Dr. Vera Usuelli FO.DI.RI Fellowship Scientific Report (2015-2016)

Main project

Unveiling the antigen specific Bregs defect in autoimmune diabetes

Objective

B lymphocytes (B cells) are relevant antigen-presenting cells that contribute to the onset of type 1 diabetes (T1D), and their depletion prevents and cures autoimmune diabetes in non-obese diabetic (NOD) mice[1,2]. Recent findings indicated B-cells as potential regulatory cells[1,2]. In T1D patients treated with Rituximab (anti-CD20) B-cell depletion slows C-peptide loss but does not cure T1D[3]. Our group and others showed that a pool of B-cells, re-emerging after B-cell depletion, exhibits regulatory function in NOD mice[1,2] and that B-cell-depleted patients display a paradoxical increase in the T-cell anti-islet immune response[4]. Our hypothesis is that a defect in Breg generation exists in T1D and that antigen-specific Bregs may be expanded, newly generated and used to re-establish tolerance toward islet autoantigens in T1D. We plan to characterize Bregs and the mechanism behind their defect in T1D in order to establish a reliable and reproducible method to generate Bregs from T1D individuals, aiming at a novel cure for T1D.

Background and current status of research

Although T1D has been described as a $CD4^+$ T cell-mediated disease, B-cells appear to play a crucial role in the autoimmune destruction of pancreatic islets[1,5,6]. B-cells may promote T1D by (i) presenting islet-derived peptides to autoreactive T-cells, (ii) producing autoantibodies against β-cell antigens and (iii) secreting pro-inflammatory cytokines[1,6-10]. B-cell depletion in NOD mice, as well as neutralization of the B-cell activating factor, delays diabetes onset in pre-diabetic NOD mice and re-establishes normoglycemia in hyperglycemic NOD mice[1,2,11-13], while B-cell depletion in individuals with new-onset T1D prevents decline of the islet mass[3]. Recent evidence supports the existence of subpopulations of Bregs, which, like regulatory T-cells (Tregs), are able to modulate immune responses in an antigen-specific manner[14-18]. Preclinical and clinical studies confirm that immature, reemerging B-cells display an immunosuppressive phenotype, which may contribute to the clinical efficacy of B-cell depletion therapies[1,2,14]. To date, several populations of murine Bregs have been described based on the expression of various surface markers[16,18,19], however IL-10 expression appears to convey Breg immunosuppressive functions[14,20].

Specific aims

In long-term normoglycemic NOD mice, naturally occurring Bregs arise within a repertoire of highly activated antigen-specific B-cells, thereby protecting NOD mice from developing autoimmune diabetes. Interestingly, the B-cell phenotype/function of T1D individuals and of siblings with anti-islet autoantibody but without T1D parallels that of hyperglycemic and naturally protected NOD mice, respectively. Overriding the defect of antigen specific Bregs in T1D individuals may be used to re-establish tolerance, thus providing the proof-of-principle to translate to the clinic an antigen-specific Breg-based cell therapy for T1D.

To test this hypothesis, we propose the following Specific Aims:

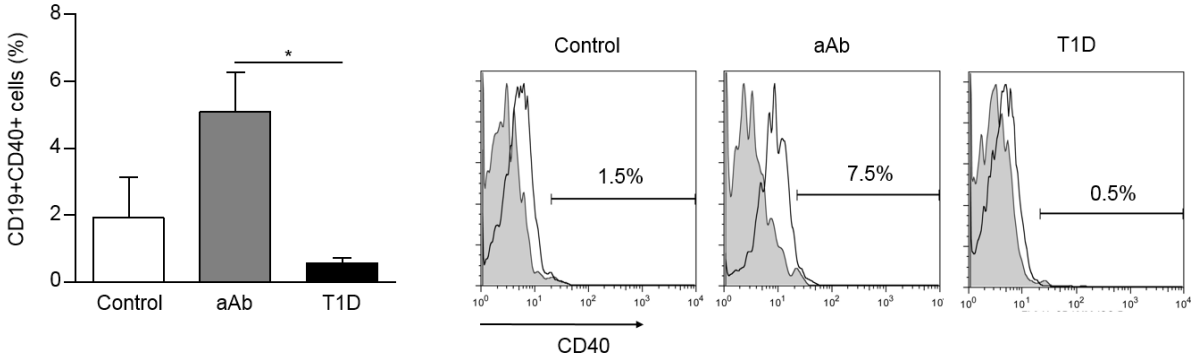
Specific Aim 1. To profile naturally occurring Bregs in T1D individuals;

Specific Aim 2. To establish a protocol for antigen-specific Bregs generation *ex vivo* and test Bregs immunoregulatory properties in clinically relevant autoimmune assays.

