-inflammatory cytokines by effector T cells or APC.

**Spontaneous pancreatic islet cytokine production is time dependent and reflects production by stimulated splenocytes.** To determine the relevance of cytokine production stimulated from splenocytes, we isolated pancreatic islets from NOD mice between the ages of 4 and 15 weeks, extracted RNA from whole islet tissue and quantitated IL-1β, TNF, IL-6 and IFN-γ RNA by Taqman real-time PCR. Islet IL-1β and IL-6 mRNA expression increased between 4 and 15 weeks (p<0.05, p<0.0001 respectively, 1-way ANOVA with post-hoc analysis for linear trend); reflecting the time course of stimulated splenocytes (Figure 5). IL-17 signal was too low to amplify from whole islets, likely due to a small proportion of infiltrating Th17, as IL-17 was amplified from stimulated NOD.Lt splenic T cells (not shown). Thus, the Th17-promoting cytokines, IL-1β and IL-6, are also expressed by inflamed islets.

**IL-17 production is promoted by addition of Tregs to Teff in an IL-1β dependent manner at the time of peak insulitis.** We therefore tested the relationship between NOD mouse age, Treg, Teff, IL-1β, and IL-17 production, by adding Treg from NOD or NOD IL-1R1−/− mice to syngeneic Teff stimulated by DC and anti-CD3 (Figure 6). IL-17 increased approximately 3 fold with the addition of Treg cells to Teff from 5 week NOD.Lt mice (Figure 6A). IL-17 secretion was dependent on IL-1β signaling in vitro and in vivo, as anti-IL-1β mAb blocked the secretion of IL-17 in vitro, and NOD.IL1R1−/− T cells did not secrete IL-17. By 10 weeks of age, both Teff and Treg secreted IL-17 and this was no longer dependent on IL-1β in vitro (Figure 6B). However, since T cells isolated from 10
week NOD.IL1R1−/− mice did not secrete IL-17 ex vivo, the data suggest that IL-1β (and concomitant IL-6) produced in response to antigen presentation, promotes the development of Th17 from a young age in NOD.Lt mice through FoxP3+ Treg cell conversion to Th17 (47). To assess this in vitro, Teff and Treg purified from 6 week CD45.1 NOD.Lt, CD45.2.NOD and CD45.1 NOD.IL-1R1−/− mice were stimulated with DC and anti-CD3. Purified CD45.2 CD25− Teff included 4% CD4+FoxP3+ cells (of which <1% were CD25+), and purified CD45.1 CD25+ Treg comprised 86% FoxP3hi and 14% FoxP3lo cells. After 72h, IL-17 was measured in supernatants, and PMA-restimulated cells were stained for CD4, CD45.2, FoxP3 and IL-17. Teff and Treg secreted IL-17 in an IL-1-dependent manner. When Teff were mixed with Treg, IL-17 was secreted if either Teff or Treg but not both lacked IL-1R1 (Figure 6C). The proportion of CD45.1+ FoxP3hi cells fell to 30% in culture (Figure 6D, (i)). Approximately 0.6% of the input CD45.2+ Teff and 1.8% of input CD45.1+ Treg expressed IL-17 (Figure 6D, (ii)). Of the IL-17+ Treg, the majority were FoxP3lo. When all cells were IL-1R1−/−, 0.3% of Teff and 0.1% of Treg cells expressed IL-17 in culture (data not shown). However, if IL-1R1−/− Teff were mixed with wt Treg, 4% of input wt FoxP3lo and FoxP3hi Treg expressed IL-17 (Figure 6D, (iii)), and if IL-1R1−/− Treg were mixed with wt Teff, 0.9% of Teff expressed IL-17 (Figure 6D, (iv)). Thus, IL-1R1−/− Treg were unable to prevent low level of IL-17 production by IL-1R1+ Teff. Moreover, FoxP3 expression is unstable after activation in the presence of effector cells, and conversion of FoxP3hi and FoxP3lo Th17 is IL-1-dependent.

