Immunity
Article

A Subset of Interleukin-21$^+$ Chemokine Receptor CCR9$^+$ T Helper Cells Target Accessory Organs of the Digestive System in Autoimmunity

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SUMMARY

This study describes a CD4$^+$ T helper (Th) cell subset marked by coexpression of the cytokine interleukin 21 (IL-21) and the gut-homing chemokine receptor CCR9. Although CCR9$^+$ Th cells were observed in healthy mice and humans, they were enriched in the inflamed pancreas and salivary glands of NOD mice and in the circulation of Sjögren's syndrome patients. CCR9$^+$ Th cells expressed large amounts of IL-21, inducible T cell costimulator (ICOS), and the transcription factors *Bcl6* and *Maf*, and also supported antibody production from B cells, thereby resembling T follicular B helper (Tfh) cells. However, in contrast to Tfh cells, CCR9$^+$ Th cells displayed limited expression of CXCR5 and the targets of CCR9$^+$ Th cells were CD8$^+$ T cells whose responsiveness to IL-21 was necessary for the development of diabetes. Thus, CCR9$^+$ Th cells are a subset of IL-21-producing T helper cells that influence regional specification of autoimmune diseases that affect accessory organs of the digestive system.

INTRODUCTION

Accessory organs of the digestive system are connected to the small intestine by excretory ducts and include the pancreas and salivary glands, which are targets of the autoimmune diseases type 1 diabetes (T1D) and Sjögren's syndrome, respectively. T1D is a chronic autoimmune disease leading to the destruction of the insulin-producing β cells in the islets of Langerhans of the pancreas. In nonobese diabetic (NOD) mice, an initially mild pancreatic infiltration progresses over a period of 2–3 months, culminating in the loss of insulin-producing islets and clinical T1D (Bach and Mathis, 1997). However, islet inflammation varies considerably and the infiltrate may exhibit features of lymphoid neogenesis with ectopic germinal centers (GC) (Astorri et al., 2010; Jansen et al., 1993; Kendall et al., 2007). Studies collectively demonstrate that T cells of both the CD4$^+$ and CD8$^+$ lineages as well as B cells are necessary for the development of T1D in the NOD mouse (Serreze et al., 1996; Wicker et al., 1994). However, their individual roles and interactions remain poorly understood.

The autoimmune disease that develops in NOD mice also targets other accessory organs of the digestive system, namely the salivary glands and gallbladder. Similar to the pancreas, the reduced function of the salivary glands is due to lymphocytic infiltration, in which autoreactive T cells are thought to play a major role (Goillot et al., 1991). Indications of gall bladder involvement include diminished motility in NOD mice (Graewin et al., 2004), and a congenic NOD strain has been shown to spontaneously develop a fatal autoimmune biliary ductular disease (Koarada et al., 2004).

Effector and memory T cells can be classified based upon their ability to traffic through peripheral tissues, a property controlled by the expression of tissue-specific adhesion and chemoattractant receptors (Kunkel et al., 2003). CD4$^+$ T helper (Th) cells activated in intestinal lymph nodes selectively acquire responsiveness to the ligand (known as TECK or CCL25) for the G protein-coupled chemokine receptor 9 (CCR9) (Papadakis et al., 2000; Vicari et al., 1997; Wurbel et al., 2000) and express high amounts of α4β7 integrin (Campbell and Butcher, 2002). Lymphocytes infiltrating the islets in both human T1D and the NOD mouse express α4β7-integrin, supporting a link between T1D and the gastrointestinal immune system (Vaarala, 2002). Furthermore, antibodies blocking either α4β7-integrin or its ligand, the mucosal addressin cell adhesion molecule (MadCAM-1), prevent diabetes in NOD mice (Hänninen et al., 1998; Phillips et al., 2005).

Cytokines produced by Th cells influence the maintenance of the islet infiltrate and can contribute to β cell death either directly or through their effects on immune cells. The cytokine interleukin 21 (IL-21), produced by several Th cell subsets, has a critical role in both autoimmune diseases and inflammatory diseases of the gastrointestinal mucosa (Monteleone et al., 2009). IL-21 is thought to deliver a costimulatory signal that activates signaling pathways affecting the proliferation, differentiation, and survival of lymphocytes (Spolski and Leonard, 2008), but how IL-21 mediates its effect on autoimmune disease pathogenesis remains an important unanswered question.

In this study, we describe a subset of IL-21-producing CCR9-expressing CD4$^+$ T cells with a Tfh cell-like phenotype that

infiltrate accessory organs of the digestive system and provide help to CD8+ T cells for the development of type 1 diabetes.

