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RAE1ε Ligand Expressed on Pancreatic Islets Recruits NKG2D Receptor-Expressing Cytotoxic T Cells Independent of T Cell Receptor Recognition

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SUMMARY

The mechanisms by which cytotoxic T lymphocytes (CTLs) enter and are retained in nonlymphoid tissue are not well characterized. With a transgenic mouse expressing the NKG2D ligand retinoic acid early transcript 1ε (RAE1ε) in β-islet cells of the pancreas, we found that RAE1 expression was sufficient to induce the recruitment of adoptively transferred CTLs to islets. This was dependent on NKG2D expression by the CTLs and independent of antigen recognition. Surprisingly, the recruitment of CTLs resulted in the subsequent recruitment of a large number of endogenous lymphocytes. Whereas transgenic mice did not develop diabetes, RAE1 expression was sufficient to induce insulitis in older, unmanipulated transgenic mice that was enhanced by viral infection and pancreatic inflammation. These results demonstrate that the expression of an NKG2D ligand in islets is sufficient to recruit CTLs regardless of their antigen specificity and to induce insulitis.

INTRODUCTION

The expression of NKG2D ligands on β-islet cells of the pancreas is proposed to play a key role in the pathogenesis of type 1 diabetes. In the human, genetic linkage studies demonstrate a positive association between a polymorphism in the gene encoding NKG2D ligand MHC class 1 chain-related protein A (MICA) and autoimmune diabetes (Nikitina-Zake et al., 2004), and altered NKG2D expression is observed in type 1 diabetic patients (Rodacki et al., 2007). In the nonobese diabetic (NOD) mouse model, the NKG2D ligand retinoic acid early transcript 1 (RAE1) was detected on β-islet cells, and treatment with a blocking NKG2D antibody in this model inhibited $CD8^+$ T cell infiltration into islets and diabetes development (Ogasawara et al., 2004). These data suggest that NKG2D expression in the pancreas may be a causative step in the development of autoimmune diabetes via engagement of NKG2D on $CD8^+$ T cells. However, it was unclear how NKG2D would function in this setting.

NKG2D is expressed by all natural killer (NK) cells in both human and mouse (Bauer et al., 1999; Wu et al., 1999) and on subsets of T cells. In the human this includes all $CD8^+$ T cells (Bauer et al., 1999), all γδ T cells (Bauer et al., 1999), NKT cells, and small subsets of $CD4^+$ T cells (Dai et al., 2009; Groh et al., 2003, 2006). In the mouse NKG2D expression is limited to activated (not naive) $CD8^+$ T cells (Jamieson et al., 2002), subsets of γδ T cells (Jamieson et al., 2002), NKT cells, and a small number of $CD4^+$ T cells (Hyka-Nouspikel et al., 2007).

NKG2D binds to a wide variety of ligands, all of which are related to MHC class I in sequence. In the human, the ligands are MICA and MICB (Bauer et al., 1999) and the UL-16 binding protein (ULBP), or retinoic acid early transcript 1 (RAET1) family member molecules (Bacon et al., 2004; Chalupny et al., 2003; Cosman et al., 2001). In the mouse, the ligands include the RAE1 (RAE1α−ε) protein family, the H60 (H60a-c) proteins, and mouse UL16-binding protein-like transcript 1 (MULT1) (Carayannopoulos et al., 2002a; Cerwenka et al., 2000; Diefenbach et al., 2000, 2003; Takada et al., 2008; Whang et al., 2009). These ligands are thought to be expressed not in most normal tissue, but rather their expression is induced under conditions of cell stress, such as viral infection, cellular transformation, or DNA damage (reviewed in Champsaur and Lanier, 2010).

In NK cells, NKG2D is an activating receptor, with engagement of NKG2D leading to cell killing (Bauer et al., 1999; Wu et al., 1999). In contrast, the function of NKG2D on $CD8^+$ T cells is less clear. Most studies suggest that NKG2D can act as a costimulatory receptor for cytotoxic T lymphocytes (CTLs), enhancing T cell receptor (TCR)-driven responses (Bauer et al., 1999; Chalupny et al., 2003; Cosman et al., 2001; Diefenbach et al., 2000; Markiewicz et al., 2005; Sutherland et al., 2002). However, other studies suggest that NKG2D alone has no effect on T cell activation (Champsaur and Lanier, 2010; Ehrlich et al., 2005). Our own studies of NKG2D function on T cells (Cemerski et al., 2007; Markiewicz et al., 2005) support the idea that NKG2D can costimulate T cells, but that it can also have functions independent of antigen recognition. Namely, we showed that NKG2D engagement could induce immunological synapse formation in CTLs independent of antigen. The significance of this finding was unclear, because NKG2D-mediated synapse formation did not result in activation of CTLs.

The recruitment of CTLs into tissue plays a critical role in the immunity to pathogens and also in the pathogenesis of

autoimmune diseases. The rules that govern the ability of CTLs to enter and remain in nonlymphoid tissues are complex and may be different for individual tissues. CTLs appear to have unrestricted access to many tissues (Masopust et al., 2004), but secondary signals, such as chemokine secretion from T helper cells, can enhance CTL access to tissues like the vaginal mucosa (Nakanishi et al., 2009). With respect to the pancreas, two recent reports suggest that islet-antigen specificity is required for the recruitment of both $CD4^+$ and $CD8^+$ T cells to islets (Lennon et al., 2009; Wang et al., 2010). However, the data suggesting that interaction between NKG2D and its ligands is required for $CD8^+$ T cell recruitment to islets in NOD mice (Ogasawara et al., 2004) led us to hypothesize that NKG2D may function to attract CTLs to pancreatic islets in diabetic individuals where its ligands are expressed.

To address our hypothesis, we generated a transgenic mouse with expression of the NKG2D ligand RAEε in pancreatic islets. We found that RAE1ε expression allowed for the specific recruitment of adoptively transferred CTLs to pancreatic islets independent of antigen recognition by the T cells. Demonstrating that this recruitment was through engagement of NKG2D on the CTLs, NKG2D-deficient CTLs were not recruited. Interestingly, this recruitment of CTLs resulted in the subsequent recruitment of endogenous lymphocytes via chemokine expression in the islets. Additionally, a mild insulitis, made up largely of lymphocytes, developed spontaneously in older transgenic mice that could be worsened by inducing pancreatic inflammation and increasing CTL numbers in the mice. Taken together, these results demonstrate that sterile expression of NKG2D ligands on β-islet cells can lead to antigen-independent CTL recruitment and insulitis. This suggests that aberrant NKG2D ligand expression by islet cells could be an initial trigger for lymphocyte recruitment during the development of autoimmune diabetes.