In the IL-1-rich insulitic environment, RelBlo DC reduce suppressive capacity of Treg cells at the expense of Th17 cells. Given the IL-1-dependent dysregulation of Treg, coincident with the failure of RelBlo DC to prevent T1DM, we analyzed the relationship between RelBlo DC administration and Treg number and function. As NOD mice age, Treg lose expression of FoxP3 (48). The frequency of FoxP3+ Treg in spleen and intensity of FoxP3 expression 4 weeks later were not affected by RelBlo DC administration to 4 or 12 week mice (Figure 7A). Treg cells purified from untreated 8 week-old NOD mice suppressed Teff purified from naïve 6 week-old NOD mice, but this was significantly reduced by administration of RelBlo DC 4 weeks previously (Figure 7B). Suppressive capacity of Treg cells purified from 16 week-old NOD mice was reduced relative to the activity of Treg cells purified from 8 week-old mice, and significantly reduced by administration of RelBlo DC 4 weeks previously (Figure 7B). In contrast, suppressive activity of Treg cells isolated from 16 week-old NOD.IL-1R1−/− recipients of RelBlo DC administered 4 weeks earlier and untreated NOD.IL-1R1−/− mice was equivalent (Figure 7B). The data demonstrate that RelBlo DC exacerbate the age-dependent decline in Treg cell function. Similarly, when 6 week wt or IL-1R1−/− mice were untreated or injected with RelBlo DC, IL-17 secretion by splenic Teff and Treg was promoted by RelBlo DC in an IL-1-dependent manner four weeks later (Figure 7C). IL-1 dependent IL-17 secretion by anti-CD3-stimulated pancreatic draining lymph node cells from these mice was greater after treatment with RelBlo DC (Figure 7C). Consistent with this IL-1-dependence, promotion of IL-17 production by RelBlo DC was not altered by treatment of wt recipients with anti-IL-17 mAb (Figure 7D). Consistent with the inability of IL-1R1−/− Treg to suppress IL-17 production by wt cells in vitro (Figure 6), adoptive transfer of wt or IL-1R1−/− Treg purified from 4 week-old donors to 6 week-old recipients did not impact IL-17 secretion in the presence of RelBlo DC (Figure 7D). By staining, IL-17 was almost all produced by host FoxP3lo Treg (data not shown). The data demonstrate that RelBlo DC exacerbate the
age-dependent decline in Treg cell function at the expense of conversion to Th17 in NOD mice including in pancreatic draining lymph node, in an IL-1-dependent manner.

**IL-17 impairs Treg function systemically.** Although IL-1 impairs the suppressive function of Treg (Figure 4)(17), a recent publication showed that inhibition of IL-17 for 10 days in 10 week NOD mice was sufficient to impair progression to diabetes, associated with increased Treg infiltration of islets (49). When we treated 6 week NOD with anti-IL-17, Teff were suppressed by splenic Treg significantly better than those of untreated mice (Figure 8). The data support the conclusion that, in addition to the broad impact of IL-1, IL-17 itself impacts suppression by FoxP3⁺ Treg.

**DISCUSSION**

Many newly-diagnosed T1DM patients exhibit high levels of IL-1β or a microarray signature including IL-1β (22; 50). Although metabolic disturbance is implicated in the IL-1β expression by such patients, there are suggestions of IL-1 over-expression earlier in the disease course. For example, sera from at-risk first-degree relatives induced an IL-1 related gene expression signature, when incubated with healthy PBMC (15). We show here that as NOD mice age, IL-1 is produced systemically, including in the pancreatic islet, and is implicated in the immune dysregulation that occurs as islet autoantigen is presented in at-risk mice, by virtue of their predisposing MHC class II alleles. Tolerant pINS.NOD mice also fail to upregulate IL-1. These data suggest that IL-1 production by self-reactive peripheral blood T cells could be a useful early prognostic indicator in children with high risk MHC alleles – even before development of islet autoantibodies. Not all T1DM patients show elevated IL-1β levels or gene signature. Delayed diabetes progression in NOD.IL1R1⁻/⁻ mice suggests IL-1β is not necessary for this form of diabetes; however, high IL-1 might predict a more aggressive disease course associated with high-risk MHC alleles.