RESULTS

An Abundance of IL-21 from NOD CD4+ T Cells

Previous studies have shown that NOD mice exhibit increased IL-21 mRNA expression relative to NOD$^{B6.Idd3}$ mice (King et al., 2004; McGuire et al., 2009) that carry the gene encoding IL-21 within the *Idd3* T1D susceptibility locus from C57BL/6 mice (Lyons et al., 2000). IL-21 protein was similarly increased 2- to 3-fold from naive CD4+ T cells from NOD mice after polyclonal activation in vitro (Figure 1A). ICOS is upregulated on CD4+ T cells after activation and provides a critical costimulatory signal for T helper cell differentiation (Dong et al., 2001). Partitioning of the CD44^{hi} memory cells according to ICOS expression indicated that although ICOS-expressing memory CD4+ T cells were the major cellular source of IL-21 from both NOD and NOD$^{B6.Idd3}$ cells, NOD ICOS+ memory CD4+ T cells produced approximately 7-fold more IL-21 (Figure 1B).

IL-21 protein was also detected in memory phenotype (CD44^{hi}) CD4+ T cells directly ex vivo by intracellular immunostaining, in which the specificity of IL-21 detection was tested against *Il21*$^{-/-}$ NOD CD4+ T cells (Figures 1C and 1D). The results demonstrated an age-related increase in both the fractions and numbers of CD4+ T cells spontaneously producing IL-21 in the pancreatic lymph node (PLN) (Figures 1E and 1F), pancreatic infiltrate (Figures 1G and 1H), spleen, mesenteric lymph nodes (MLN), and blood (Figure S1 available online) of NOD mice compared with NOD$^{B6.Idd3}$ mice. These findings indicated that CD4+ Th cells from the lymphoid organs and inflamed tissues of NOD mice produced an abundance of IL-21.

IL-21-Producing T Helper Cells in the Islet Lesion Coexpress CCR9

Because of the close association of the pancreas and gut, we analyzed the expression of the gut-homing molecules CCR9 and $\alpha4\beta7$ integrin on IL-21-producing CD4+ T cells in the islet infiltrate. Remarkably, the IL-21+ population in the islet lesion coexpressed CCR9, with little evidence for a defined population that expressed high amounts of either marker alone (Figure 1I). The gut-homing characteristics of this population were supported by the observation that approximately 50% of cells also expressed $\alpha4\beta7$ integrin (Figure 1J). In addition, a small fraction of cells were observed in the lamina propria of the small intestine (Figure 1K). As noted previously, the chronic inflammation in NOD mice is not restricted to the pancreas and includes the salivary glands (Goillot et al., 1991). Therefore, it was of interest to observe that approximately half of the CCR9-expressing CD4+ T cells in the salivary gland infiltrates also produced IL-21 (Figure 1L).

CCR9+ Th cells were enriched in the pancreatic infiltrates, salivary gland infiltrates, and the PLN of NOD mice compared with the tissues of congenic NOD$^{B6.Idd3}$ strain and C57BL/6 mice in which infiltration into the pancreas and salivary glands was not observed (Figure 1M). CCR9+ Th cells constituted a substantial fraction of the total IL-21-producing CD4+ T cell population in the pancreas and salivary gland infiltrates (Figure 1N) and approximately 25% of IL-21-producing T cells in the MLN and lamina propria coexpressed CCR9 (data not shown). In addition, CCR9+ Th cells were observed in the other accessory organs of the digestive system, namely the gall bladder, biliary duct, and liver of PBS-perfused NOD mice (data not shown). CCR9 expression was not restricted to CD4+ T cells; it was observed on approximately 15% of activated/memory phenotype CD8+ T cells and expressed highly on 10% of B cells in the islet infiltrate (see also Figure S1) and was not dependent on IL-21 because CCR9+ Th cells were observed in secondary lymphoid organs of *Il21*$^{-/-}$ and *Il21r*$^{-/-}$ NOD mice (data not shown). Taken together, these findings indicate that CCR9+ Th cells were present throughout secondary lymphoid organs but were enriched in the inflamed accessory organs of the digestive system.

CCR9+ Th Cells Are Increased in the Blood of Patients with Sjögren's Syndrome

To investigate the presence of CCR9-expressing T helper cells in human autoimmune diseases that affect accessory organs of the digestive system, we analyzed peripheral blood from a cohort of patients with Sjögren's syndrome. Memory phenotype CCR9+ Th cells were detected in small numbers in both human tonsillar tissues and peripheral blood (Figure 2A). By contrast, CCR9+ Th cells were expanded in the circulation of most Sjögren's syndrome patients (Figure 2B), and 9/15 Sjögren's syndrome patients exhibited a 4- to 5-fold increase in memory phenotype CCR9+ Th cells relative to controls (Figure 2C). The increased fraction of CCR9+ Th cells in Sjögren's syndrome patients was not simply due to an increase in the fraction of CD4+ T cells (Figure 2D) or memory phenotype CD4+ T cells (Figure 2E), which were equivalent in the blood of patients and controls. However, there were no significant differences ($p > 0.05$) in clinical or laboratory parameters and no relationship between the proportion of circulating CCR9+ Th cells and CXCR5+ Tfh cells in a subset of patients in which both parameters were measured (data not shown). These findings demonstrated that IL-21-producing CCR9+ Th cells were observed in the peripheral blood of most Sjögren's syndrome patients.