RESULTS

Generation of Transgenic Mice with Constitutive RAE1ε Expression in Pancreatic Islets

The expression of RAE1 in the β cells of the islet is suggested to be an initial trigger for the development of diabetes in the NOD mouse (Ogasawara et al., 2004). We hypothesized this may be via CTL recruitment to the pancreas. To test this hypothesis, we generated a conditional transgenic mouse in which expression of RAE1ε is controlled by the tissue-specific expression of Cre recombinase (Figure 1; Figure S1 available online). We cloned the RAE1ε cDNA into a plasmid (pCCALL2) that contains the constitutive chicken β-actin promoter, followed by a loxP-flanked gene encoding β-galactosidase-Neomycin (β-geo) (Novak et al., 2000). In the presence of Cre, the gene encoding β-geo is removed, allowing for RAE1ε expression. The construct was electroporated into embryonic stem (ES) cells and clones were screened for the expression of β-galactosidase as well as expression of RAE1ε after transient expression of Cre. Blastocyst injection was used to generate a transgenic mouse line. We confirmed the expression of β-galactosidase activity in most tissues in this mouse line (data not shown).

To induce the constitutive expression of RAE1ε in the β cells of the pancreatic islets, we bred the pCCALL2-RAE1ε mice to mice expressing Cre recombinase under the control of the rat insulin promoter (RIP-Cre) (Postic et al., 1999). We easily detected RAE1ε expression in double-transgenic (RIP-RAE1ε), but not single-transgenic (pCCALL2-RAE1ε or RIP-CRE), mice (Figure 1). Importantly, unlike transgenic models in which NKG2D ligands are expressed ubiquitously (Coudert et al., 2008; Oppenheim et al., 2005; Wiemann et al., 2005), the level of NKG2D expression on splenic NK cells was unaffected in RIP-RAE1ε mice (data not shown).

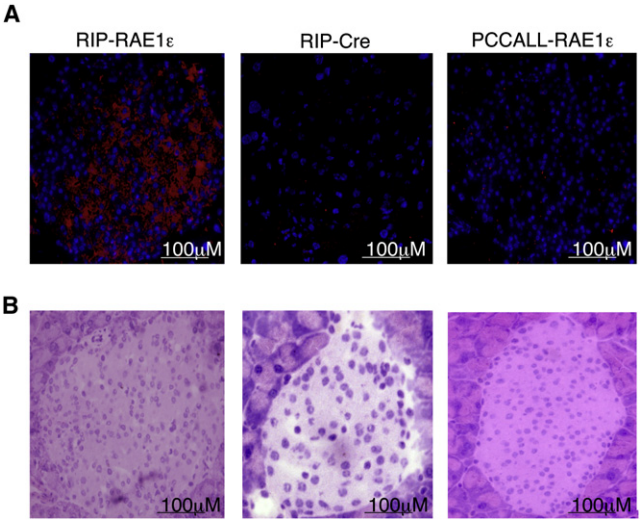

Figure 1. Specific Expression of RAE1ε in β-Islet Cells of RIP-RAE1ε Mice

(A) Pancreases from RIP-RAE1ε, RIP-Cre, and PCCALL-RAE1ε mice were sectioned and stained with a RAE1ε-specific antibody (red) and nuclei counterstained with Hoescht (blue).

(B) Serial sections of those shown in (A) were stained with H&E.

See also Figure S1.

Antigen-Independent Recruitment of CTLs to RAE1ε-Expressing Islets via NKG2D

The expression of RAE1 in the β-islet cells of NOD mice is thought to potentiate injury mediated by NKG2D-expressing $CD8^+$ T cells (Ogasawara et al., 2004). Because of the ability of NKG2D engagement to induce CTL synapse formation (Markiewicz et al., 2005), we hypothesized that expression of RAE1ε in islets might function to capture and retain $NKG2D^+$ CTLs in the pancreas in an antigen-independent manner. To directly test this, we adoptively transferred ovalbumin (OVA)-specific CTLs (OT-I) with the assumption that this TCR has no reactivity against any antigen in the islet. In-vitro-generated OT-I CTLs (Figure S2A) were labeled with CFSE and then adoptively transferred into RIP-RAE1ε or control (RIP-Cre and pCCALL2-RAE1ε) mice. As a positive control, OT-I CTLs were transferred into transgenic mice with OVA expression in the islets (RIP-OVA) (Kurts et al., 1996). Twenty-four hours later, islets were purified and single-cell suspensions analyzed for the presence of the CFSE-labeled CTLs. We detected transferred CTLs in the double-transgenic mice and RIP-OVA mice, but not in the single-transgenic control mice (Figures 2, S2C, and S2D). The presence of similar numbers of CFSE-labeled CTLs in the spleen of all mice verified that the mice had received similar numbers of CTL (Figure 2A).

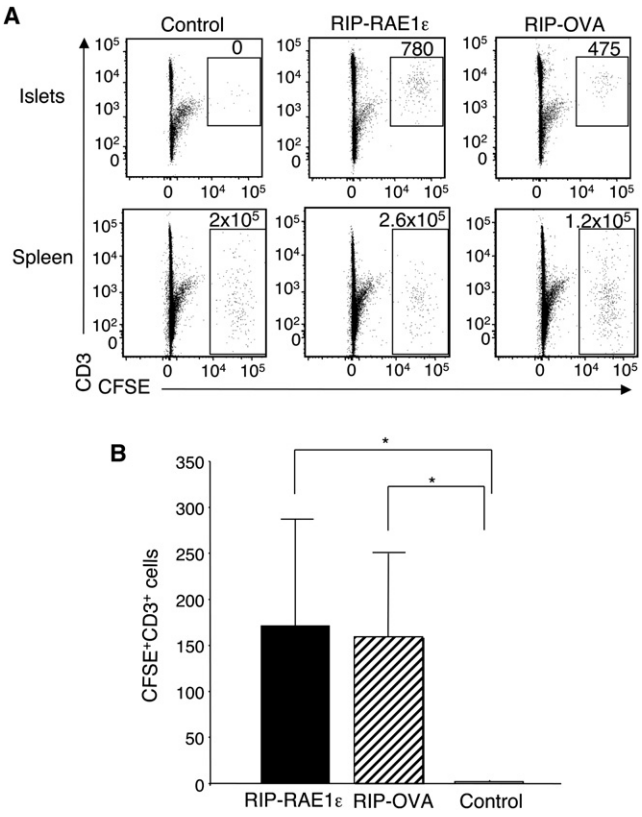

Figure 2. Antigen-Independent Recruitment of Adoptively Transferred CTLs to Islets of Mice with β-Islet Cell-Specific RAE1ε Expression

(A) Flow cytometric plots demonstrate the presence of adoptively transferred OT-I CTLs ($CFSE^+CD3^+$) in purified islets (top) from RIP-RAE1ε and RIP-OVA but not control mice. The plots shown are gated on live ($7\text{-}AAD^-$) cells. Similar analyses demonstrated similar numbers of $CFSE^+$ cells present in the spleens (bottom). The numbers shown are the total number of transferred CTLs present in the organ per mouse.

