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Expression level of a pancreatic neo-antigen in beta cells determines degree of diabetes pathogenesis

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Abstract

It is not fully understood how the expression level of autoantigens in beta cells impacts autoimmune diabetes (T1D) development. Earlier studies using ovalbumin and also insulin had shown that secreted antigens could enhance diabetes development through facilitated presentation by antigen presenting cells. Here we sought to determine how the expression level of a membrane bound, non-secreted or cross-presented neo-antigen, the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV), would influence T1D. We found that a RIP-LCMV transgenic mouse line exhibiting higher levels of beta cell GP expression developed more severe diabetes after LCMV infection or transfer of high numbers of activated autoreactive T cells. Importantly, all beta cells were lost and a significant increase in morbidity and mortality from T1D was noted. Insulinitis and accumulation of autoaggressive CD8 cells was more profound in the RIP-LCMV-GP high-expressor line. Interestingly, the additional introduction of neo-antigen-specific CD4⁺ helper or regulatory T cells was able to influence diabetogenesis positively or negatively. We conclude that a higher degree of autoantigen expression results in increased diabetes susceptibility. Therefore, autoantigens such as insulin that are expressed at higher levels in beta cells might have a more profound impact on diabetes pathogenesis.

Keywords

autoimmune diabetes; tolerance; transgenic mice; T cells; immunomodulation; lymphocytic choriomeningitis virus glycoprotein

1. Introduction

Juvenile or Type 1 Diabetes (T1D) is the most common autoimmune disease affecting 20 million people worldwide. During pathogenesis, autoaggressive CD4⁺ and CD8⁺ T cells

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infiltrate pancreatic islets and destroy insulin-producing beta cells. When over 80% of pancreatic beta cells have been destroyed or are dysfunctional, a rise in blood glucose levels is observed eventually leading to T1D and only life-long insulin therapy can keep the patient alive [1–4]. However, insulin therapy cannot fully protect against the long-term complications such as nephropathy, neuropathy, retinopathy and cardiovascular diseases, which can lead to a significantly reduced life span [3]. In recent years, alternative therapies modulating the immune response of T1D patients have yielded promising results. For example, anti-CD3 immune therapy depletes autoaggressive T cells but helps augmenting regulatory T cell numbers and thus allows prolonged beta cell mass survival [5–7]. However, permanent tolerance to pancreatic beta cell autoantigens has not been achieved yet and thus a detailed understanding of T1D pathogenesis and the factors influencing T1D outcome are essential to allow for the development of successful T1D therapies in the future.

During the past 20–30 years, transgenic mice expressing a neo-antigen under control of the rat insulin promoter (RIP) have helped in understanding the pathogenesis of T1D and the search for alternative T1D therapies [8–16]. For example, introducing autoantigen-specific effector T cells into RIP-autoantigen expressing mice can result in ignorance to the autoantigen [9, 10], deletion of autoantigen-specific T cells [17–19] or spontaneous development of insulinitis or diabetes [13,14,16,20–22], thus defining parameters that are required for breaking T cell tolerance and development of autoimmune diabetes. Over the past, the importance of autoantigen levels on autoimmune disease development has been studied in some experimental diabetes models. For example, using double transgenic mice expressing MHC class II-restricted hen egg lysozyme (HEL)-specific CD4⁺ T cells and different amounts of membrane-bound HEL in pancreatic beta cells, Unanue and colleagues showed a direct correlation between CD4⁺ T cell-mediated T1D incidence and pancreatic autoantigen levels [14,16]. In a parallel study, using double transgenic mice expressing ovalbumin (OVA)-specific CD8⁺ T cells and different amounts of secreted OVA in pancreatic islets, Kurts and colleagues demonstrated the impact of autoantigen levels on the cross-presentation pathway: high dose autoantigens induced deletional tolerance via cross-presentation, in contrast, low dose autoantigens were ignored by naïve T cells but could still sensitize activated effector cells [15,19]. So far, the impact of membrane-bound, non-cross-presented or secreted autoantigen expression levels on stimulating MHC class I-restricted CD8⁺ T cell responses and T1D susceptibility has not been elucidated yet. Underlining the importance of understanding the influence of autoantigen levels on cytotoxic T lymphocyte (CTL)-mediated T1D susceptibility, Peakman and colleagues demonstrated elegantly human beta cell killing by preproinsulin (PPI)-specific CTLs providing evidence for beta cell destruction not only by mouse but also human CD8⁺ T cells [23]. Interestingly, increased glucose concentration as a result of beta cell death led to increased PPI production by the remaining beta cells thereby enhancing PPI-specific CTL activation and beta cell killing [23].

In the present study, we took advantage of the RIP-GP system to address the role of membrane-bound autoantigen expression levels on CD8⁺ T cell-dependent T1D pathogenesis. RIP-GP Armstrong and RIP-GP Berlin mice express the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) from the LCMV-Armstrong and LCMV-WE strains, respectively, under control of the rat insulin promoter and thus express LCMV-GP in pancreatic beta cells [9,10]. Following a non-viral T1D induction protocol, which leaves the LCMV-GP as the only variable in these two mouse strains, we demonstrate that both RIP-GP strains develop T1D with different kinetics and incidence. We show that the contrasting T1D pathogenesis is due to different pancreatic LCMV-GP protein but not mRNA expression levels, concluding that autoantigen expression can determine T1D susceptibility. The antigen effect was even still notable in the virally-induced RIP-LCMV diabetes model. Our results also demonstrate that LCMV-GP-specific CD4⁺ T helper and regulatory cells can strongly influence T1D outcome.