Expression of CCR9 Is Important but Not Necessary for CCR9+ Th Cell Migration into the Pancreatic Islets

The expression of CCR9 suggested that IL-21-producing Th cells in the islet lesions of NOD mice had originated from the small intestinal mucosa and/or had the potential to home to tissues that express its ligand, CCL25 (TECK) (Wurbel et al., 2000). Previous studies have shown that CCL25 is expressed at high amounts in the thymus and small intestine (Wurbel et al., 2000). Immunoblot analyses also detected CCL25 in isolated islet preparations from NOD mice and confirmed the presence of CCL25 protein in the NOD intestine (Figure 3A).

To determine whether CCR9 was a functional chemokine receptor on CCR9+ Th cells, we performed a chemotaxis assay in which migration of total lymphocytes, from PLN and pancreas infiltrate, toward CCL25 was assessed. Lymphocytes observed to migrate toward CCL25 in vitro (Figure 3B) were reduced by CCL25 antibody blockade of CCR9:CCL25 interactions (Figure 3B). Flow cytometric analyses of the migrated populations demonstrated enrichment for CCR9+ Th cells from both the PLN (Figure 3C) and pancreas (Figure 3D). These observations were

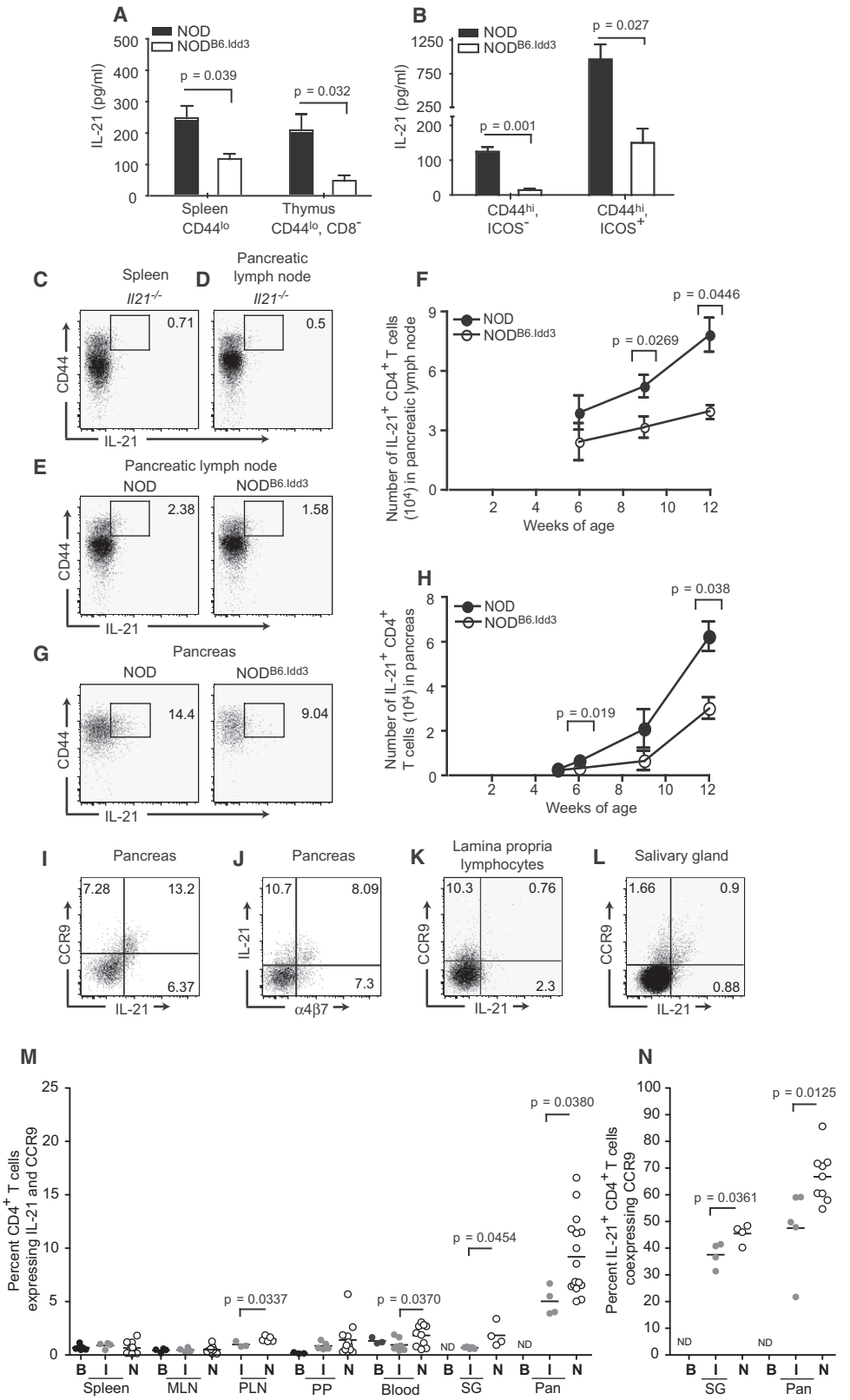

Figure 1. Increased Production of IL-21$^+$CD4$^+$ T Cells in NOD Mice

(A and B) Naive (CD44^{lo}) CD4$^+$ T cells from spleen and thymus (A) and memory (CD44^{hi}), ICOS$^-$ and ICOS$^+$, CD4$^+$ T cells from NOD and NOD$^{B6.Idd3}$ spleen (B), cultured for 4 days with CD3 and CD28 mAb. IL-21 was measured by ELISA and data presented as means ± SEM; n = 6 mice per group from two experiments.

