Short communication

CD8 T-cell reactivity to islet antigens is unique to type 1 while CD4 T-cell reactivity exists in both type 1 and type 2 diabetes

Ghanashyam Sarikonda a,1, Jeremy Pettus a,b,1, Sonal Phatak b, Sowbarnika Sachithanantham a, Jacqueline F. Miller a, Johnna D. Wesley c, Eithon Cadag d, Ji Chae b, Lakshmi Ganesan b, Ronna Mallios a, Steve Edelman b, Bjoern Peters a, Matthias von Herrath a,c,*

* Corresponding author: La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA
1 These authors contributed equally.

1. Introduction

Epidemiological studies indicate that the incidence of type 1 (T1D) and type 2 diabetes (T2D) is increasing [1,2]. While both conditions share the end result of hyperglycemia, they are classically defined as distinct clinical entities. Generally, T1D is considered an autoimmune disease [3] while T2D is thought to be a metabolic disorder driven by insulin resistance. However, recent postmortem studies suggest that β-cell destruction occurs even in T2D subjects [4,5]. Additional studies have found evidence of islet autoimmunity in patients with T2D [6,7]. Collectively, these findings support the hypothesis that islet-directed autoimmunity could play a role in both T1D and T2D. This is evident clinically with the existence of overlapping syndromes such as latent autoimmune diabetes of adulthood (LADA), lean type 2 diabetics, and ketosis-prone T2D. Our ability to detect autoimmunity in diabetes is currently limited to determining the presence and titer of classic T1D autoantibodies. While these antibodies have proven invaluable in predicting disease onset, their ability to predict disease course post-onset is severely limited (discussed in Ref. [8]). Therefore, a need exists to develop a biomarker that will indicate underlying autoimmunity, changes as the disease progresses, and can be followed serially for evidence of therapeutic efficacy.

In T1D subjects, frequencies of islet antigen reactive CD4+ and CD8+ T-cells [9–12] are higher compared to healthy donors (HD), and thus, have the potential to function as such a biomarker (discussed in Ref. [13]). However, how these parameters differ between patients with T1D and T2D is largely unknown. Further, previous cross-sectional studies have not taken into account the inherent biological variation within a given subject that could lead to misinterpretation of results due to stochastic fluctuations (discussed in Ref. [14]). In the current report, we followed subjects with T1D or T2D and HDs over a one-year period with monthly blood draws. At each visit, we determined IFN-γ and IL-10 cytokine...
production by CD4+ T-cells in response to diabetes-associated antigens, and frequencies of autoreactive CD8+ T-cells against HLA-A2-restricted antigenic epitopes. To improve the robustness of the data and reduce the influence of inherent subject-specific variation, data from all available visits for each subject were averaged. Our intent in this study was to determine features of autoimmunity that are potentially shared between both disease states and those that are unique to T1D. If a unique feature of T1D could be identified, this would help to define a true biomarker for T1D. We found that CD4+ T-cell reactivity to islet antigens was shared between T1D and T2D while increased CD8+ T-cell autoreactivity was unique to subjects with T1D.

2. Materials and methods

2.1. Subject recruitment, study design, and scheduled blood draws

We enrolled subjects clinically defined as having T1D (n = 33) or T2D (n = 15), along with HD (n = 10) as controls. Patients were classified as T1D or T2D by their referring practitioner using commonly accepted clinical criteria including age at diagnosis, initial presentation, autoantibody status, family history, dependence on exogenous insulin, and BMI. Diabetic donors were recruited at the Veterans Affairs (VA) hospital in San Diego, La Jolla Institute for Allergy and Immunology (LIAI), and at Taking Control of Your Diabetes educational conferences. Protocols were approved by the University of California-San Diego and LIAI Institutional Review Boards. Informed consent, study identification numbers, clinical case histories, and other information were collected and recorded by clinical investigators. HDs were recruited from a normal blood donor program at LIAI and did not require separate consent forms. To maintain the anonymity of HDs, only the age and sex of the donors were recorded. Use of glucocorticoids at the time of study served as an exclusion criterion. HD and T1D cohorts in this study have also been described in a related paper (Sarikonda et al., submitted to *PLoS ONE*). Each subject had ~40 mL non-fasting blood drawn once every month for 12 months.