Results

Human Studies

We evaluated the B-cells phenotype in patients with T1D. We performed, a head-to-head comparison between B-cells obtained from PBMCs of patients with T1D (n=3), of subjects with autoantibodies (aAb) but without T1D (n=4), and of healthy controls (n=4) using FACS analysis for the most relevant surface markers (CD40, CD80, CD86, MHC class II). In patients with T1D, we observed reduced CD19+CD40+ cells expression (%) on peripheral B-cells as compared to those with autoantibodies but without T1D and to healthy controls.



We then assessed *the B-cells regulatory function in patients with T1D.* We sorted $CD19^+CD24^+CD27^+$ Bregs cells (5% of B-cells) and tested for their immunoregulatory properties *ex vivo*. During a preliminary anti-CD3/-CD28 assay, we found that Bregs from patients with T1D were less capable of abrogating IFN-γ production from stimulated T-cells, thus suggesting a possible defect in Bregs function.

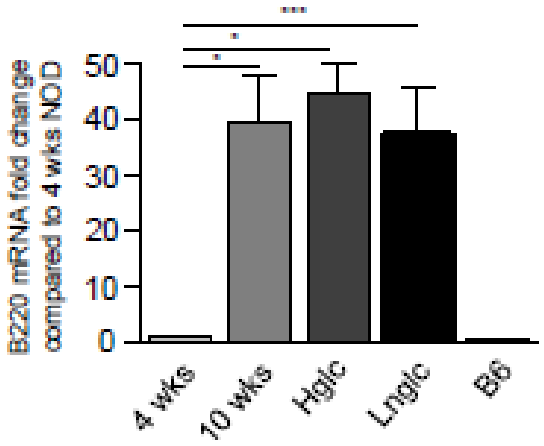
Murine studies

While conducting human studies, we also performed *in vivo and ex vivo* experiments on the NOD mouse, the murine model of autoimmune T1D.

The data published by our group suggest that naturally occurring Bregs may arise within pancreatic islets in long-term normoglycemic NOD mice in the presence of an antigen-driven clonal selection and with maintenance of CD40 signaling, thus preventing the onset of spontaneous autoimmune diabetes. These islet-harbored Bregs have been characterized in our lab, and they represent the proof-of-concept that the biological platform for Breg generation does exist *in vivo.* We will thus move to peripheral Bregs ($CD19^+CD1d^{hi}CD5^+$ cells), which appear capable of being more easily and more practically used, particularly from a clinical point of view.

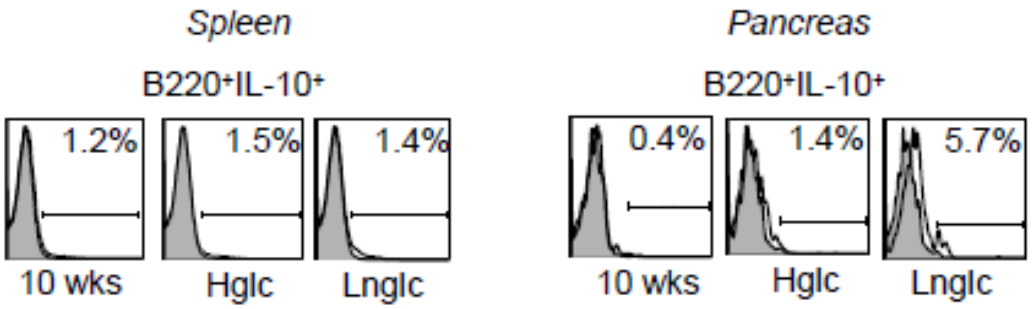
Ex vivo studies

Long-term normoglycemic NOD mice exhibit preserved islet B-cell infiltrate compared to hyperglycemic NOD mice: Long-term normoglycemic NOD mice display preserved islet morphology compared to hyperglycemic mice and are protected from severe infiltration. When B220 expression was analyzed by rt-PCR, a ~40 fold increase in B220 expression was detected in hyperglycemic (hglc) and long-term normoglycemic NOD mice (Lnglc) compared to 4- and 10-week-old normoglycemic NOD mice ($p<0.01$)