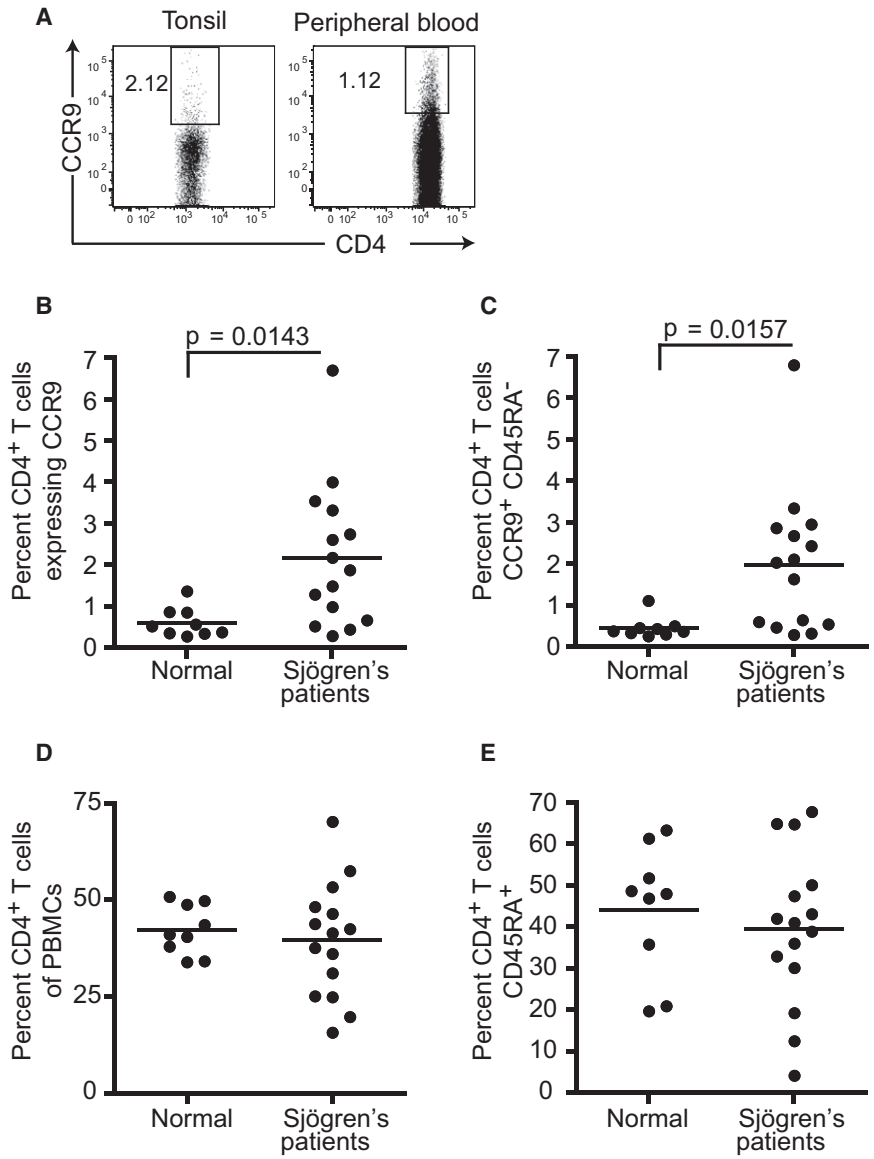

Figure 2. Human CCR9+CD4+ T Cells Are Expanded in Sjögren's Patients

(A) Representative dot plots of CCR9 expression in human tonsil and peripheral blood.

(B–E) Flow cytometric analyses of blood from healthy individuals (normal) and Sjögren's syndrome patients showing $CCR9^+$ cells as a percentage of $CD4^+$ T cells (B) and $CCR9^+$ $CD45RA^-$ cells as a percentage of $CD4^+$ T cells (C); p values from nonparametric unpaired t tests. Also shown are $CD4^+$ T cells as a percentage of peripheral blood mononuclear cells (PBMCs) (D) and $CD45RA^+$ cells as a percentage of $CD4^+$ T cells (E), where n = 9–15.

borne out in vivo where blockade of CCR9:CCL25 interactions with CCL25 Ab reduced the number of CFSE-labeled $CCR9^+$ Th cells recovered after 5 days from the pancreas and MLN, but not PLN or spleen, of NOD recipients (Figure 3E). CCL25 antibody treatment also reduced the number of endogenous $CCR9^+$ Th cells and $CD8^+$ T cells in the islet lesion observed on day 7 of antibody treatment (Figure 3F) and reduced the frequency of infiltrated islets (Figure 3G), and continued administration inhibited the development of diabetes in NOD mice (Figure 3H).