In NOD mice, early IL-1 over-production has functional consequences: systemic (including islet) induction of IL-6 and IFN-γ, reduction in Treg suppressor function, FoxP3 instability, reprogramming of Treg to Th17, and dysregulation of the mechanisms by which tolerizing DC suppress autoimmunity. Similar to NOD mice, IL-6 downregulated FoxP3 expression and (with IL-1 and TGF-β) reprogrammed Treg to Th17 (47; 51-53). Reprogramming is of particular concern in the context of autoimmunity, because FoxP3⁺Treg are selected in the thymus for their reactivity towards self-antigens, and FoxP3⁺Th17 could contribute to pathogenic self-reactivity (54). In EAE, approximately 10% of splenic FoxP3⁺ cells produced IL-17 (47). Th17 differentiation is initiated but not maintained by IL-1 in NOD mice, and can be initiated by other factors in IL-1R1⁻/⁻ NOD mice. Thus, IL-1β accelerates diabetes by reprogramming Treg to Th17 at the expense of suppressor function, between 6 and 18 weeks in NOD mice. Moreover, IL-1 enhances T cell IFN-γ, which drives insulitis, and must be contained by FoxP3⁺Treg (6; 17).

Many immunotherapeutic strategies, which induce Treg, prevent diabetes when administered to young NOD mice but not during insulitis (25; 55-58). This is likely reflected in human T1DM, where the outcome of preventive trials of antigen-specific tolerizing immunotherapy has varied (59; 60). Nasal insulin, which was ineffective, was administered to very high risk children in the late pre-clinical phase (60) when IL-1 was likely already expressed. Similar to previous studies, RelB⁻/⁻ DC immunotherapy in young NOD mice did not depend on presentation of pro-insulin antigen (25). This could be because non-transgenic RelB⁻/⁻ DC present a range of self-antigens which tolerize relevant autoreactive T cells, or because of non-
specific Th2 immune-deviation by DC cultured in FCS, presenting FCS-derived epitopes (61; 62). The latter is less likely because DC cultured without NF-κB inhibitor were effective, and neither Teff nor Treg secreted more IL-4 after administration of RelBlo DC (not shown) (61; 62).

In a recent study, anti-IL-17 mAb administered to 10 week but not 4-5 week NOD mice prevented diabetes (49). The current studies demonstrate that 10 weeks is ideal and 5 weeks is too early to block IL-17. Furthermore, IL-17 may impact the capacity of Teff to be suppressed by Treg. This may be indirect, as anti-IL-17 reduces DC differentiation and promotes IL-10 production (63; 64).

Tolerising DC delivered to young mice likely regulate autoantigen presentation, preventing expression of proinflammatory triggers for Th17. RelBlo DC, which suppress inflammatory arthritis, also prevent diabetes in NOD mice when administered at 4 weeks, but not 14 weeks. At 10-14 weeks, RelBlo DC exacerbated the IL-1-dependent decline in Treg cell function and conversion to Th17. When IL-1β and IL-6 are over-expressed by NOD mice, reprogramming Th17, the ground is no longer fertile for therapies which induce Treg. In contrast, GAD-specific Ig-GAD-immunotherapy, which promotes IFN-γ and blocks IL-17 was only effective when delivered after 8 weeks of age (65). Our data in NOD mice have important implications for appropriate timing of immunotherapy in humans at risk of T1DM. IL-1 and IL-17 would be interesting early biomarkers in children at risk of T1DM to identify similar pathogenetic stages and to stratify treatment to tolerizing immunotherapy, or cytokine blockade (66).

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**Figure Legends**

**Figure 1:** RelBlo tolerizing DC inhibit diabetes when administered to young, but not insulitic NOD mice.
RelBlo DC were generated from BM of NOD or pINS.NOD transgenic mice in the presence of GM-CSF, IL-4 and Bay11-7082. Control DC were generated from the same mice in the presence of cytokines and the absence of Bay11-7082. Twenty eight day-old (A, B) or 100 day-old female NOD (C, D) mice were injected s.c. with 5 x 10⁵ DC. Mice were screened weekly for diabetes until 250 days of age. Diabetes incidence curves are shown, for groups each containing 12 mice. * P<0.05 (Kaplan Meier survival analysis with Bonferroni correction for multiple groups). Insulitis was assessed at 12 weeks (E) in 4 female NOD mice per group treated at 4 weeks of age with DC as shown. Data represent mean ± SD.