(B) The number of adoptively transferred OT-I CTLs (±SEM) present in purified islets per mouse from RIP-RAE1ε, RIP-OVA, or PCCALL-RAE1ε and RIP-Cre mice. The number shown is the average of eight independent experiments (±SEM) (RIP-RAE1ε and control) or four independent experiments (RIP-OVA). *$p < 0.0001$ in a two-sided Student's t test.

See also Figure S2.

Confirmatory experiments done with OT-I CTLs generated from RAG-1-deficient (*Rag1*$^{-/-}$) mice ruled out any role for antigen recognition in the recruitment of the CTL to islets in the RIP-RAE1ε mice (Figures S2E and S2F).

To determine whether the recruitment of CTLs to the islets in RIP-RAE1ε mice was dependent on NKG2D, we adoptively transferred CFSE-labeled OT-I CTLs from *DNAX*-activating protein of 10 KDa (DAP10)-deficient (*Hcst*$^{-/-}$) mice. DAP10 is an adaptor protein required for both NKG2D surface expression and signaling on T cells (Gilfillan et al., 2002); *Hcst*$^{-/-}$ CTLs therefore do not express NKG2D on the surface, nor can they be stimulated by NKG2D stimulation (Markiewicz et al., 2005). Confirming that the recruitment of CTLs to the islets was dependent on NKG2D expression, CFSE-labeled *Hcst*$^{-/-}$ CTLs were not detected in islets expressing RAE1ε (Figures 3A and 3B). Experiments with OT-I CTLs generated from mice completely deficient in NKG2D (*Klrk1*$^{-/-}$) (Zafirova et al., 2009) further confirmed a requirement for NKG2D surface expression for the recruitment of the adoptively transferred CTLs (Figure S3). These results demonstrate that RAE1ε expression in islets can recruit NKG2D-expressing CTLs in the absence of TCR recognition of antigen.

CTL Recruitment to RAE1ε-Expressing Islets Stimulates the Recruitment of Endogenous Lymphocytes

During the flow cytometric analysis of the islets for the presence of adoptively transferred CTLs, we noted that when CTLs were detected in the islets, there was also a much larger accumulation of endogenous immune cells (Figures 3A and 3C). Flow cytometric analysis showed that this endogenous infiltrate was largely composed of T lymphocytes (Figure 3C). Analysis of the single-transgenic control mice after adoptive transfer of CTLs, as well as analysis of the RIP-RAE1ε mice that received *Hcst*$^{-/-}$ CTLs or did not receive CTLs, showed few numbers of inflammatory cells (Figure 3C). We did not detect NKG2D expression on any of the infiltrating cells, suggesting that the recruitment of these other cells was not mediated by the presence of RAE1 (data not shown). This is not surprising because most murine $CD4^+$ T cells and naive $CD8^+$ cells don't express NKG2D (Champsaur and Lanier, 2010). Together, these results suggest that the recruitment of the NKG2D-expressing CTLs stimulated the recruitment of other T cells in an NKG2D-independent manner.

CTL Recruitment to RAE1ε-Expressing Islets Leads to CCL5 Expression in Islets

We hypothesized that the recruitment of endogenous lymphocytes to RAE1-expressing islets after CTL recruitment was due to the induction of chemokine expression in the islets. To test this, we extracted RNA from islets purified from RIP-RAE1ε and single-transgenic control mice that had received OT-I CTLs as well as RIP-RAE1ε mice that did not receive T cells. We then determined the chemokine genes differentially expressed among the islets by using a PCR mouse chemokine array. We found multiple chemokines upregulated in the islets purified from RIP-RAE1ε adoptive transfer recipient mice compared with islets purified from control adoptive transfer recipient mice or RIP-RAE1ε mice that did not receive CTLs, the most highly expressed being CCL5 (RANTES) (Figure 4A and data not shown).

We next set out to determine which cell type was responsible for chemokine expression in the islets of RAE1-expressing adoptive transfer recipient mice. First, islets were purified from RIP-RAE1ε mice that had or had not received OT-I CTLs, single-cell suspensions were generated, the cells were separated into $CD45^+$ (immune) and $CD45^-$ (nonimmune) cell populations, and the chemokines expressed by each of these populations were determined. Multiple chemokine transcripts were detected in both cell populations by this method, suggesting that multiple cell types might be producing chemokines (Figure 4B). To determine whether any of these chemokines were detectable at the protein level, purified islets were cultured overnight and the supernatants analyzed for the presence of chemokines. Only one chemokine, namely CCL5, was increased in the supernatants of RAE1-expressing islets from CTL adoptive transfer recipient mice compared with those from RAE1-expressing islets that had not received CTLs or control mice

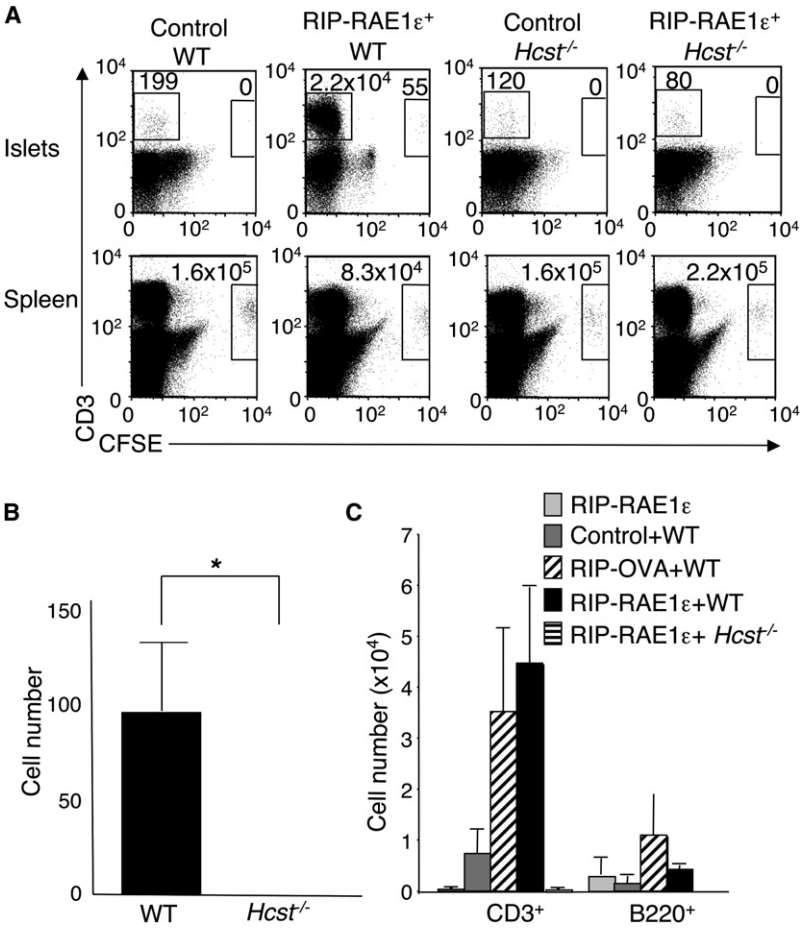

Figure 3. Recruitment of Endogenous Lymphocytes to Islets of Mice with β-Islet Cell-Specific RAE1ε Expression after Recruitment of Adoptively Transferred CTLs via NKG2D