Immunofluorescence of histological sections of NOD pancreata identified cells that coexpressed CD4 and CCR9 within the islet infiltrate (Figure 3I). Small numbers of cells expressing both CD8 and CCR9 and B220 B cells expressing CCR9 were also detected in the islet lesion of NOD mice (Figure 3I). By contrast, 1 week after treatment, pancreatic islets of CCL25 Ab-treated NOD mice frequently exhibited few infiltrating lymphocytes (Figures 3I and 3G). Therefore, CCR9:CCL25 interactions induced the migration of $CCR9^+$ Th cells, supported the pancreatic migration and/or retention of $CCR9^+$ Th cells, and were important for diabetes development in NOD mice.

$CCR9^+$ Th Cells Are Distinct from Th1, Th2, and Th17 Cells

$CCR9^+$ Th cells in NOD mice had all the hallmarks of a $CD4^+$ Th effector memory population, with high expression of CD44 (Figure 1) and little CD62L or CCR7 expression (Table S1), but they were not NK T cells because they failed to bind the CD1 tetramer αGalCer (Figure S2). However, the cytokine profile of $CCR9^+$ Th cells from the inflamed pancreas and PLN was largely restricted to IL-21 because, in contrast to $CCR9^-$ Th cells, $CCR9^+$ Th cells were observed to produce little, if any, IL-4, IL-17A, IFN-γ, or TNF-α after restimulation in vitro (Figure S2).

(C and D) Representative dot plots of intracellular immunostaining for IL-21 in negative control *Il21*$^{-/-}$ $CD4^+$ T cells in spleen (C) and pancreatic (D) lymph nodes.
(E–H) IL-21 in NOD and $NOD^{B6.Idd3}$ $CD4^+$ T cells in pancreatic lymph node, representative dot plot (E) and absolute number of $CD4^+$ T cells at ages shown (F), and in pancreas as representative dot plot (G) and absolute number of $CD4^+$ T cells at ages shown (H), where n = 16–22 mice per group from eight experiments.
(I–L) Representative dot plots of $CD4^+$ T cells. CCR9 and intracellular IL-21 (I) and IL-21 and α4β7 (J) on $CD4^+$ T cells from NOD pancreata and representative dot plots of CCR9 and IL-21 in $CD4^+$ T cells from small intestine lamina propria (K) and salivary gland (L).
(M and N) Percentage of $CD4^+$ T cells that coexpress CCR9 and IL-21 (M), and percentage of $IL\text{-}21^+CD4^+$ T cells that express CCR9 (N) from 9-week-old NOD mice; n = 6–15 from four experiments; ND, not detected.