2.2. PBMC isolation

Isolation of PBMCs using Ficoll–Paque (Sigma) density gradient centrifugation was performed as described [15]. PBMCs were adjusted to a concentration of 8 × 10^4/mL in AIM-V medium (Invitrogen Life Sciences, San Diego, CA) and used fresh for ELISpot or cryopreserved in AIM-V medium containing 40% FCS and 10% DMSO and stored in liquid nitrogen for up to 18-months prior to analysis.

2.3. HLA-typing

Donors were first screened using an anti-HLA-A2 antibody (eBioscience, San Diego, CA) and flow cytometry. Those who were positive were further typed using genomic DNA. All enrolled donors had HLA-DR typing performed. HLA-A2 and HLA-DR typing from genomic DNA was performed as described [16].

2.4. ELISpot

The ELISpot assay was performed according to the original standardized reports [9] with minor modifications. High purity (~95%, Genscript) DR4-restricted epitopes of IA-2 (752–775) and proinsulin (C19–A3), as well as the DR3-restricted GAD65 epitope (335–352), were used to stimulate PBMCs [15]. A pool of immunogenic viral peptides (CEF) was used as a positive control (Mabtech Inc., Mariemont, OH) and cells incubated only in AIM-V medium served as a negative controls. Development of IFN-γ and IL-10 spots was performed using U-Cytech antibodies and reagents as described [15]. Spots above 65-μM in size were counted as

Table 1

<table>
<thead>
<tr>
<th>Subject</th>
<th>HD</th>
<th>T1D</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of males (%)</td>
<td>1 (10)</td>
<td>18 (55)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>Age (Yrs)</td>
<td>31.3 ± 5.27</td>
<td>46.06 ± 15.91</td>
<td>60.47 ± 8.48</td>
</tr>
<tr>
<td>Disease duration (Yrs)</td>
<td>N/A</td>
<td>18.55 ± 14.93</td>
<td>14.57 ± 1.98</td>
</tr>
<tr>
<td>BMI</td>
<td>N/A</td>
<td>25.39 ± 3.04</td>
<td>35.43 ± 7.00</td>
</tr>
<tr>
<td>Insulin dose (U/kg)</td>
<td>N/A</td>
<td>40.48 ± 16.56</td>
<td>90.09 ± 75.11</td>
</tr>
<tr>
<td>TDD insulin (U/kg)</td>
<td>N/A</td>
<td>0.525 ± 0.22</td>
<td>0.75 ± 0.49</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>N/A</td>
<td>7.039 ± 1.22</td>
<td>7.45 ± 0.26</td>
</tr>
<tr>
<td>Number of males (%)</td>
<td>1 (10)</td>
<td>18 (55)</td>
<td>10 (67)</td>
</tr>
</tbody>
</table>

Mean ± SD values are shown. N/A represents not available (BMI and HbA1c for HD) or not applicable (Duration and Insulin usage for HD).

Please cite this article in press as: Sarikonda G, et al., CD8 T-cell reactivity to islet antigens is unique to type 1 while CD4 T-cell reactivity exists in both type 1 and type 2 diabetes, Journal of Autoimmunity (2013), http://dx.doi.org/10.1016/j.jaut.2013.12.003
cytokine spots using the KS-ELISPOT reader [16] (Zeiss, Munich, Germany). All epitopes were run in triplicate and averaged.