Taken together, we established that expression levels of a membrane-bound autoantigen can determine T1D susceptibility.

2. Material and Methods

2.1. Mice

Transgenic RIP-LCMV-GP Berlin (RIP-GP Berlin) mice [9] were a kind gift from Dr. Pamela S. Ohashi. RIP-LCMV-GP Armstrong (RIP-GP Armstrong or RIP-GP Arm) mice [10] and Smarta TCR transgenic mice [24] were kind gifts from Dr. Michael B. A. Oldstone. P14 TCR transgenic mice [25], Rag-deficient C57BL/6 and C57BL/6J (B6) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All animal procedures were approved by the IACUC of the La Jolla Institute for Allergy and Immunology, and The Scripps Research Institute.

2.2. Viral and non-viral Type 1 Diabetes induction protocol

LCMV Armstrong clone 53b was prepared by a single passage on BHK-21 cells. RIP-GP Berlin and RIP-GP Armstrong mice were infected with 10^4 plaque-forming units (pfu) LCMV-Armstrong intraperitoneally (i.p.).

For the non-viral T1D induction protocol, RIP-GP mice were injected intravenously with 1.5×10^7 naïve P14 spleen cells followed by i.p. immunizations with 2mg gp33 (Abgent, NH₂-KAVYNFATM-COOH) in ultrapure water containing 50µg of CpG adjuvant (Sigma) on days 1 and 3 after P14 transfer. On day 6 after P14 transfer, mice received an i.p. injection of 500µg polyinosinic:polycytidylic acid (polyIC, Sigma). Blood glucose levels were measured in regular intervals up to day 21. Mice with blood glucose levels above 300mg/dl were considered diabetic.

2.3. In vitro culture of CD4⁺ Smarta cells

1×10^7 naïve Smarta spleen cells were stimulated *in vitro* for three days in 24-well-plate wells with 1µg/ml gp61 peptide (Abgent, NH₂-GLNGPDIYKGVYQFKSVEFD-COOH) and 100U/ml rhIL-2 in 2ml RPMI containing 10% FCS. After three days in culture (CD4⁺ fraction was confirmed CD69⁺CD44^{hi}CD62L^{int-lo}, activated phenotype), 1×10^7 total cells were administered intravenously into RIP-GP Armstrong recipients four days after P14 transfer.

For the *in vitro* generation of Smarta CD4⁺ regulatory T cells (Tregs), 3×10^7 MACS-purified CD4⁺CD25⁻ Smarta spleen and lymph node cells were cultured for seven days with 3×10^7 T cell-depleted B6 spleen cells as antigen-presenting cells (APCs), 1µg/ml gp61 peptide, 50U/ml rhIL-2, 1×10^{-7} M Vitamin D3 and 3×10^{-8} M Dexamethasone in 40ml RPMI containing 10% FCS. 1×10^6 cultured cells were subsequently administered intravenously into RIP-GP Berlin recipients eight days after P14 transfer.

2.4. Flow cytometry

Flow cytometry antibodies were purchased from BD Pharmingen and Biolegend (San Diego, CA). For intracellular stains, single-cell suspensions from pancreatic draining lymph node (PDLN) and pancreas were restimulated for 5 – 6 h with gp33 peptide in the presence of brefeldin A (Sigma) followed by staining for surface expression of CD8, Vα2 and Vβ8.1/2 (specific for P14 TCR), CD44 and CD62L. Cells were then fixed with 3% formaldehyde, permeabilized with 0.05% saponin, and stained for intracellular IFN-γ and TNF. Samples were acquired on a LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.5. Immunohistochemistry

Pancreata were immersed in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) and quick-frozen on dry ice. 6–10- μ m pancreatic tissue sections were acetone-fixed and stained with CD8-specific and insulin-specific antibodies. Immunohistochemical detection was done using biotinylated secondary antibodies and Avidin-D coupled to HRP, followed by enzymatic development with DAB or AEC chromogen (Vector Laboratories), or using AP-labeled secondary antibodies followed by enzymatic development with Vector Blue (Vector Laboratories).

2.6. Immunofluorescence

Acetone-fixed pancreatic tissue sections were stained with insulin-specific antibody (guinea pig anti-swine insulin, 1:250-diluted, DAKO) and LCMV-GP-specific ascites (WE33.6, mouse IgG2a, 1:50 dilution, kind gift of Dr. Michael Buchmeier). Immunofluorescent detection was done using AF568-labeled goat anti-guinea pig and AF488-labeled goat anti-mouse polyclonal IgG (both 1:400 dilution, Invitrogen) secondary antibodies. After the samples were mounted with Immuno-Fluore mounting medium (MP Biomedicals Inc.), coverslips were visualized by fluorescence microscopy with a Nikon Eclipse E800 fluorescence microscope.

2.7. RT-PCR

Organs were snap-frozen, homogenized in Trizol reagent (Invitrogen) and total RNA was extracted. To prevent its degradation, pancreatic RNA was re-extracted a second time to ensure complete removal of RNases. Residual genomic DNA was eliminated by DNase digestion for 20 min with the TURBO DNA-free kit (Ambion). Reverse transcription was carried out with 15 μ g total RNA using the first-strand cDNA synthesis kit (Amersham Biosciences) with the oligo dT-primer provided in the kit according to the manufacturer's instructions. PCR primers were as follows: β -actin, 5' GACGGCCAGGTCATCACTAT 3' and 5' ACATCTGCTGGAAGGTGGAC 3'; LCMV-GP, 5' TCAGTGGAGTTTGATATGTC 3' and 5' CTCTGATACTGAGGTGTAG 3'.