(A) NKG2D is required for CTL recruitment to the islet. OT-I CTLs were prepared from wild-type (WT) and *Hcst*$^{-/-}$ mice and transferred after labeling with CFSE. Representative flow cytometric plots demonstrate the presence or absence of endogenous T cells (CD3$^+$CFSE$^-$) and adoptively transferred WT CTLs (CD3$^+$CFSE$^+$) (left two columns) or *Hcst*$^{-/-}$ CTLs (right two columns) in purified islets (top) or spleens (bottom) from RIP-RAE1ε and control PCCALL-RAE1ε and RIP-Cre mice. The numbers shown are the total number of adoptively transferred CTLs or endogenous T cells present per mouse.

(B) The number of adoptively transferred WT or *Hcst*$^{-/-}$ OT-I CTLs (±SEM) present in purified islets per mouse from RIP-RAE1ε mice. The number shown is the average of three independent experiments (±SEM). *$p < 0.0001$ in a two-sided Student's t test.

(C) The number of endogenous T cells (CD3$^+$CFSE$^-$) and B cells (B220$^+$) per mouse in purified islet preparations from RIP-RAE1ε, RIP-OVA, or control OT-I CTL adoptive transfer recipient mice, unmanipulated RIP-RAE1ε mice, and RIP-RAE1ε *Hcst*$^{-/-}$ OT-I CTL adoptive transfer recipient mice. The number shown is the average (±SEM) of seven independent experiments (RIP-RAE1ε and control) or three independent experiments (RIP-OVA).

See also Figure S3.

that had received CTLs (Figure 4C). Given that CCL5 is a potent T cell chemoattractant (Appay and Rowland-Jones, 2001), these data suggest that CCL5 expression in the RAE1-expressing adoptive transfer recipient mice was probably responsible for the recruitment of endogenous T cells.

CCL5 is predominantly secreted by activated CD8$^+$ T cells (Appay and Rowland-Jones, 2001). Therefore, we wondered whether the adoptively transferred CTLs themselves could be responsible for the CCL5 production detectable in the RAE1-expressing islets of CTL adoptive transfer recipients. Indeed, CCL5 was detected in supernatants from overnight cultures of OT-I CTL alone and this was not increased further by stimulation through NKG2D (Figure 4C and data not shown). Taken together, these data suggest that CCL5 expression by the recruited OT-I CTLs in the RAE1-expressing adoptive transfer recipient mice was probably responsible for the subsequent recruitment of endogenous T cells.

Transgenic Expression of RAE1ε in Islets Leads to Lymphocytic Infiltrates into the Pancreas of Older Mice

Although young (up to 3 months) RIP-RAE1ε mice did not contain pancreatic immune infiltrates in the absence of CTL transfer, we wondered whether spontaneous lymphocyte recruitment could occur as the mice aged. We hypothesized that this might then lead to islet destruction and diabetes development. To test this, we screened RIP-RAE1ε mice for the development of diabetes by regular blood glucose readings for up to 1 year. Over this period, we did not detect any glucose abnormalities in any of our double-transgenic mice. Histological examination of older mice (8–10 months), however, demonstrated that all RIP-RAE1ε mice analyzed developed a mild insulitis (nine of nine mice) (Figure 5). In contrast, little to no insulitis was seen in PCCALL2-RAE1ε or RIP-Cre mice (Figure 5D) or in young (6–8 weeks) RIP-RAE1ε mice (data not shown). To determine the composition of the inflammatory infiltrate in older RIP-RAE1ε mice, islets were isolated and analyzed by flow cytometry (Figure 6). This analysis showed that the immune infiltrates were a mixture of immune cells composed mainly of lymphocytes (Figure 6C). These results demonstrate that expression of RAE1 in the islets caused spontaneous recruitment of lymphocytes and the development of a mild insulitis.

Inflammation-Induced Infiltration of Lymphocytes into RAE1ε-Expressing Islets

We hypothesized the lack of diabetes development in RIP-RAE1ε mice could be due to poor access of lymphocytes to uninflamed tissue. Our adoptive transfer experiments demonstrated that RAE1 expression on β-islet cells was sufficient to induce the recruitment of a small number of NKG2D-expressing CTLs to the islets. We wondered whether inflammation could increase CTL recruitment to islets further in RIP-RAE1ε mice. To test this, we first treated young RIP-RAEε and singly transgenic control mice with the β-islet cell-specific toxin streptozotocin (STZ) to cause pancreatic inflammation prior to

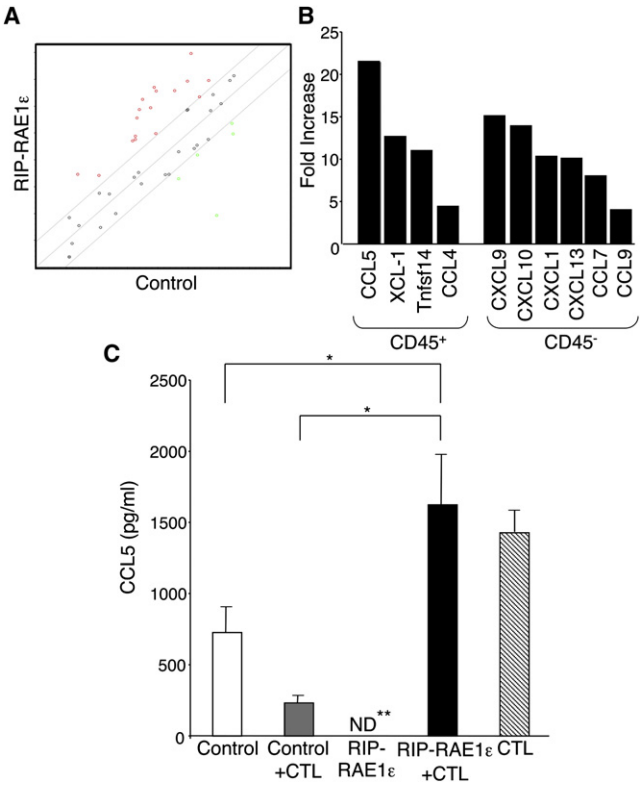

Figure 4. CTL Recruitment to RAE1ε-Expressing Islets Results in CCL5 Expression in Islets

(A) Differential chemokine gene expression in islets purified from RIP-RAE1ε and control RAE1-negative (PCCALL-RAE1ε and RIP-Cre) mice that received OT-I CTLs 24 hr previously. Genes upregulated greater than 3-fold are shown in red circles and genes decreased more than 3-fold are shown in green circles.