2.5. Qdot-HLA-A2-multimer assay

HLA-A2-restricted peptides (>95% purity, Genscript) from islet antigens and their labeling with quantum dots (Qdots) have been described previously [12]: insulin (B-chain, aa 10–18), IA-2 (aa 797–805), IGRP (aa 265–273), PPI (aa 15–23), GAD65 (aa 114–123), and pp1APP (aa 5–13). Peptide-loaded HLA-A*0201 monomers were produced by the NIH Tetramer Core Facility (Emory University, Atlanta, GA), according to standard protocols, http://tetramer.yerkes.emory.edu/client/protocols. Streptavidin-conjugated Qdots (Qdot-585, -605, -655, -705, and -800; Invitrogen, San Diego, CA) were added to achieve a 1:20 streptavidin-Qdot:biotinylated-pHLA ratio to generate multimeric HLA-A2-peptide complexes. Each HLA-A2-monomer was labeled with two different Qdots and each peptide-multimer had its own unique Qdot combination [12]. For each HLA-A2+ subject, samples from all available visits were labeled, acquired and analyzed simultaneously, as described [12]. Briefly, cryopreserved PBMC were thawed and 2 x 10^6 cells were immediately stained first with all Qdot-labeled multimers (0.1 μg each multimer) followed by Alexa Flour-700-CD8 along with FITC-labeled anti-CD4, -CD14, -CD20, -CD40, and -CD16 antibodies (eBiosciences, San Diego, CA) and resuspended in PBS supplemented with 0.5% BSA and containing 7-AAD (eBioscence, San Diego, CA, USA). Sample data were acquired using an LSRII (BD Biosciences, San Diego, CA) with the following filter settings; for the 488-nm laser: Qdot 655, 635LP, and 655/20. For the 405-nm laser: QD800, 770LP, 800/30; QD705, 685LP, 710/40; QD605, 595LP, 605/20 and QD585, blank, and 585/22.

2.6. Statistical analysis

All data were analyzed and statistical analyses performed using GraphPad Prism 5 software (GraphPad Software, Inc.). Additional topological-based data analyses were conducted using the Ayasdi IRIS software (Ayasdi, Inc.). Statistical significance was determined using Spearman’s Rho test, Mann–Whitney test, unpaired t-test, and ANOVA.

3. Results and discussion

Herein we described CD4+ and CD8+ T-cell islet antigen reactivity in a cohort of patients with T1D compared to T2D and HDs. Our study design provided a robust amount of data in that each patient was followed for one year with monthly blood draws, and to our knowledge, is the first study comparing these immunological parameters between disease states longitudinally. We found that only subjects with T1D consistently had increased numbers of circulating, autoreactive CD8+ T-cells yet both T1D and T2D subjects had similar autoreactive CD4+ T-cell-derived cytokine production.

We recruited 33 subjects with T1D, 15 with T2D, and 10 HD as controls. A summary of participants’ baseline characteristics is shown in Table 1. Detailed subject profiles with age, duration of disease, HLA phenotypes, and autoantibody status are listed in Table S1. A higher proportion of T1D donors were HLA-DR4+ and HLA-A2+ (58% and 49%) compared to T2D donors (27% each) or HDs (14% and 40%); this was expected given the known association between HLA and T1D [17–19].

Extensive validation of the indirect ELISpot assay [9] showed low intra- and inter-assay co-efficient of variation in assay performance (7.3% and 13%, respectively, data not shown). We then determined IFN-γ and IL-10 cytokine production from CD4+ T-cells after stimulation with two DR4-restricted peptides (PI19–32 & IA-2/752–775) and one DR3-restricted epitope (GAD65335–352). As previous studies have shown that these peptides are also reactive in some DR4/DR3-negative donors, we included data from all donors regardless of their DR3/4 status in this first analysis. Net spot numbers (spots with antigen – spots in media) against all three epitopes were pooled at each visit and averaged over all visits. Surprisingly, T2D subjects had significantly more IFN-γ producing CD4+ T-cells than controls (Fig. 1A, left panel). In contrast, T1D subjects (but not T2D subjects) had more IL-10 producing CD4+ T-cells than control subjects (Fig. 1A, right panel). Importantly, while cytokine production differed between control subjects and those with diabetes, there was no statistically significant difference between the T1D and T2D cohorts in either IFN-γ or IL-10 production. Further, in T2D subjects, higher IFN-γ production by CD4+ T-cells suggests the existence of either general inflammation or a degree of underlying autoimmunity. In support of the latter, a
Fig. 3. Distinct populations comprise the T1D and T2D cohorts and highlight the existence of unique immune profiles. A: Distinct sub-groups within each disease type were identified when the data (ELISpot + Clinical Parameters + Additional Immune Response Data) were used to construct the topological network. The similarity metric and mathematical functions used to generate the topological network were normalized cosine (metric) and principal and secondary principal components (embedded). B: The subgroups identified in A differed from each other in the mean total spot count, regardless of stimulating antigen.
significant proportion of T2D patients exhibited T-cell proliferation when exposed to human islet preparations [20].