2.8. Quantitative Real-Time PCR

Total RNA was prepared from homogenized pancreata by two sequential extractions with Trizol reagent (Invitrogen). Residual genomic DNA was removed from the total RNA using the Turbo DNA-free kit (Ambion). cDNA was then synthesized with the first-Strand cDNA synthesis kit (Amersham Biosciences). For each reaction, 1 μ l of a 1:10 dilution of cDNA (for β -actin reaction) or undiluted cDNA (for LCMV-GP reaction), 11.25 μ l of dH₂O, 12.5 μ l of SYBR Green PCR master mix (Qiagen, Valencia, CA), and 500nM of forward and reverse primers were mixed in 25 μ l of total volume. Quantitative Real-time PCR (Q-PCR) was performed in triplicate using an ABI PRISM 7700 Sequence Detector (Perkin Elmer, Waltham, MA) under the following conditions: 50°C, 2min, and 95°C, 15min, followed by 45 cycles of 95°C, 15s; 60°C, 30s, and 72°C, 1min. The delta-delta crossing threshold (Ct) method was used for data analysis (according to the Applied Biosystems manual). mRNA expression levels were quantified relative to that of β -actin mRNA. Confirmation of single amplicons and lack of primer-dimer formation was performed by melting-curve analysis.

3. Results

3.1. Reduced degree of T1D pathogenesis in RIP-GP Armstrong compared to RIP-GP Berlin mice following LCMV infection or peptide/adjuvant induction

RIP-GP Armstrong and RIP-GP Berlin mice express the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) from the LCMV-Armstrong and LCMV-WE strains, respectively, under control of the rat insulin promoter resulting in pancreatic LCMV-GP expression [9,10].

The two glycoproteins each have a length of 498 amino acids and differ only by 45 amino acids (Supplementary Fig. 1). Importantly, the two main CD8 (LCMV-GP₃₃₋₄₁, gp33) and CD4 epitopes (LCMV-GP₆₄₋₈₀), recognized by the P14 TCR [25] and the Smarta TCR [24], respectively, are identical between the LCMV-GP Armstrong and LCMV-GP WE protein (Supplementary Fig. 1), and it has been demonstrated previously that these responses are crucial for T1D pathogenesis in the RIP-GP T1D model [9,26]. Without LCMV infection or any other manipulation, these mice do not develop diabetes since naïve LCMV-GP-specific T cells sufficiently ignore the neo-self-antigen expressed in pancreatic beta cells. Upon LCMV infection, however, activated LCMV-GP-specific T cells migrate into the pancreas and destroy LCMV-GP-expressing pancreatic beta cells resulting in insulin loss and diabetes development [9,10]. Interestingly, RIP-GP Armstrong and RIP-GP Berlin mice infected with the same dose of LCMV-Armstrong (10^4 pfu intravenously) developed T1D with different kinetics and incidence (Fig. 1A). RIP-GP Berlin mice showed earlier onset of diabetes and diabetes incidence was 100%. In contrast, RIP-GP Armstrong mice developed diabetes two days later and maximally 80% of the mice turned diabetic (Fig. 1A). In order to address the mechanisms explaining the different T1D pathogenesis in the two RIP-GP strains without the added complexities associated with a viral infection system, we established a virus-free T1D induction protocol. Our protocol targets exclusively one autoantigen (gp33) and provides identical numbers of autoantigen-specific effector T cells (P14 cells), thereby keeping pancreatic LCMV-GP expression as the only variable in the two strains (Fig. 1B). P14 CD8 TCR transgenic mice were used as a source of autoantigen-specific T cells, and RIP-GP Armstrong and RIP-GP Berlin mice served as autoantigen-expressing recipients. P14 mice are TCR transgenic mice that express CD8⁺ T cells specific for the LCMV-GP₃₃₋₄₁ (gp33) peptide presented on MHC class I molecules H-2D^b [25]. On day 0, 1.5×10^7 P14 spleen cells were transferred intravenously into RIP-GP Armstrong or RIP-GP Berlin recipients, followed by intraperitoneal immunizations on days 1 and 3 with 2mg gp33 peptide containing 50µg of the TLR9-ligand CpG [27] as adjuvant. On day 6 after P14 transfer, mice received 500µg TLR3-ligand polyinosine-polycytidylic acid (polyIC) [28] to induce inflammation (Fig. 1B). Thus, the virus-free T1D induction protocol involved the adoptive transfer of autoantigen-specific P14 cells into RIP-GP recipient mice followed by the antigen-specific immunization with a peptide/adjuvant mixture resulting in specific activation of P14 T cells, destruction of LCMV-GP-expressing beta cells and diabetes development. As a side note, peptide treatment alone was not sufficient to induce T1D (data not shown) due to the lack of proper activation of antigen presenting cells (APCs) and likely an insufficient inflammatory response [29–33].