(B) Chemokine gene transcripts increased at least 3-fold in islets from RIP-RAE1ε mice that received OT-I CTLs compared with controls and the cell type in which they were detected (average fold increase, n = 3).

(C) Levels of secreted CCL5 (average ± STD, n = 4) measured from islets purified from RIP-RAE1ε or control OT-I CTL adoptive transfer recipient mice, unmanipulated RIP-RAE1ε, or control mice, and OT-I CTL prior to adoptive transfer. *p < 0.03; **ND (not detectable).

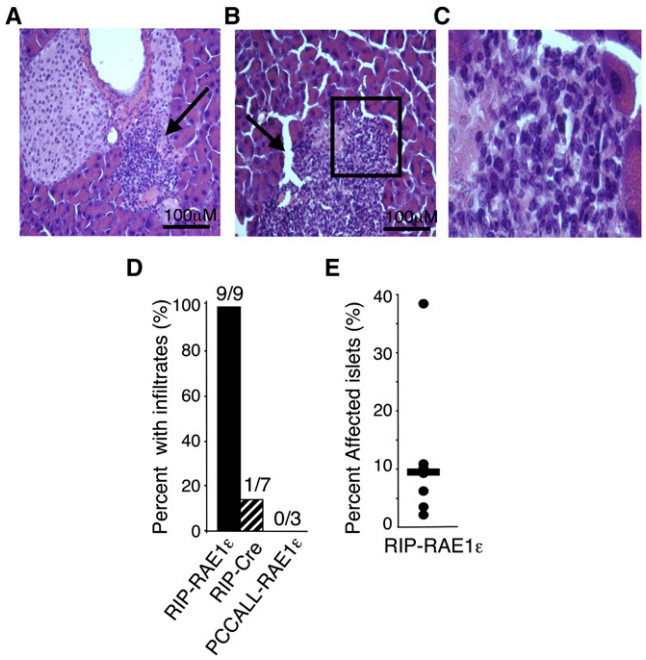

Figure 5. Mice with β-Islet Cell-Specific RAE1ε Expression Develop a Mild Insulitis as They Age

(A and B) Examples of immune infiltrates (arrows) inside pancreatic islets of two 8-month-old RIP-RAE1ε mice.

(C) The boxed portion of (B) magnified.

(D) The percentage of 8- to 10-month-old RIP-RAE1ε (n = 9), RIP-Cre (n = 7), and PCCALL-RAE1ε (n = 3) mice analyzed with immune infiltrates in the pancreas.

(E) The percentage of islets affected in the pancreata of the 8- to 10-month-old RIP-RAE1ε mice shown in (D).

adoptive transfer of OT-I CTLs. Treatment with STZ resulted in greater recruitment of both OT-I CTLs and endogenous T cells to the islets of RIP-RAE1ε mice compared with untreated RIP-RAE1ε mice or control mice (Figure 7A). These results suggest that pancreatic damage and islet RAE1 expression can synergize to induce the recruitment of T cells to the islets.

We next tested whether the increased T cell recruitment seen in STZ-treated RIP-RAE1ε mice could lead to the development of overt diabetes. RIP-RAE1ε and singly transgenic control mice were treated with a subdiabetic dose of STZ, blood glucose levels were monitored weekly, and the mice were sacrificed after 6 weeks. Although none of the mice developed diabetes, flow cytometric analysis of islets showed that the RIP-RAE1ε mice were very sensitive to the low dose of STZ and developed a greater inflammatory infiltrate compared to single-transgenic controls (Figures 7B–7D). We wondered whether we could enhance this infiltrate further by increasing the number of circulating CTLs in the mice. Therefore, to increase CTL numbers, we infected RIP-RAE1ε and singly transgenic mice with influenza A along with STZ treatment. RIP-RAE1ε mice infected with influenza alone showed a larger inflammatory infiltrate than the single-transgenic controls (Figures 7B–7D); however, the RIP-RAE1ε mice that received both STZ as well as influenza developed the greatest lymphocyte recruitment (Figures 7B–7D). These results suggest that the ability of islet-specific expression of RAE1ε to induce considerable lymphocytic infiltration requires both the presence of CTLs as well as an inflammatory stimulus that enhances the recruitment of cells into the islet.

DISCUSSION

NKG2D and its ligands are implicated in autoimmune diabetes in both humans and mice (Nikitina-Zake et al., 2004; Ogasawara et al., 2004). However, it was not clear how NKG2D and NKG2D ligands could contribute to diabetes development. Our current study offers one possible mechanism. We demonstrate that NKG2D engagement on CTLs with its ligands expressed on islet cells can lead to the recruitment of the CTL to pancreatic islets. Through the secretion of chemokine, this can then lead to the recruitment of additional T cells that do not express NKG2D. This suggests that at least one function of NKG2D in diabetes may be to mediate the recruitment of T cells to pancreatic islets.

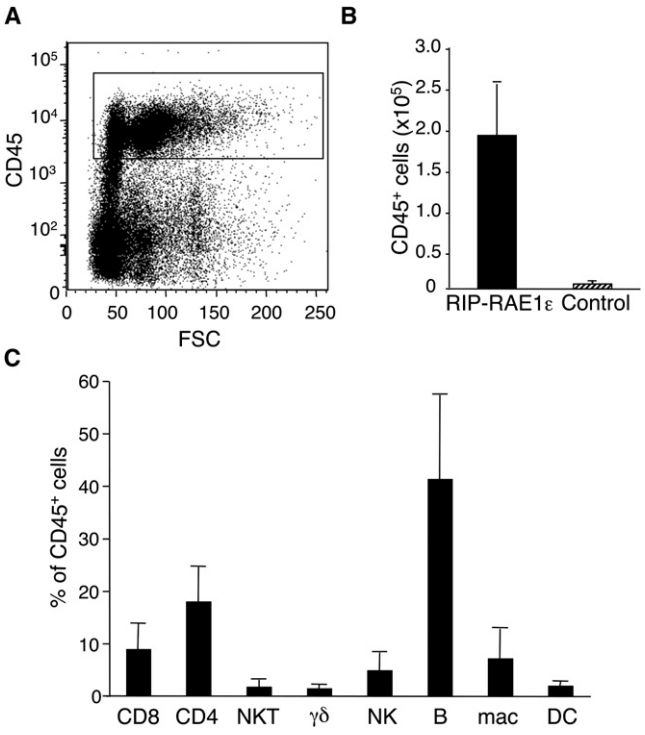

Figure 6. Inflammatory Infiltrate in Older RIP-RAE1ε Mice Is Composed Largely of Lymphocytes

(A) Representative flow cytometry plot demonstrating the presence of immune cells in islet preparations from RIP-RAE1ε mice. Islets purified from an 8-month-old RIP-RAE1ε mouse was stained with a CD45-specific antibody. The plot shown is gated on live (7-AAD$^-$) cells.