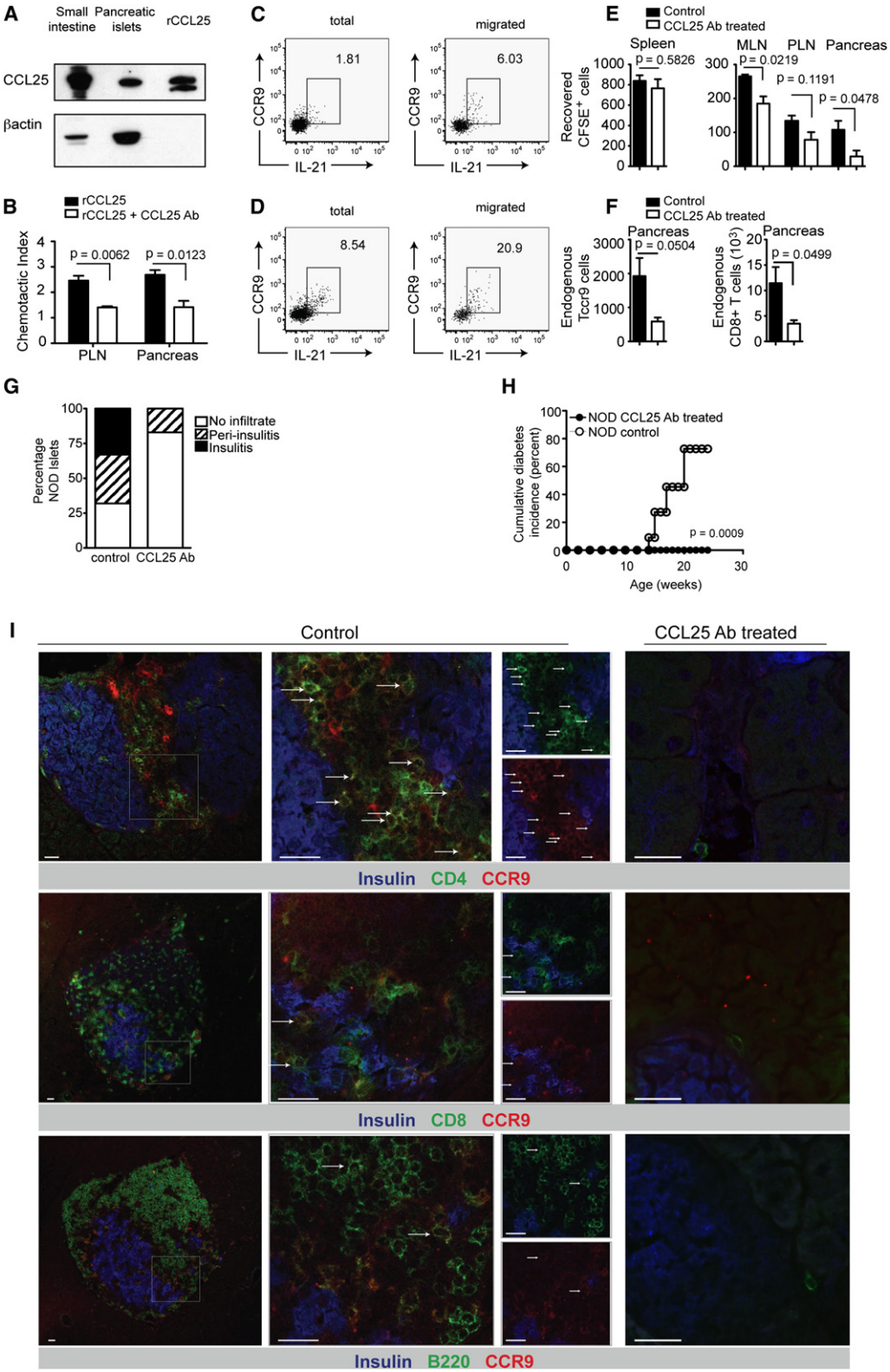

Figure 3. A Role for CCR9:CCL25 Interactions in CCR9+ Th Cell Migration and the Development of Type 1 Diabetes

(A) Representative immunoblot showing CCL25 in NOD small intestine and pancreatic islets, compared to rCCL25, representative of three experiments.

(B) Chemotactic index calculated as the number of cells isolated from 11-week-old NOD pancreatic lymph nodes and pancreas that migrated across 3 μm membrane toward rCCL25 (10 μg/ml), in the presence or absence of CCL25 Ab (4 μg/ml) over background migration.

Transcription factors act as master controllers of cytokine gene expression to establish lineage-specific transcriptional programs during Th cell differentiation (Ivanov et al., 2006; Pai et al., 2004). Analyses of the transcription factors expressed by CCR9$^+$ Th cells and CCR9$^-$ Th cells from the pancreas and PLN revealed that the expression of *Tbx21* (encoding T-bet) in both groups was similar to the levels observed in Th1 cells differentiated in vitro (Figure 4A). By contrast, the Th2 transcription factor GATA-3 mRNA was lower in both CCR9$^+$ Th cells and CCR9$^-$ Th cells relative to Th2 cells (Figure 4B). Despite the minimal IFN-γ production in CCR9$^+$ Th cells, T-bet protein was detected in both CCR9$^+$ Th cells and CCR9$^-$ Th cells by immunoblot (Figure 4C; see also Figure S2), whereas there was little if any GATA-3 protein detected in CCR9$^+$ Th cells relative to CCR9$^-$ Th cells (Figure 4C). In accordance with the lack of IL-17 production from CCR9$^+$ Th cells, the Th17 cell transcription factor *Rorc* (encoding ROR-γt) (Ivanov et al., 2006) was expressed at similar low amounts in CCR9$^+$ Th cells and CCR9$^-$ Th cells, relative to Th17 cells (Figure 4D). Thus, CCR9$^+$ Th cells expressed transcription factors typically associated with Tfh cells.

Pancreatic CCR9$^+$ Th Cells Express High Amounts of IL-21, *Bcl-6*, and *Maf* but Are Distinct from Tfh Cells

It was of interest to observe the Tfh cell transcription factors *Maf* (Bauquet et al., 2009) and *Bcl6* (Yu et al., 2009; Johnston et al., 2009; Nurieva et al., 2009) in abundance in CCR9$^+$ Th cells from the pancreas and PLN compared with both CCR9$^-$ Th cells and Tfh cells (Figures 4E and 4F). In addition, the high expression of both IL-21 and Bcl-6 was not restricted to murine CCR9$^+$ Th cells, as shown by the fact that CCR9$^+$ Th cells from human tonsillar tissue similarly expressed high amounts of both Bcl6 (Figure 4G) and IL-21 (Figure 4H) compared with memory phenotype CD4$^+$ T cells that did not express CCR9.