Following the peptide/adjuvant treatment, only RIP-GP Berlin mice showed comparable T1D kinetics and incidence as observed after viral infection; RIP-GP Berlin mice developed diabetes between 7–12 days after P14 transfer and showed 100% diabetes incidence (Fig. 1B). In addition, diabetic mice had to be sacrificed three days after diabetes detection due to deteriorating health. In contrast, none or only very few RIP-GP Armstrong mice developed T1D following the peptide/adjuvant treatment and the few mice that developed diabetes showed a mild and often transient form of hyperglycemia (Fig. 1B). In summary, our non-viral T1D induction protocol induced a very different T1D outcome in the two RIP-GP strains tested; fast onset and high T1D incidence in RIP-GP Berlin recipients and slower onset and only low to non-existing T1D development in RIP-GP Armstrong recipients.

3.2. Reduced destructive CD8⁺ T cell infiltration into pancreatic islets of RIP-GP Armstrong compared to RIP-GP Berlin recipients following peptide/adjuvant treatment

We next performed a kinetic analysis of the pancreas of RIP-GP Armstrong and RIP-GP Berlin mice to compare the degree of CD8⁺ T cell infiltration and insulin loss between both recipient groups (Fig. 2). CD8⁺ T cell infiltrations started to appear around day 7 after P14 transfer in both recipient groups. However, whereas RIP-GP Armstrong mice only showed an

accumulation of CD8⁺ T cells around pancreatic islets (peri-insulitis), RIP-GP Berlin mice showed a strong infiltration of CD8⁺ T cells into pancreatic islets (insulitis). The destructive potential of CD8⁺ T cells in the latter group was also apparent through some insulin loss, whereas pancreatic islets of RIP-GP Armstrong mice still remained intact (Fig. 2). On day 9 after P14 transfer, both recipient groups showed CD8⁺ T cell infiltration into pancreatic islets. However, RIP-GP Berlin recipients had fewer CD8⁺ T cells in the pancreatic islets, likely since most of the beta cells were already destroyed at that time and the insulin loss was so advanced that three out of five mice were diabetic by day 9. In contrast, none of the RIP-GP Armstrong mice analyzed were hyperglycemic at this time point (Fig. 2). On day 11 after P14 transfer, all RIP-GP Berlin mice were diabetic as evidenced by the almost complete loss of insulin stain. In contrast, only two out of five RIP-GP Armstrong mice were hyperglycemic and compared to RIP-GP Berlin mice, the hyperglycemic mice showed overall lower blood glucose values, more detectable insulin stain (Fig. 2) and appeared healthier. However, CD8⁺ T cells were still present in pancreatic islets (Fig. 2), but RIP-GP Armstrong mice, which had not turned diabetic by day 11, remained normoglycemic and showed only mild CD8 infiltration into the pancreatic islets and only little insulin loss (data not shown). In summary, the histological analysis of pancreatic sections of peptide/adjuvant-treated RIP-GP recipients showed different kinetics and severity of CD8⁺ T cell infiltration into pancreatic islets of RIP-GP Armstrong and RIP-GP Berlin recipients explaining the differences in T1D pathogenesis (Supplementary Table 1).

3.3. Peptide/adjuvant-treated RIP-GP recipients of both strains show pronounced and comparable P14 activation in the PDLN and pancreas

In order to address whether decreased CD8⁺ T cell infiltration and reduced T1D incidence in RIP-GP Armstrong mice was due to an overall inefficient effector P14 CD8⁺ T cell response, we isolated pancreatic draining lymph nodes (PDLN) and pancreata of peptide/adjuvant-treated RIP-GP Armstrong and RIP-GP Berlin recipients ten days after P14 transfer (Fig. 3). Untreated RIP-GP mice showed maximally 2% P14 CD8⁺ T cells in PDLN and no detectable CD8⁺ T cells in the pancreas (data not shown). In contrast, peptide/adjuvant-treated RIP-GP Armstrong and RIP-GP Berlin recipients showed a strong expansion of P14 CD8⁺ T cells in the PDLN and pancreas (Fig. 3). While RIP-GP Berlin recipients always showed a higher P14 expansion in the spleen (data not shown) and PDLN, both RIP-GP recipients showed comparable P14 expansion in the pancreas (Fig. 3). All P14 CD8⁺ T showed an activated/memory phenotype as evidenced by strong CD44 upregulation and partial (PDLN) to almost complete (pancreas) CD62L downregulation (Fig. 3). Further, P14 CD8⁺ T cells in both RIP-GP recipients were functional since they secreted IFN- γ (Fig. 3) and TNF (not shown) after *in vitro* restimulation with gp33. These data indicate that decreased T1D pathogenesis in peptide/adjuvant-treated RIP-GP Armstrong recipients was not due to an impaired P14 effector T cell response or their inability to reach the pancreatic islets.

3.4. Decreased pancreatic LCMV-GP protein but not mRNA expression in RIP-GP Armstrong recipients explains decreased T1D pathogenesis

First, albeit unlikely based on our systemic T cell response data, we addressed whether leakiness of LCMV-GP expression in other organs could explain the decreased T1D pathogenesis observed in RIP-GP Armstrong mice. As shown in previous reports, autoantigen expression in the thymus and secondary lymphoid organs can result in complete or partial central and peripheral tolerance, respectively, of autoantigen-specific T cells [11,14–16,34]. LCMV-GP-specific RT-PCR of the pancreas, thymus, spleen and lymph nodes showed that LCMV-GP-specific mRNA expression was restricted exclusively to the pancreas in both RIP-GP Armstrong and RIP-GP Berlin mice (Fig. 4A) concluding that decreased T1D pathogenesis in the former mice was not due to leaky LCMV-GP expression. Further, the absence of detectable LCMV-GP mRNA in the primary and secondary lymphoid organs together with our

previous results showing a functional autoantigen-specific T cell response (Fig. 3), demonstrated that no tolerizing mechanisms were taking place in both RIP-GP strains.