(B) The number of immune cells present in islet preparations from RIP-RAE1ε mice compared with controls. Islets were purified from 8- to 10-month-old RIP-RAEε and RIP-Cre and PCCALL-RAE1ε control mice and pooled within groups. The islets were stained with a CD45-specific antibody and analyzed by flow cytometry. The number of live (7-AAD$^-$) CD45$^+$ cells present per mouse was determined. The data shown is the average of four independent experiments (±SEM) with a total of 14 animals in each group.

(C) The majority of cells present in islet preparations from older RIP-RAE1ε mice are lymphocytes. The CD45$^+$ cells shown in (B) were stained with antibodies specific for CD8α, CD4, NK1.1, CD3ε, TCRγδ, B220, Cd11b, and CD11c and analyzed by flow cytometry. The percentage of CD45$^+$ cells present with the given cellular phenotype is shown. Abbreviations: CD8, CD8$^+$ T cell; CD4, CD4$^+$ T cell; NKT, NK T cell; γδ, γδ T cell; NK, NK cell; B, B cell; mac, macrophage; DC, dendritic cell.

The mechanisms by which CTLs are recruited to inflammatory sites are only beginning to be characterized. Although once thought to have unrestricted access to all tissues, it is now becoming clear that CTLs require specific signals to enter certain organs. Signals described to date include specific chemokine expression in tissues after CD4$^+$ T cell recruitment (Masopust et al., 2004) and antigen recognition (Wang et al., 2010). Here we demonstrate a mechanism by which CTLs can be recruited to NKG2D ligand-expressing pancreatic islets. This recruitment was dependent on NKG2D expression by the CTLs and independent of cognate antigen recognition.

Although it was first realized that NKG2D was expressed on CD8$^+$ T cells 12 years ago (Bauer et al., 1999), the function of NKG2D on CD8$^+$ T cells is still being delineated. NKG2D was originally described as a costimulatory receptor for CTLs (Groh et al., 2001), with no function in the absence of coincident TCR signaling. Since this time, NKG2D signaling on CTLs has been demonstrated to have some function in the absence of antigen recognition by the TCR under the correct conditions (Meresse et al., 2004; Verneris et al., 2004). Our early studies demonstrated that NKG2D engagement could induce immunological synapse formation in CTLs independent of antigen (Markiewicz et al., 2005). However, the significance of this finding was unclear, because NKG2D-mediated synapse formation did not result in any measurable CTL effector function. Our present data suggest that this may lead to the accumulation of CTLs in sites of NKG2D ligand expression in the absence of antigen recognition by the TCR.

Unexpectedly, recruitment of CTLs to islets was accompanied by a much larger recruitment of additional lymphocytes. Chemokine analysis showed that after T cell adoptive transfer, CCL5, a potent T cell chemoattractant, was induced in RAE1-expressing islets. Given that the CTLs themselves secreted a large amount of CCL5, this was probably what was responsible for the recruitment of additional lymphocytes. We cannot conclude from our studies whether the additional lymphocytes recruited after NKG2D-mediated CTL recruitment were specific for islet antigens or not. Nonetheless, RAE1 expression by islets led to recruitment of cells triggered by an antigen-independent CTL response.

Although insulitis was not seen in younger RIP-RAE1ε mice, a mild insulitis did develop as the mice aged. The absence of insulitis in young mice suggests that despite constitutive expression of NKG2D by NK cells, NK cells are not able to traffic into RAE1-expressing islets by themselves and/or require additional factors to kill islet cells. Similar to our findings, Strid et al. (2008) demonstrated that acute upregulation of RAE1-β in the epidermis induced immune infiltration. However, in contrast to the slower infiltration of immune cells we observed in the pancreas of the RIP-RAE1ε mice, the acute expression of RAE1-β in skin rapidly induced immune infiltration of CD4$^-$CD8$^-$ αβ unconventional T cells, a cell type that we did not observe in the pancreas. It is not clear why the character of the infiltrate in response to RAE1 expression is so different between these two models. It could be a differential response in skin versus pancreas, to acute versus chronic RAE1 expression, or to different RAE1 isoforms. Additionally, the receptor responsive to RAE1-β in the epidermis was not determined, so the response seen in the skin may have involved a receptor in addition to NKG2D.

Because NKG2D is expressed only on CTLs and not on naive CD8$^+$ T cells, we suspected that the delayed development of insulitis in our transgenic mice was due to low numbers of CTLs present in our mice, which are maintained in specific-pathogen-free conditions, and the absence of inflammation to promote cell entry. We tested these hypotheses by treating mice with a small dose of STZ to damage and inflame the islets. Although this dose of STZ resulted in little to no cell infiltration in wild-type mice, it was able to induce inflammation in RAE1-expressing mice. To increase the number of CTLs, we infected mice with influenza virus. Infection resulted in increased numbers of infiltrating lymphocytes in the nontransgenic islet, suggesting that viral infection may have multiple effects (including increasing CTL numbers) that may allow for immune cell infiltration into