To analyze our data more stringently, we assessed cytokine production against the DR4-restricted epitopes (PLC19-A3 & IA-2β275-277) in only DR4+ subjects (T1D n = 19, T2D n = 4). As with the analysis of entire cohort above, there were no significant differences in cytokine production between T1D and T2D cohorts in this subgroup whether we assessed the average of net spots or Stimulation Index (SI) values over all visits (Fig. 1B). Thus, CD4+ T-cells from HLA-DR4+ T1D or T2D subjects appear to elicit similar cytokine production in response to islet antigens. This finding argues for a potential shared component of immune dysregulation between the disease states.

Next, to determine antigen-specific CD8+ T-cell frequencies, we employed HLA-A2-Qdot-multimer assay [12]. Mean frequencies of each of the six epitope-specific CD8+ T-cells averaged over all visits were higher in the T1D cohort compared to HD or T2D cohorts (Fig. 2A and data not shown). Using a positivity cutoff of 0.01% of CD8+ T-cells [12], the T1D cohort had greater number of visits (events) positive, in all but one of the antigen epitopes, compared to HD and T2D cohorts (Fig. 2B and data not shown). Further, when all antigen specificities were pooled, the distribution and mean number of positive events were higher in T1D subjects (Fig. 2C); however, these differences were not statistically significant. Upon setting a higher positivity threshold (>0.02% of CD8+ T-cells), the increase in CD8+ T-cell frequencies in T1D donors became apparent as they had significantly higher number of positive events compared to T2D and control donors. Taken together, this data suggests the increase in frequency of islet antigen-specific CD8+ T-cells is specific to T1D subjects, compared to HDs or T2D subjects.

While both T1D and T2D result in relative insulin deficiency, the underlying β-cell destruction in T1D is much more rapid and severe (reviewed in Ref. [21]). Additional analyses using a topological approach for detecting nonlinear patterns in high-dimensional data in an unsupervised fashion [22,23] revealed the possibility of distinct subpopulation clusters within the T1D and T2D cohorts, but not the HD group (Fig. 3A). These unique clusters were characterized by cytokine response, hinting at underlying differences in immune function within both T1D and T2D populations that may impact disease progression (Fig. 3B).

We hypothesize that CD4+ T-cell activation is common to both forms of diabetes resulting in a mild degree of β-cell loss and/or dysfunction. There is a range of inflammation that was easily seen in the topological analyses but has not been appreciated previously. In contrast, CD8+ T-cell activation is a feature unique to T1D and leads to the massive β-cell destruction characteristic of this disease. We propose a simplified model (Fig. S1) in which a primary insult to the islet results in release of autoantigens. This initial insult may be a metabolic derangement such as obesity-induced lipotoxicity and/or dysregulated K+ ATP channel [24–26] for T2D; whereas in T1D it may be environmental or viral infections [27,28]. The exact mechanisms for β-cell injury are multifactorial and complex but, regardless of the insult, the released autoantigens are taken up by antigen presenting cells (APCs) that migrate to pancreatic lymph nodes and activate T-cells. CD4+ T-cells are activated in both T1D and T2D and result in inflammation that promotes functional β-cell decline. The initiation of CD8+ T-cell activation, in contrast, is specific to the T1D disease process. It has been shown that the majority of patients with T1D have CD8+ T-cell infiltrates in the islets. This is most pronounced in patients close to diagnosis but also in a portion of patients with longstanding disease [29]. Similarly, cadaveric pancreatic samples from patients with T1D display upregulation of MHC class I up to 8-years post-diagnosis [29]. We propose that a possible secondary insult in individuals genetically predisposed to develop T1D leads to upregulation of MHC I on the islets and directed CD8+ T-cell killing. The resulting release of β-cell antigens perpetuates the attack by recruiting more CD8+ T-cells with different antigen specificities. This process could happen slowly over time in response to repeated insults as has recently been proposed [30]. While a proportion of T1D subjects did not have significant, detectable circulating autoantibody CD8+ T-cells in our study, the presence of such cells was almost exclusively found in T1D. In those T1D subjects without detectable autoantibody CD8+ T-cells, the frequency of these T-cells may wane as more islets are destroyed, reducing the antigenic load. This would explain previously published observation that some, but not all, pancreatic samples of T1D patients had insulitis [29].