We then analyzed whether differences in autoantigen expression in RIP-GP Armstrong and RIP-GP Berlin recipients could explain the decreased T1D pathogenesis observed in RIP-GP Armstrong mice. Different autoantigen expression levels could be explained for example by decreased LCMV-GP mRNA and/or decreased LCMV-GP protein expression due to the transgenic construct, transgene integration site and copy numbers or the difference in protein sequence between LCMV-Armstrong and LCMV-WE glycoproteins. First, to assess whether RIP-GP Armstrong mice expressed lower levels of LCMV-GP mRNA, we performed quantitative LCMV-GP-specific RT-PCR (Taqman PCR). Interestingly, quantitative LCMV-GP-specific RT-PCR from pancreatic samples showed similar levels of LCMV-GP mRNA in RIP-GP Armstrong and RIP-GP Berlin mice (Fig. 4B). Although LCMV-GP mRNA levels were comparable between both strains, altered LCMV-GP protein expression could still occur due to differences in post-transcriptional and translational regulation [35]. To address whether LCMV-GP protein expression levels were indeed different in both RIP-GP strains, we performed LCMV-GP protein staining with an LCMV-GP-specific monoclonal antibody (clone WE33.6, mouse IgG2a; kind gift of Dr. Buchmeier) [36] recognizing the identical amino acid sequence in LCMV-GP Armstrong and LCMV-GP WE protein (Supplementary Fig. 1). RIP-GP Berlin mice showed much stronger LCMV-GP-specific staining in pancreatic islets than RIP-GP Armstrong mice (data not shown). However, the LCMV-GP-specific stain did not work satisfactorily due to unspecific binding of the secondary antibody (goat anti-mouse polyclonal IgG or goat anti-mouse IgG2a) to endogenous IgGs resulting in a very high background stain. We therefore repeated these stains with pancreatic sections from RIP-GP Armstrong and RIP-GP Berlin mice bred onto a Rag-deficient background thereby eliminating synthesis of endogenous IgGs. Pancreatic sections from Rag-deficient B6, RIP-GP Armstrong and RIP-GP Berlin mice showed strong insulin staining in pancreatic islets (Fig. 4C, upper row). As expected, pancreatic islets from Rag-deficient B6 mice did not show any LCMV-GP-specific stain. Surprisingly, only a few pancreatic islet cells stained positive for LCMV-GP in RIP-GP Armstrong mice whereas islets from RIP-GP Berlin pancreata were highly positive for LCMV-GP (Fig. 4C, middle row). Although not all pancreatic beta cells in RIP-GP Armstrong mice stained positive for the LCMV-GP protein, we suspect that they do express low levels of the protein, which are below the detection limit of this assay. These results showed that RIP-GP Armstrong mice had decreased LCMV-GP protein expression in pancreatic islets compared to RIP-GP Berlin mice providing a possible explanation for decreased T1D pathogenesis in RIP-GP Armstrong mice.

In summary, RIP-GP Armstrong and RIP-GP Berlin mice expressed similar levels of LCMV-GP mRNA restricted exclusively to the pancreas. However, RIP-GP Armstrong mice showed reduced LCMV-GP protein expression resulting in slower T1D kinetics, decreased T1D incidence and overall decreased T1D pathogenesis after peptide/adjuvant treatment.

3.5. Presence of antigen-specific effector or regulatory CD4⁺ T cells modulates T1D pathogenesis of peptide/adjuvant-treated RIP-GP recipients

Following our non-viral T1D induction protocol, absence of or only slow and transient T1D development can be seen in RIP-GP Armstrong mice, whereas RIP-GP Berlin mice exhibit rapid and high T1D incidence. Thus, our novel finding that different LCMV-GP protein expression levels alter T1D pathogenesis can now be exploited to identify factors that affect T1D development. Specifically, we can identify risk factors inducing/increasing T1D pathogenesis in RIP-GP Armstrong recipients and identify protective/therapeutic factors suppressing/decreasing T1D pathogenesis in RIP-GP Berlin recipients. To illustrate this, we addressed whether LCMV-GP-specific effector or regulatory CD4⁺ T cells would

increase or decrease T1D pathogenesis in our RIP-GP Armstrong and RIP-GP Berlin recipients, respectively. Smarta mice are TCR transgenic mice that express CD4⁺ T cells specific for the LCMV-GP_{64–80} peptide presented on MHC class II molecules I-A^b [24].