**Figure 2.** Secretion of IL-1β by NOD splenocytes in response to anti-CD3ε antibody during the phase of insulitis does not occur in the absence of autoantigen presentation and responding T cells.
A: Splenocytes from NOD.Lt, C57Bl/6, NOD.I-A<sup>k</sup> or pINS.NOD mice of different ages were incubated for 24 hours in presence of anti-CD3ε antibody, IL-1β was assayed in the supernatants of 3 mice per group by ELISA. B: NOD.Lt splenocytes were incubated with anti-CD3ε antibody for 24 hours, the last 4 hours in presence of Brefeldin A 5µg/mL, before staining for surface markers as shown and intracellular IL-1β. Representative of 4 experiments

**Figure 3. Transient inhibition of Treg cell function is IL-1β-dependent, corresponding to the peak of insulitis.**

CD11c<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> cells were purified from spleens and lymph nodes of NOD mice of different ages (4 mice pooled per group), using immunomagnetic separation. One hundred thousand CD11c<sup>+</sup> DC, 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>−</sup> T cells and 7.5x10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells were incubated for 3 days in presence of anti-CD3ε antibody before determination of the T cell proliferative response by [³H]thymidine uptake. The regulatory capacity is expressed as % suppression of T cell proliferation in presence of CD4<sup>+</sup>CD25<sup>−</sup> T cells relative to the maximum proliferation observed in presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells and DC alone. Data from 3 separate experiments are expressed as mean ± SEM % suppression. *** p <0.0001 analyzed by 2-way ANOVA comparing NOD and IL-1R1<sup>−/−</sup> NOD over time.

**Figure 4. IL-1β drives IL-6, TNF, IL-10 and IL-17, which are not subject to Treg suppression, as insulitis develops in NOD mice.**

Cytokines were assayed in supernatants from the T cell proliferation assays (Figure 3) from mice of the ages indicated, using CBA kits and IL-17 ELISA. ○ cytokine production by CD4<sup>+</sup>CD25<sup>−</sup> T cells in the absence of CD4<sup>+</sup>CD25<sup>+</sup> Treg, ♦ cytokine production by CD4<sup>+</sup>CD25<sup>−</sup> T cells in the presence of CD4<sup>+</sup>CD25<sup>+</sup> Treg. A: wt mice, B: IL-1R1<sup>−/−</sup> mice.

**Figure 5. Age-dependent expression of pro-inflammatory cytokines by pancreatic islets**

Pancreatic islets were purified from NOD.Lt mice (n=5 per group) of the ages shown and RNA was analyzed by Taqman PCR for relative expression of IL-1β, IL-6, TNF, and IFN-γ. * p <0.05, *** p <0.0001 (one-way ANOVA with post-hoc analysis for linear trend).

**Figure 6: IL-17 is produced by Teff and reprogrammed Tregs in an IL-1β dependent manner during insulitis.**

IL-17 was assayed at 5 (A) or 10-14 weeks (B) in supernatants from the T cell proliferation assay by ELISA, from NOD.Lt cells with or without anti-IL-1 or anti-IL-17 mAb (10µg/mL), or from NOD.I-L1R<sup>−/−</sup> cells. C: CD4<sup>+</sup>CD25<sup>−</sup> Teff and CD4<sup>+</sup>CD25<sup>+</sup> Treg purified from 6 week CD45.1 NOD.Lt, CD45.2.NOD and CD45.1 NOD.I-L1R<sup>−/−</sup> mice were stimulated with DC and anti-CD3. After 72h, IL-17 was measured in supernatants. *** p <0.0001 (1 way ANOVA). D: Cells from the same experiment were restimulated with PMA in the presence of brefeldin A and stained for CD4, CD45.2, FoxP3 and IL-17 (D). Cells are gated on CD4 and the relevant congenic marker to analyze Teff and Treg FoxP3 and IL-17 expression individually.