Consistent with the presence of Tfh-like cells, histological analyses of NOD pancreata and flow cytometric analyses of the islet infiltrate revealed infrequent organized lymphoid aggregates and PNA staining clusters (GC) with a small percentage of GC B cells in nonimmunized NOD mice housed under SPF conditions (Figure S3). However, further examination identified markers that distinguished CCR9$^+$ Th cells in the pancreatic lesion from Tfh cells (King et al., 2008). For instance, the expression of the signaling lymphocyte activation molecule (SLAM)-associated protein, SAP, was reduced compared with Tfh cells (Figure 4I), and relatively few CCR9$^+$ Th cells or CCR9$^-$ Th cells in unmanipulated NOD mice expressed the B follicle homing marker CXCR5 (Figure 4J). By contrast, in the Peyer's patches and salivary glands of NOD mice, approximately 30% and 15% of CD4$^+$CD44^{hi} T cells, respectively, coexpressed CCR9 and CXCR5, indicating the presence of CCR9-expressing Tfh-phenotype cells at these sites (Figure 4J). In support of this, SAP expression was also more highly expressed in CXCR5$^+$CCR9$^+$ Th cells than CXCR5$^-$CCR9$^+$ Th cells from the Peyer's patches (Figure S2). However, in contrast to Tfh cells, the coinhibitory molecules PD-1 and BTLA were not consistently expressed on either CCR9$^+$ or CCR9$^-$ Th cells (Figures 4K and 4L). The costimulatory molecule ICOS, in turn, was more highly expressed on CCR9$^+$ Th cells than CCR9$^-$ Th cells (Figure 4L). Thus, pancreatic CCR9$^+$ Th cells were distinguished from Tfh cells by differential expression of CXCR5, PD-1, and SAP.

CCR9$^+$ Th Cells Resist Upregulation of CXCR5 In Vitro

Recent studies have highlighted the plasticity of T helper cell subsets (Wei et al., 2009). Therefore, we questioned whether, given appropriate signals, CCR9 could be upregulated on Tfh cells and whether CXCR5 could be upregulated on CXCR5$^-$CCR9$^+$ Th cells. Culture of Tfh cells purified from immunized NOD mice and naive CD4$^+$ T cells with dendritic cells purified from the MLN (Elgueta et al., 2008) demonstrated that Tfh cells were relatively refractory to CCR9 upregulation in vitro (Figure 4M). Likewise, CCR9$^+$ Th cells from the PLN and pancreas cultured under Tfh cell-inducing conditions (Nurieva et al., 2008) upregulated CXCR5 poorly in comparison to both CCR9$^-$ Th cells and naive CD4$^+$ T cells (Figure 4N). Furthermore, expression of CXCR5 and PD-1 were increased on CCR9$^-$ Th cells but not CCR9$^+$ Th cells in vivo after immunization with a polyvalent antigen (Figure S3).

Reciprocal Relationship between CCR9$^+$ Th Cells and B Cells

B cells have been shown to be important for the development of T1D in the NOD mouse (Serreze et al., 1996), and the increased expression of ICOS and IL-21 by CCR9$^+$ Th cells indicated the potential for superior interaction with B cells. Similar to CCR9-expressing CD4$^+$ T cells from human blood (Papadakis et al., 2003), pancreatic CCR9$^+$ Th cells supported antibody production from B cells. However, CCR9$^+$ Th cells and CD4$^+$ ICOS^{hi} (CCR9$^-$) T cells from the spleen equally supported the production of IgM, total IgG, and IgG1 and IgG2c isotypes from B cells purified from the pancreas and spleen in vitro (Figure 5A). Both Th cell populations were more effective than naive CD4$^+$ T cells, but the amounts of antibody were modest compared with B cells stimulated with CD40 ligand (Figure 5A; Figure S4).

Because any interaction of CCR9$^+$ Th cells and B cells was likely to have reciprocal effects, IL-21 expression was measured in NOD mice that had been made genetically deficient in B cells (*Ighm*$^{-/-}$). Both IL-21 mRNA and IL-21 protein production were

(C and D) Representative dot plots of the total and migrated CD4$^+$ T cells (CCR9/IL-21) from pancreatic lymph node cells (C) and pancreatic infiltrate (D); n = 4–5, from two experiments.
(E and F) 10- to 13-week-old NOD mice treated with CCL25 Ab, or control Ab, 20 μg, i.v. 3 times per week. n = 4 from two experiments.
(E) CFSE-labeled pancreas and pancreatic lymph nodes sorted CCR9$^+$ Th cells recovered from spleen, mesenteric lymph node (MLN), pancreatic lymph node (PLN), and pancreas, on day 5.
(F) Numbers of endogenous CCR9$^+$ Th cells and CD8$^+$ T cells on day 7. n = 4 from two experiments.
(G) Insulitis index day 14, n = 4–6 from two experiments.
(H) Cumulative incidence of diabetes, n = 8–11.
(I) Representative sections of pancreata on day 14, stained for insulin (blue) and CCR9 (red). Row 1 CD4 (green), row 2 CD8 (green), and row 3 B220 (green). Arrows indicate the position of CCR9 costaining on immune cells. Scale bars represent 20 μm.