We first analyzed whether the additional presence of LCMV-GP-specific Smarta CD4⁺ T cell help would be sufficient to increase T1D pathogenesis in RIP-GP Armstrong mice. For this purpose, P14 spleen cells were transferred into RIP-GP Armstrong recipients followed by the peptide/adjuvant treatment. One group of mice additionally received 1×10^7 *in vitro* activated LCMV-GP-specific Smarta cells four days after P14 transfer (Fig. 5A). As shown in Figure 5A, RIP-GP Armstrong recipients, who had not received any CD4⁺ T cell help remained normoglycemic over the whole examination period. In contrast, up to 80% of RIP-GP Armstrong recipients, who had received LCMV-GP-specific Smarta CD4⁺ T cell help, developed diabetes (Fig. 5A). Importantly, RIP-GP Armstrong recipients only showed high T1D incidence when high numbers of activated autoantigen-specific cells were transferred; transfer of naïve Smarta⁺ cells did not increase T1D pathogenesis in RIP-GP Armstrong recipients (data not shown). In order to follow the impact of CD4⁺ T cell help on cell infiltration into pancreatic islets, we isolated the pancreas of peptide/adjuvant-treated RIP-GP Armstrong mice with or without activated Smarta cell transfer 21 days after P14 transfer. As shown in Figure 5B, presence of CD4⁺ T cell help resulted in a strong increase of CD8⁺ and CD4⁺ T cell and CD11b⁺ APC infiltrations into pancreatic islets. We also found increased infiltration of B220⁺ B cells and CD11c⁺ dendritic cells into pancreatic islets (data not shown). These results suggest that the presence of antigen-specific CD4⁺ T cell help was necessary to recruit sufficient effector T cells and APCs to the autoantigenic site thereby enabling more severe pancreatic beta cell destruction, autoantigen presentation to effector T cells and T1D development. Presence of cellular infiltrates might in addition generate a sufficiently high inflammatory environment necessary to keep the autoimmune process alive by increasing beta cell destruction and thus expression of the neo-autoantigen in the PDLN.

Conversely, we addressed whether presence of autoantigen-specific CD4⁺ regulatory T cells (Tregs) might be sufficient to suppress T1D pathogenesis in high autoantigen-expressing RIP-GP Berlin recipients. For this purpose, we transferred P14 cells into RIP-GP Berlin recipients followed by the peptide/adjuvant treatment. One group of mice additionally received 1×10^6 *in vitro* generated autoantigen-specific Smarta Tregs on day eight after P14 transfer (Fig. 5C). We chose this time point such that the Tregs would not interfere with the initial priming of the autoantigen-specific CD8⁺ T cells but should still be able to interfere with the active beta cell destruction process. As shown in Fig. 5C, presence of Smarta⁺ Tregs indeed drastically reduced T1D incidence of RIP-GP Berlin recipients; the one mouse that was already diabetic on day 8, did not revert, however, all other RIP-GP Berlin recipients remained normoglycemic from day 8 on. In contrast, all RIP-GP Berlin recipients who had not received any regulatory T cells developed T1D (Fig. 5C) and had to be sacrificed five days after diabetes development. Future work will address the mechanisms by which these regulatory T cells suppressed T1D development. Possible explanations could be suppression of cellular infiltration into pancreatic islets, reduction of beta cell killing and/or induction of a shift from an inflammatory to an immuno-suppressive environment via cytokine modulation.

Taken together, our results have shown that the additional transfer of autoantigen-specific effector or regulatory CD4⁺ T cells influenced T1D pathogenesis in our peptide/adjuvant-treated RIP-GP recipients by either increasing or decreasing T1D development, respectively.

4. Discussion

Our results have shown that RIP-GP Armstrong and RIP-GP Berlin recipients showed different T1D kinetics and incidence after LCMV infection. This prompted us to investigate the

difference between the two RIP-GP recipients in more detail by developing a non-viral T1D induction protocol, which consisted of the adoptive transfer of equal numbers of autoantigen-specific effector T cells followed by repeated autoantigen-specific peptide stimulation in the presence of adjuvants. The advantages of this protocol are several-fold. Most importantly, it eliminates the complexity of a viral system. Further, by using a constant number of identical autoantigen-specific effector cells and by priming with only one autoantigen, it leaves the LCMV-GP as the only variable in this system and hence allows addressing the influence of LCMV-GP levels on T1D pathogenesis. Following this treatment, RIP-GP recipients showed a clear difference in T1D pathogenesis: RIP-GP Armstrong mice either did not develop diabetes or only a mild and often transient form of T1D; in contrast, all RIP-GP Berlin mice developed diabetes and succumbed to it a few days later. Interestingly, the reason for this strong difference in T1D pathogenesis was the result of decreased LCMV-GP protein but not mRNA expression in pancreatic islets of RIP-GP Armstrong mice. Reduced LCMV-GP protein expression in RIP-GP Armstrong mice can be explained by differences in post-transcriptional or translational regulation between the two RIP-GP strains. Capping, splicing, addition of a poly(A) tail, sequence-specific nuclear export rates, sequestration of the RNA transcript, secondary mRNA structure, differential codon usage, and/or antisense RNA or protein binding decide on the overall mRNA stability and thus on how much mRNA is translated into protein [35]. The generation of the two RIP-GP strains involved transgenic constructs differing not only in the LCMV-GP cDNA but also in the region providing RNA splicing and polyadenylation signals: The SV40 t intron was used as RNA splicing signal in the transgenic construct for the generation of RIP-GP Armstrong mice while a genomic fragment including the exons 2 and 3 from the beta-globin gene provided the RNA splicing and polyadenylation signals in the transgenic construct for the generation of RIP-GP Berlin mice [9,10]. These differences in RNA splicing signals but also additional above-mentioned factors influencing translation of the mRNA into protein may therefore well explain the different LCMV-GP protein expression levels observed in RIP-GP Armstrong and RIP-GP Berlin mice.