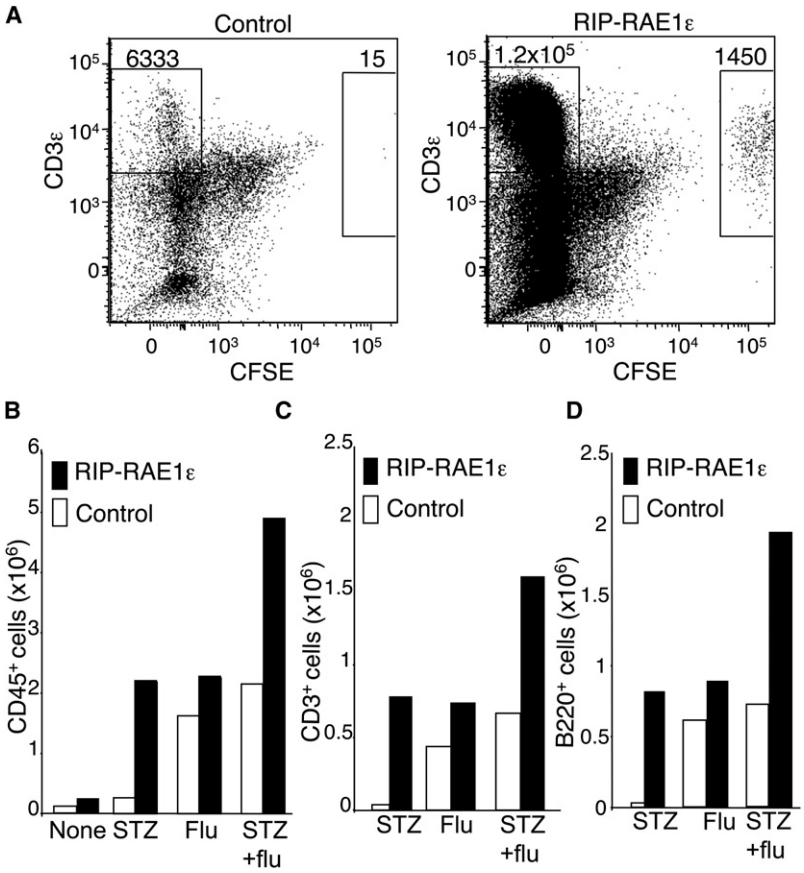

Figure 7. Inflammation-Induced Recruitment of Endogenous Lymphocytes to Islets of Mice with β-Islet Cell-Specific RAE1ε Expression

(A) STZ treatment increases recruitment of T cells to islets. RIP-RAE1ε and control PCCALL-RAE1ε and RIP-Cre mice were treated with STZ (2 doses of 75 mg/kg 24 hr apart), and 10^7 CFSE-labeled OT-I CTLs were adoptively transferred along with the second dose of STZ. Twenty-four hours later, the pancreases were harvested and pooled within groups, and islets purified. Single-cell suspensions of the purified islets were analyzed by flow cytometry. The plots shown are gated on live (7-AAD$^-$) cells. The numbers shown are the total number of transferred CTLs and endogenous T cells present per mouse.

(B) STZ treatment and viral infection increase immune infiltration into the pancreas of RIP-RAE1ε mice. Islets were purified from RIP-RAE1ε or control PCCALL-RAE1ε and RIP-Cre mice 6 weeks after STZ injection (2 doses of 50 mg/kg), intranasal infection with influenza A (100 PFU), or both STZ treatment and influenza A infection. The islets were stained with a CD45-specific antibody, analyzed by flow cytometry, and the number of live (7-AAD$^-$) CD45$^+$ cells present per mouse determined.

(C) The CD45$^+$ cells shown in (B) were stained with antibodies specific for CD3 and analyzed by flow cytometry.

(D) The CD45$^+$ cells shown in (B) were stained with antibodies specific for B220 and analyzed by flow cytometry. The number of cells present per mouse is shown. These results are representative of two independent experiments.

peripheral organs including the pancreas. The expression of RAE1ε resulted in a moderately enhanced number of infiltrating lymphocytes when the mice were infected with influenza. Combining STZ and infection with influenza resulted in a synergistic effect with a much greater lymphocyte infiltration detected in RAE1ε-expressing animals compared to the single-transgenic controls. This suggests that in the pathogenesis of diabetes, a virus that could infect the islets and induce NKG2D ligand expression might be able to both stimulate islet inflammation as well as induce CTL infiltration.

Analysis of the pancreatic cellular infiltrate in older RIP-RAE1ε mice demonstrated that CTLs constituted only a minority of the cells. Our results from the adoptive transfer studies allow for the possibility that it may be these cells that were originally recruited because of their expression of NKG2D and that they then recruited other cells via chemokines. This would suggest that the development of insulitis in our older mice might be determined not only by the infectious history of the mouse but also by an inflammatory trigger in the islets. However, our current data do not allow us to definitively determine the cell type(s) driving this spontaneous recruitment of lymphocytes. In addition, although the influx of lymphocytes was able to generate appreciable inflammation, it, however, did not result in the development of diabetes. This suggests that additional factors, including the participation of antigen-specific T cells capable of killing islet cells, may be required for the development of disease.

In contrast to our findings, two recent reports (Lennon et al., 2009; Wang et al., 2010) demonstrated that recruitment of T cells to islets was a cell-autonomous event that required islet-antigen specificity. The first (Lennon et al., 2009) used an elegant system of retrogenic mice that expressed both islet-specific and nonspecific CD4$^+$ T cells. In this system there was no recruitment of T cells specific for irrelevant antigen to islets even when there was significant recruitment of diabetogenic T cells. One caveat to these studies is that the nonspecific T cells were naive. T cell activation alters receptor expression, including integrins and chemokine receptors, allowing for T cell trafficking to tissues (Denucci et al., 2009). It may be that only activated T cells, or subsets of these cells, are able to be recruited in an antigen-independent fashion because of differential receptor expression. Our data suggest that NKG2D may be one of these receptors. In the mouse, NKG2D is expressed only on activated CD8$^+$ T cells (Raulet, 2003), a cell type not investigated in the retrogenic mouse study.

The authors of the second study (Wang et al., 2010) generated a gene-targeted NOD mouse with a mutated islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) so that diabetogenic CD8$^+$ T cells normally responsive to an epitope derived from IGRP were no longer responsive. These mice developed diabetes normally, but no IGRP-specific CD8$^+$ T cells were found in inflamed islets. These authors did test the possibility that activation was required for recruitment of bystander T cells by adoptively transferring IGRP-specific CD8$^+$ T cells activated in vitro for 3 days into the mice. These activated CD8$^+$ T cells were also not recruited to the islets early

after transfer. However, NKG2D expression is not upregulated on T cells early in culture (Markiewicz et al., 2005). Consistent with our data, small numbers of these cells were recruited to islets by 5 days after transfer, at a time when they presumably expressed higher levels of NKG2D.

A major unanswered question is why NKG2D ligands would be expressed in pancreatic islets. Are there circumstances that induce expression of NKG2D ligands in islets normally, or does pancreatic expression of these proteins occur only in diabetic-prone individuals as a result of some dysregulation? It may be that NKG2D ligands are normally expressed during times of pancreatic stress, but diabetes ensues only when there are additional defects in immune tolerance resulting in the presence of cells capable of killing β cells. One such stress may be the perinatal wave of β-cell death that is required for proper tissue remodeling of the pancreas and a proposed precipitating event for the development of diabetes (Finegood et al., 1995; Kassem et al., 2000; Petrik et al., 1998; Scaglia et al., 1997; Trudeau et al., 2000; Turley et al., 2003). Another pancreatic stress proposed to play a role in autoimmune diabetes development, viral infection (Hober and Sauter, 2010), could also induce expression of NKG2D ligands. Future studies will be required to determine whether these or other stimuli induce expression of NKG2D ligands in normal or diabetic-prone individuals.