The amount of remaining β-cells clearly differs between type 1 and type 2 diabetics, although recent investigations have shown that some β-cells regularly survive even in type 1 patients over long periods of time [31]. Induction of autoreactive T cells and their maintenance clearly has to occur after exposure to islet antigens. Two aspects of our study are, in this respect, novel and remarkable: First, CD8+ T-cells reacting with islet antigens are only found in type 1 patients, indicating over-expression of MHC class I on β-cells in T1D [29] is instrumental for their sensitization. Second, CD4 autoreactivity is present in both types of diabetes, suggesting that mere β-cell stress can lead to presentation of autoantigens via the MHC class II pathway.

The current report indicates that CD4+ T-cell autoreactivity may be common to both T1D and T2D, and could represent a shared mechanism of β-cell dysfunction. Conversely, autoreactive CD8+ T-cells appear to be unique to T1D and may be responsible for the accelerated loss of β-cell mass. This evidence gives credence to the concept that the different forms of diabetes represent a continuum from autoimmunity to metabolic disturbances with some overlapping and some unique features for each disease. Thus, measuring CD8+ autoreactivity in T1D subjects could serve as a more specific immune biomarker to study disease progression and, possibly, response to therapeutics in future clinical trials.

In conclusion, the data presented here demonstrates that there is a shared autoimmune component between T1D and T2D. The complexity of both diseases is further highlighted by topological analyses; the emergence of distinct subgroups underscores the need for better understanding of the impact of the diabetogenic immune response on disease progression and therapeutic outcomes. Identifying and understanding immune-based differences within the spectrum of diabetes—beyond T1D and T2D—will ultimately facilitate the development and use of more targeted, personalized therapies.

Funding

GS was supported by a postdoctoral fellowship from the T1D center of San Diego. This work was supported by a subaward number 3002074563 from University of Michigan, Ann Arbor (UMich), to MvH, issued under NIH award number 3UL1RR024986-05S1 to UMich under the direction of Dr. Thomas P. Shanley, PI.

Conflict of interest

The authors declare that there are no conflicts of interest pertaining to this manuscript.

Acknowledgments

We thank Ken Coppeters, (NNRC, Seattle, WA) and Bart Roep (LUMC, Leiden, Netherlands) for many helpful discussions and advice in manuscript preparation; Joana RF Abreu and Bart Roep (LUMC, Leiden, Netherlands) for assistance with the Qdot-HLA-
multimer assay; Tobias Boettler, Darius Schneider, Renee Parker and Mark Bouchard (LIAI) and the nurses at UCSD VA hospital for their assistance in obtaining blood samples for this study; Denise Baker and Duy Le (LIAI) for their assistance with HLA typing. Finally, we would like to thank the NIH tetramer core facility at Emory University for their help in providing HLA-A2–peptide monomers.

GS, JP, SS, SP JFM, and JC participated in obtaining samples and performing assays. GS, JP, RM, BP, DW, EC, and MVH performed the data analysis. GS, JP, BP and MVH wrote the manuscript. GS, SE and MVH conceptualized and designed the project. GS, JP and MVH are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of data analysis.

Parts of the data shown in this manuscript were presented at the Immunology of Diabetes Society (IDS) conference 2012, held in Victoria, British Columbia, Canada.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2013.12.003

References