Importantly, the differential LCMV-GP protein expression levels in the two RIP-GP strains demonstrate how the level of a membrane-bound autoantigen can determine the degree of autoimmune diabetes susceptibility: Low autoantigen levels may not lead to autoimmune disease or only to a mild and transient form of autoimmune disease; in contrast, high autoantigen levels result in severe autoimmune disease. The importance of autoantigen expression levels for autoimmune disease development had been reported for other T1D mouse models as well, however not for membrane-bound [37,38] CD8 targets. For example, Kurts et al. have shown that high but not low autoantigen dose facilitates cross-presentation to naive T cells and thus increases autoimmune susceptibility in transgenic mice expressing secreted OVA in pancreatic beta cells [15]. Further, in a MHC class II-restricted T1D mouse model using double transgenic mice expressing HEL-specific TCR transgenic CD4⁺ T cells and different amounts of membrane-bound HEL in pancreatic beta cells, already a 10-fold difference in pancreatic HEL expression levels made the difference between T1D incidence and protection [16]. Finally, Peakman and colleagues confirmed the influence of autoantigen levels on T1D pathogenesis also in the human setting: At high glucose concentrations, an increase in the presentation of preproinsulin by the remaining beta cells was observed resulting in increased beta cell killing by preproinsulin-specific CTLs [23].

Importantly, our model also shows that the autoantigen level alone is not the only factor that determines autoimmune diabetes development: Other important factors include but are not limited to the nature of the antigenic stimulus (viral versus non-viral stimulus) [9,30,32,33, 39], the proliferative capacity, quantity and quality of the effector T cell response [21,40–44], antigen-specific CD4⁺ T cell help [45,46] versus regulatory T cells [47–51], bystander activation and bystander suppression [52,53], presence of co-stimulation [29,54,55], nature of the chemokine and cytokine milieu (inflammatory versus suppressive) [26,56–61],

concomitant infection [62] etc. As shown here, augmentation of activated Smarta CD4⁺ effector T cells, specific for the GP neo-autoantigen, enabled to overcome autoimmune diabetes resistance in low autoantigen-expressing RIP-GP Armstrong recipients, probably by recruiting high numbers of autoantigen-specific effector T cells and APCs to the autoantigenic site thereby keeping the autoimmune process alive. Similar results were observed in the NOD-LCMV-nucleoprotein (NP) model where only presence of high numbers of activated LCMV-NP-specific but not LCMV-GP-specific CD8⁺ T cells induced T1D [43] and in the RIP-OVA model where T1D was only induced when activated CD4⁺ T cells were also present [46]. Conversely, administration of Smarta CD4⁺ regulatory T cells suppressed autoimmune diabetes susceptibility in high autoantigen-expressing RIP-GP Berlin recipients.

Taken together, autoantigen expression levels plus the immunological environment (stimulatory versus suppressive) determine T1D outcome: If the autoantigen-specific stimulus is sufficiently high, autoimmune disease will ensue. In this study, sufficiently high autoantigen-specific stimulus was achieved through either high autoantigen expression in an inflammatory environment (peptide/adjuvant-treated RIP-GP Berlin recipients) or decreased autoantigen expression in an inflammatory environment in the presence of enough numbers of activated autoantigen-specific cells (peptide/adjuvant-treated RIP-GP Armstrong mice having received activated Smarta cells). Conversely, if the autoantigen-specific stimulus is weak, autoimmune disease will not develop or only in a mild form. Low autoantigen-specific stimulus in this case was achieved through low autoantigen expression in an inflammatory environment (peptide/adjuvant-treated RIP-GP Armstrong mice) or high autoantigen expression in an inflammatory environment with the additional presence of regulatory T cells (peptide/adjuvant-treated RIP-GP Berlin mice having received Smarta Tregs). It is therefore essential to identify and understand the mechanism of action of these factors, which can shift a low autoantigen environment without autoimmune disease to a strong stimulatory environment leading to autoimmune attack and overt autoimmune disease.

Finally, our T1D model in combination with ENU mutagenesis could help in the identification of novel genetic T1D protective and risk factors. N-Ethyl-N-nitrosurea (ENU) is a highly potent mutagen targeting spermatogonial stem cells [63,64]. The ENU-induced point mutations can either be gain-of-function or loss-of-function mutations and can thus be used in forward (phenotype based) genetic screens with which one can identify and study the phenotype of interest, in our model either increased or decreased T1D incidence in the RIP-GP Armstrong and RIP-GP Berlin recipients, respectively. To determine, which genes are essential for CD8 effector function (activation, expansion, migration into pancreatic islets and beta cell killing) in our T1D model, one could perform ENU mutagenesis on P14 cells followed by the adoptive transfer of these cells into RIP-GP Berlin recipients. Peptide-adjuvant-treated RIP-GP Berlin mice not developing T1D could then further be analyzed to identify the genes mutated in P14 cells resulting in loss of T1D susceptibility. Conversely, ENU mutagenesis of RIP-GP recipients would target endogenous cells and thus allow addressing pancreatic beta cell genes necessary for beta cell survival and protection against cytokine- or effector T cell-mediated attack etc., but also address the role of endogenous regulatory T cells or the inflammatory milieu during the autoimmune process. Finally, mutagenesis of P14xRIP-GP recipients would allow the analysis of both mutated effector and endogenous cells thereby allowing the identification of novel genes critically involved in T1D disease pathogenesis.