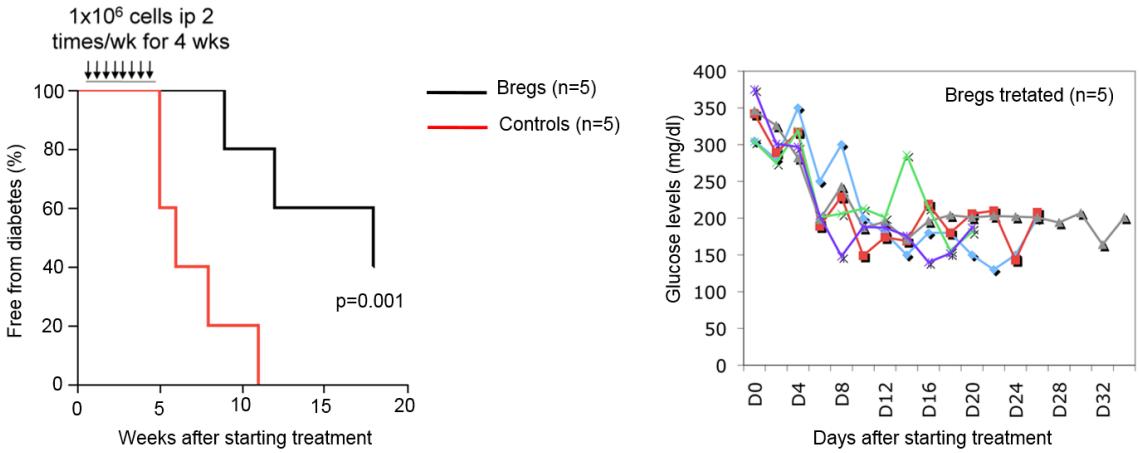
Long-term normoglycemic NOD mice exhibit greater percentages of Bregs compared to hyperglycemic NOD mice: Bregs ($B220^+IL\text{-}10^+$ cells) were quantified by FACS. 10-week-old normoglycemic, hyperglycemic and long-term normoglycemic NOD mice display a similarly low percentage of splenic $B220^+IL\text{-}10^+$ cells (Bregs). However, the percentage of islet-harbored Bregs was significantly higher in long-term normoglycemic NOD mice compared to both hyperglycemic animals and 10-week-old normoglycemic (10-week-old): (0.4% 10-week-old, 1.4%, hyperglycemic

5.7% long-term normoglycemic NOD mice). A similar pattern was evident in the population of $B220^+CD93^+CD23^+IgM^+$ cells (anergic B-cells), which were found in greater numbers in long-term normoglycemic NOD mice compared to hyperglycemic and 10-week-old normoglycemic mice.

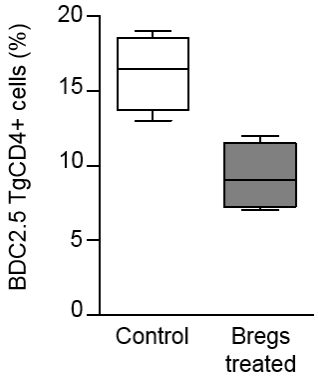


In vivo studies

We evaluated the effects of the peripheral Bregs cells on the diabetes incidence in overtly hyperglycemic NOD mice. We treated overtly hyperglycemic NOD mice (with glucose levels higher than 250 mg/dl for 3 consecutive days) with $CD19^+CD1d^{hi}CD5^+$ cells (peripheral Bregs), ($1x10^6$ cells ip twice per week for 4 weeks) from normoglycemic NOD mice. We observed in 5 out of 5 Bregs-treated NOD mice reversal of hyperglycemia.