**Figure 7: In the IL-1-rich insulitic environment, tolerising DC reduce suppressor function at the expense of IL-17**

Four week-old, 12 week-old NOD mice, and 12 week-old NOD.I-L1R<sup>−/−</sup> mice were injected s.c. with RelB<sup>lo</sup> DC or saline. A: The % CD4<sup>+</sup> cells expressing FoxP3 in spleen and the mean fluorescence intensity (MFI) of FoxP3 expressed by CD4<sup>+</sup> T cells were enumerated. B: Four weeks later, CD4<sup>+</sup>CD25<sup>−</sup> T cells were isolated from spleen and varying numbers were added to 1 x 10<sup>5</sup> CD11c<sup>+</sup> splenic DC and 1x10<sup>5</sup> CD4<sup>+</sup>CD25<sup>−</sup> T cells purified from naïve 6 week NOD mice, and stimulated with 0.5µg/mL anti-CD3. Proliferation was assessed by incorporation of [³H] thymidine. Data represent the mean of triplicate wells ± SEM. 6-9 mice were pooled in each of two separate experiments. *** p < 0.0001 (two-way ANOVA). 6 week wt or IL-1R1<sup>−/−</sup> mice were
untreated or injected with RelB$^{lo}$ DC. As DC were administered, some groups of wt mice were administered anti-IL-17 mAb i.p. on alternate days for 10 days, or CD4$^{+}$CD25$^{+}$ Treg purified from either wt or NOD.IL-1R1$^{-/-}$ mice i.v. once. After 4 weeks, Teff and Treg were purified from each group and incubated with DC in the presence of anti-CD3. IL-17 levels were measured in supernatants by ELISA * p <0.05, *** p<0.0001 (one way ANOVA) (C) and cells restimulated with PMA in the presence of brefeldin A were stained for CD4, CD45.2, FoxP3 and IL-17 (D).

**Figure 8. Enhanced Treg suppressive capacity in response to anti-IL-17.**

CD4$^{+}$CD25$^{+}$ and CD4$^{+}$CD25$^{-}$ T cells purified from spleens of wt mice administered anti-IL-17 mAb i.p. on alternate days for 10 days 4 weeks prior at age 6 weeks, were incubated alone or in a 1:1 ratio with DC and anti-CD3. Proliferation was assessed by incorporation of $[^{3}H]$ thymidine and mean ± SEM; % suppression was calculated. Three mice were pooled in each of two separate experiments. * p <0.05 (t test).

**REFERENCES**


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Figure 1

A

B

C

D

E

- NOD DC
- NOD RelB<sup>lo</sup> DC
- PBS
- pNS.NOD DC
- pNS.NOD RelB<sup>lo</sup> DC

Percent diabetic

Age (days)

Percent diabetic

Age (days)

Percent diabetic

Age (days)

Percent diabetic

Age (days)

DC from pNS.NOD

DC from NOD

Pbs

DC

RelB<sup>lo</sup> DC

Insulitis score
Figure 2

A

![Graph showing IL-1β levels vs. mice age (week) with different mouse strains indicated.] (NOD, pINS.NOD, C57BL/6, NOD.I-A^k)

B

![Flow cytometry plots showing CD4, CD8, CD11c, and F4/80 staining with IL-1β levels on the x-axis.] (Values: CD4: 20.2, 9.4, CD8: 19.5, 4.6, CD11c: 2.9, 1.2, F4/80: 11.1, 4.0)
Figure 3
Figure 4

A

IL-6

IL-10

TNF

IFN-γ

IL-17

IL-12p70

IL-4

MCP-1

IL-2

○ no Tregs  ♦ 75 000 Tregs

weeks

B

IL-6

IL-10

TNF

IFN-γ

IL-17

IL-12p70

IL-4

MCP-1

IL-2

○ no Tregs  ♦ 75 000 Tregs

weeks
Figure 7

A

B

C

D

Figure 8

- NOD.Lt
- anti-IL-17 treated NOD.Lt

% suppression

[^3]H-thymidine uptake (cpm)