In summary, by using transgenic mice with expression of the NKG2D ligand RAEε in pancreatic islets, we demonstrate that expression of NKG2D ligands in the pancreastic islets can induce the recruitment of CTLs to islets. This recruitment was independent of islet antigen specificity, but was dependent on NKG2D engagement on the CTLs. Further, once CTLs are recruited to islets, a large number of additional lymphocytes can be recruited via chemokine secretion. Given that aberrant NKG2D ligand expression has been linked to diabetes development (Ogasawara et al., 2004), our results suggest that at least one role for NKG2D in the development of diabetes may be to mediate the recruitment of T cells to such pancreatic islets.

EXPERIMENTAL PROCEDURES

Mice

All mice were housed under specific-pathogen-free conditions in the Washington University School of Medicine animal facilities in accordance with institutional guidelines. OT-I TCR transgenic, RIP-Cre, and RIP-OVA mice were purchased from Jackson Laboratory. *Hcst*$^{-/-}$ mice (Gilfillan et al., 2002) were provided by M. Colonna (Washington University School of Medicine). *OT-1,-Rag1*$^{-/-}$ mice were purchased from Taconic Farms, Inc. The *Klrk1*$^{-/-}$ mice have been previously described (Zafirova et al., 2009).

To generate PCCALL-RAE1ε mice, the cDNA encoding RAE1ε (Carayannopoulos et al., 2002b) (provided by L. Carayannopoulos, Washington University School of Medicine) was inserted into the multiple cloning site of the PCCALL2 vector (Lobe et al., 1999) (provided by A. Nagy, SLRI, Toronto, Canada). This plasmid contains a chicken β-actin promoter, followed by a loxP-flanked gene encoding β-geo (lacZ and neomycin resistance), and the RAE1ε cDNA inserted 3′ of the 3′ *lox*P site. This construct was transfected into C57BL/6/129 ES cells (provided by B. Sleckman, Washington University School of Medicine). ES cell clones were tested for their ability to express RAE1 after transient expression of Cre. Selected ES cells were then microinjected into blastocysts by the Washington University School of Medicine Department of Pathology and Immunology microinjection core facility. Potential founders were screened by PCR with the primers 5′-GGAAATCCATCGCTCGACCA-3′ and 5′-TCA CATCGCAAATGCAAATGC-3′. The founder mouse was then backcrossed five generations onto C57BL/6J (Jackson Laboratory).

Tissue Sectioning and Staining

For immunofluorescent staining, pancreata were frozen in OCT freezing medium (Tissue-Tek) and 5 μm sections were cut by the Washington University School of Medicine Anatomic and Molecular Pathology Core. The sections were fixed with cold acetone and (1) stained with a RAE1ε-specific antibody (1:50) (R&D Systems) followed by a Cy3-conjugated anti-rat IgG (Jackson Immunoresearch) and Hoechst stain (1 μg/ml) or (2) hematoxylin (Sigma Aldrich) and eosin (Sigma Aldrich).

T Cell Adoptive Transfer Experiments

CTLs were generated in vitro by culturing splenocytes and lymph node cells from OT-I TCR transgenic, *OT-1- Hcst*$^{-/-}$, *OT-1-Rag1*$^{-/-}$, or *OT-1-Klrk1*$^{-/-}$ mice in IMDM + 10% FCS in 6-well plates (2.5×10^7 cells/well) with 1 μM OVA peptide (SIINFEKL) (provided by P. Allen, Washington University School of Medicine) for 5 days. Live cells were harvested with Ficoll-hypaque (GE Healthcare) and were >98% CD8$^+$TCRVα2$^+$ (Figures S2 and S3). The cells were labeled with 1 μM CFSE (Invitrogen) and injected i.v. (10^7 cells/mouse). Pancreatic islets were harvested 24 hr later.

Islet Purification

Islets were purified with a ficoll gradient by published methods (Kelly et al., 2003). Pancreata were minced and digested with collagenase IV (20 mg/ml) (Sigma Aldrich). The digested pancreata were spun through a ficoll gradient (25%–23%–20.5%–11%) and the islets harvested.

Flow Cytometry

A single-cell suspension was generated from isolated islets or spleens by homogenization through a 40 μm cell strainer. Cells were stained with antibodies purchased from BD Biosciences (CD45-PE-Cy7, CD4-PE, CD3ε-APC, B220-PE-Cy5.5, TCR-γδ-PE-Cy.5, CD11c-APC, NK1.1-FITC) or eBioscience (CD8-750, CD11b-750), and dead cells were stained with 7-AAD (BD Biosciences). The cells were then analyzed on a FACSCanto II flow cyotmeter (BD Biosciences).

Chemokine Expression Analyses

Detection of Chemokine Transcripts

Single-cell suspensions from purified islets were separated into CD45$^+$ and CD45$^-$ fractions via a magnetic bead based separation system (Cellection Biotin Binder Kit, Invitrogen; biotinylated anti-CD45, BD Biosciences). Total RNA was isolated with Trizol (Invitrogen) followed by the RNeasy Mini Kit (QIAGEN) according to the manufacturers' protocols. Reverse transcription was performed with the SABiosciences RT^2 First Stand Kit (QIAGEN). The cDNA was analyzed with the SABiosciences Mouse Chemokines and Receptors RT^2 Profiler PCR Array (QIAGEN) according to the manufacturer's protocol, a Prism 700 real-time PCR machine (Applied Biosciences), and the SABiosciences Web-Based PCR Array Data Analysis software. Transcripts in total islets were compared between RIP-RAE1ε + CTL and control + CTL (n = 2), and RIP-RAE1ε + CTL and RIP-RAE1ε without CTL (n = 1). The average fold upregulation in islets from RIP-RAE1ε + CTL is shown. The expression of these transcripts was then compared between CD45$^+$ and CD45$^-$ cell populations from RIP-RAE1ε mice + CTL (n = 2).

Detection of Chemokine Proteins

Purified islets from RIP-RAE1ε and control PCCALL-RAE1ε and RIP-Cre mice that had or had not received OT-I CTLs were cultured overnight. The supernatants from these cultures were then analyzed with the Bio-Pex Pro mouse cytokine 23-plex assay (Bio-Rad) and a Bio-Plex workstation (Bio-Rad).

Streptozotocin Injection

After 6 hr of fasting, mice were injected with streptozotocin (Sigma Aldrich) in citrate buffer (pH 4.5) i.p. Two streptozotocin injections were given 24 hr apart.

Influenza Infection

Mice were anesthetized with ketamine and xylazine. 100 PFU of influenza A, strain WSN/33 (provided by H. Virgin, Washington University School of Medicine) was given i.n.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.immuni.2011.11.014.

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