In summary, our data highlight the importance of autoantigen expression levels on diabetes pathogenesis and the use of our T1D model to identify factors that affect T1D development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Reduced degree of T1D pathogenesis in RIP-GP Armstrong compared to RIP-GP Berlin mice following LCMV infection or peptide/adjuvant induction

Age- and sex-matched RIP-GP Armstrong (RIP-GP Arm, red triangles) and RIP-GP Berlin (blue squares) mice were either infected with 10^4 pfu LCMV-Armstrong intraperitoneally (A) or received the non-viral peptide/adjuvant treatment (B). In brief, RIP-GP mice received an intravenous injection of 1.5×10^7 P14 spleen cells followed by intraperitoneal immunizations with 2mg gp33 containing 50 μ g of CpG adjuvant on days 1 and 3 after P14 transfer. On day 6 after P14 transfer, mice received an i.p. injection of 500 μ g polyinosinic:polycytidylic acid (polyIC). Blood glucose levels were measured in regular intervals up to day 21. Mice with blood glucose levels above 300mg/dl were considered diabetic. The graphs show percent diabetic mice after LCMV infection (A) or after P14 transfer (B).

Fig. 2. Reduced destructive CD8⁺ T cell infiltration into pancreatic islets of RIP-GP Armstrong compared to RIP-GP Berlin recipients following peptide/adjuvant treatment

RIP-GP Armstrong (RIP-GP Arm) and RIP-GP Berlin mice received the peptide adjuvant treatment as depicted in Figure 1B. On days 0, 4, 7, 9, and 11 after P14 transfer, pancreata were isolated and stained for CD8 and insulin. Arrows show CD8⁺ cells surrounding or infiltrating pancreatic islets. All RIP-GP Berlin mice (n=5) were diabetic by day 11. In contrast, only two out of five RIP-GP Arm mice were diabetic on day 11. Histological sections from day 9 and day 11 RIP-GP Berlin, and day 11 RIP-GP Arm mice are from diabetic mice. PI, peri-insulitis; I, Insulitis; DI, destructive Insulitis; NSS: non-sequential section. 10x magnification was used for d0 and d11 RIP-GP Arm sections; all other sections were 20x magnification.

Fig. 3. Peptide/adjuvant treatment induces P14 expansion, activation and cytokine secretion in the PDLN and pancreas of both RIP-GP Armstrong and RIP-GP Berlin recipients

RIP-GP Armstrong (RIP-GP Arm) and RIP-GP Berlin mice received the peptide/adjuvant treatment as depicted in Figure 1B. Ten days after P14 transfer, pancreatic draining lymph nodes (PDLN) and pancreata were isolated, and cells stained for expansion (first and second column), activation (third column) and IFN- γ secretion (fourth column) of P14 CD8⁺ T cells. Histogram plots in the first column show CD8⁺ staining. The second column depicts gated CD8⁺ T cells stained for transgenic P14 TCR (V α 2 and V β 8.1/2). The third column shows gated P14 CD8⁺ T cells stained for the activation markers CD44 and CD62L. The fourth column shows gated P14 CD8⁺ T cells stained for intracellular IFN- γ after 5-hour incubation with the P14-specific epitope LCMV-GP₃₃₋₄₁. Numbers in plots represent percentages of depicted populations.

Fig. 4. RIP-GP Armstrong and RIP-GP Berlin mice express similar levels of pancreas-restricted LCMV-GP mRNA but different LCMV-GP protein levels

(A) RT-PCR analysis of LCMV-GP and β -actin transcripts expressed in the pancreas (P), thymus (T), spleen (S) and lymph nodes (LN) of C57BL/6, RIP-GP Armstrong and RIP-GP Berlin mice. Samples were run with (+RT) or without reverse transcriptase (-RT).

(B) Quantitative real-time PCR of pancreatic LCMV-GP mRNA in C57BL/6, RIP-GP Armstrong and RIP-GP Berlin mice. Data shown are mean $\pm$ standard deviation of expression levels measured in triplicate relative to β -actin. -RT samples were all negative (data not shown).

(C) Pancreatic sections from Rag-deficient C57BL/6 (left column), RIP-GP Armstrong (middle column) and RIP-GP Berlin mice (right column) were stained for insulin (red, upper row) and LCMV-GP protein (green, middle row). The bottom row shows the overlay of the insulin and LCMV-GP protein stain. The pictures were taken with a 20x objective.

Fig. 5. Presence of antigen-specific effector or regulatory CD4⁺ T cells modulates T1D pathogenesis of peptide/adjuvant-treated RIP-GP recipients

(A) RIP-GP Armstrong recipients received the peptide/adjuvant treatment as depicted in Figure 1B. On day 4 after P14 transfer, one group of mice additionally received 1×10^7 *in vitro* activated Smarta spleen cells (red triangles). Blood glucose levels were measured in regular intervals up to day 21. Mice with blood glucose levels above 300mg/dl were considered diabetic. The graph shows percent diabetic RIP-GP Armstrong mice after P14 transfer.

(B) On day 21 after P14 transfer, pancreatic sections were stained for CD8, CD4, CD11b (brown) and insulin (blue). Left column shows pancreatic sections from a non-diabetic RIP-GP Armstrong mouse without additional transfer of Smarta cells, right column shows sections from a diabetic RIP-GP Armstrong mouse with additional transfer of Smarta cells. All sections are 20x magnification.

(C) RIP-GP Berlin recipients received the peptide/adjuvant treatment as depicted in Figure 1B. On day 8 after P14 transfer, one group of mice additionally received 1×10^6 *in vitro* generated Smarta regulatory T cells (green circles). Blood glucose levels were measured in regular intervals up to day 21. Mice with blood glucose levels above 300mg/dl were considered diabetic. The graph shows percent diabetic RIP-GP Berlin mice after P14 transfer.