We also assessed the peripheral Bregs effect on the autoreactive T-cells in vivo. We tracked the effects of peripheral Bregs treatment on proliferation of autoreactive BDC2.5 TCR Tg $CD4^+$ cells by using an anti-ideotypic antibody against Vβ4. NOD.SCID mice were reconstituted with splenocytes from normoglycemic NOD 10-week-old mice. After 7 days (thereby allowing reconstitution of the immune system), mice were either treated with Bregs or were left untreated, and isolated BDC2.5 TCR Tg $CD4^+$ cells were labeled with CFSE and transferred into treated or control NOD.SCID mice. After 72 hours, mice were examined for autoreactive $CD4^+$ cells, and the percentage of cells was evaluated. Interestingly, fewer Tg $CD4^+$ cells were recovered from Bregs-treated hosts.



Novelty and importance of this work
The discovery of novel therapeutics aimed at re-establishing tolerance toward islet autoantigens in T1D is necessary. No trials examining the use of Bregs in T1D can be found on the ClinicalTrials.gov database. Most trials associated with B-cells suggest B-cell depletion, without mentioning the different subsets of B-cells. This may be due to the uncertainty surrounding Bregs and to the absence of clear evidence of Bregs in T1D. Our data suggest that naturally occurring Bregs endowed with immunoregulatory properties exist and may protect NOD mice from diabetes. If successful, our approach has considerable translational potential, as the infusion of autologous *ex vivo* expanded or newly generated Bregs will be theoretically feasible.

Other projects:

1) miR-21 in chronic allograft vasculopathy
Background. Heart transplantation is the most effective therapy to prolong life expectancy in patients with end-stage heart failure. However, despite much progress in the field, allograft survival has not improved and chronic allograft vasculopathy (CAV) still limits long-term allograft survival, whose molecular pathogenesis remain largely unknown. MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression post-transcriptionally and are emerging as master regulators of the immune response.
Methods. We used a well-characterized murine model of CAV, the minor MHC Class II mismatch bm12 into C57BL/6 model, to explore the miRnome in CAV.
Results. We first profiling the miRnome in human and murine transplanted hearts. We identified 192 miRNAs that appeared specifically up- or downregulated in murine transplanted allograft. 29 of these miRNAs were upregulated in the syngeneic as well. Among the remaining 163 miRNAs, miR-21 was the most highly expressed in CAV with a 20-fold increase compared to syngeneic bm12 transplanted hearts. The data was confirmed in human transplanted heart as well. Targeting miR-21 with a specific anti-miR-21 oligonucleotides promotes long-term allograft survival in 100% of bm12 into B6 cardiac transplanted mice (compared to control anti-miR). This indefinite survival was associated with a reduced macrophage infiltration and fibrosis. Interestingly, macrophage appeared highly expressing miR-21 and *in vivo* injection of Cy3-labeled anti-miR-21 oligonucleotides showed a complete macrophage uptake. Anti-miR-21 oligonucleotides, reshape macrophage phenotype, reduced phagocytic and migration macrophage capacity, with no effect on allopresentation ability. The conditional deletion of miR-21 with the use of the miR-21$_{flox}$LysM$_{Cre}$ B6 mice is ongoing to finally confirm the role of miR-21 in CAV.
Conclusion. Macrophage miR-21 upregulation is evident in CAV and targeting miR-21 with a specific anti-miR-21 oligonucleotides, may represent a novel therapeutic tool to abrogate the onset of CAV

2) The role of P2X7 pathway in chronic allograft vasculopathy
This is a study where healthy controls, heart transplanted patients with regular function and heart transplanted patients with chronic allograft vasculopathy have been enrolled. We are exploring whether expression of P2X7 receptor is correlated with chronic allograft vasculopathy occurrence. Our preliminary results show that P2X7 receptor is up-regulated in chronic allograft vasculophaty as compared to controls and to heart transplanted patients with regular function and that P2X7R loss-of-function mutation is associated to increased Th1 and Th17 generation. Our data demonstrated that the absence or mutation of P2X7R induces alteration in the T cell metabolic state and increases Th1/Th17 cell generation, ultimately accelerating CAV and shortening the survival of cardiac allografts. We plan to address mechanisms behind this and whether targeting P2X7 receptor may cause inhibition of the pro-inflammatory alloimmune response

Dr. Vera Usuelli FO.DI.RI Fellowship Economic Report (2015-2016)

19,450 Euro: Salary for Research Fellow (Dr. Vera Usuelli) for 12 months.

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