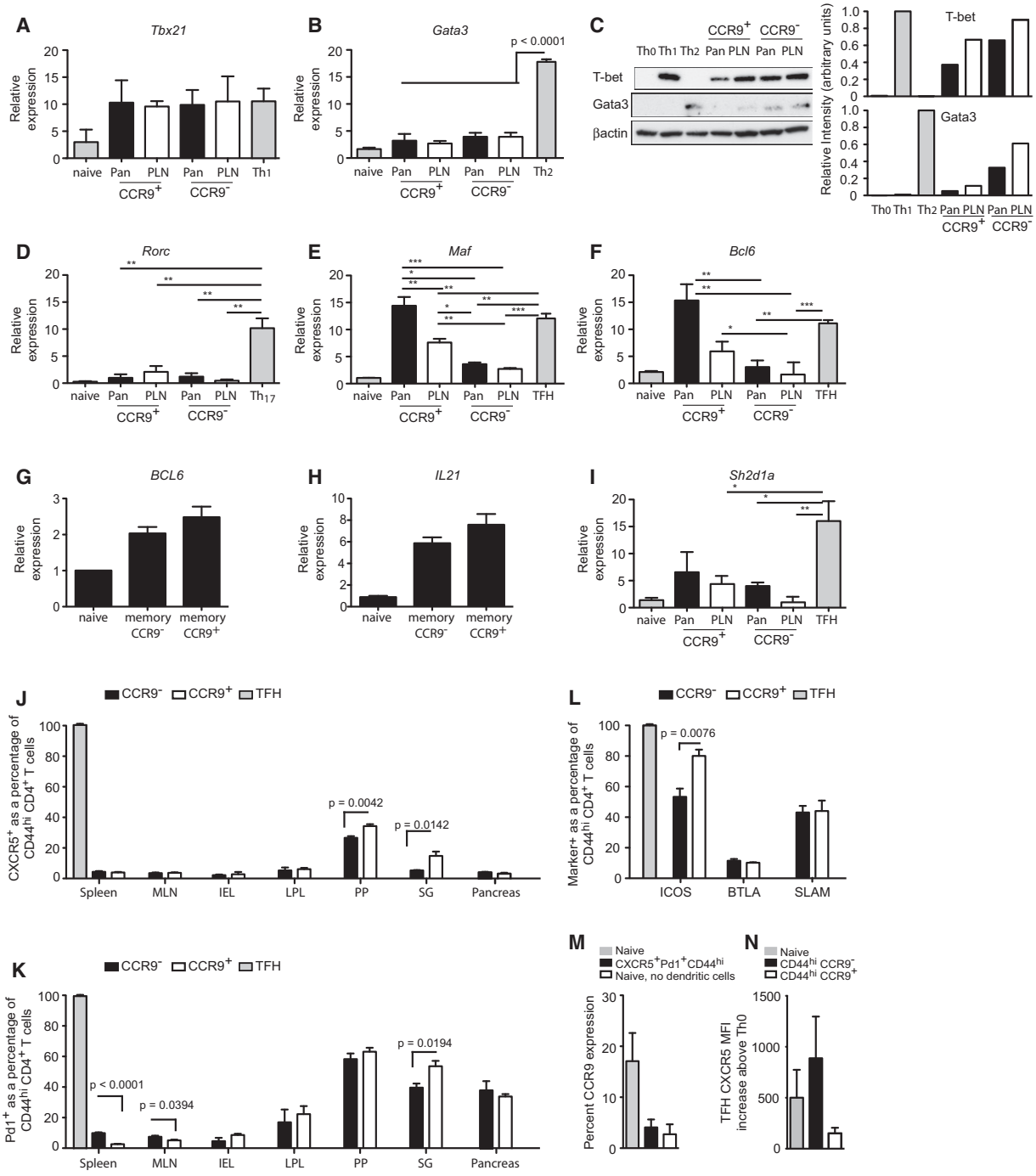

Figure 4. Transcription Factor and Surface Marker Characterization of CCR9+ Th Cells

Real-time PCR analysis and immunoblot of transcription factors and FACS analyses of cell surface markers from CCR9+CD44hiCD4+ T cells, sorted from NOD pancreas and pancreatic lymph nodes; sorted naive CD44loCD4+ T cells and CCR9−CD44hiCD4+ T cells from the pancreas and pancreatic lymph nodes; Th1, Th2, and Th17 polarized lymphocytes; and CXCR5+Pd1+CD44hiCD4+ (Tfh) cells from the spleen of day 7 SRBC immunized NOD mice.

(A and B) Real-time PCR analysis of *Tbx21* (A) and *Gata3* (B).

(C) T-bet and GATA-3 protein expression after 3 days in vitro stimulation with CD3 mAb and CD28 mAb (Th0), representative of three experiments.

(D–F) Real-time PCR analysis of *Rorc* (D), *Maf* (E), and *Bcl6* (F).

(G and H) Real-time PCR of *Bcl6* (G) and *Il21* (H) in human tonsillar CCR9+CD45RA−CD4+ T cells, from two experiments.

(I) Real-time PCR analysis of *sh2d1a* (SAP) expression. Murine RT-PCR data shown as mean ± SEM, where n = 4–8 from three experiments.

(J and K) Quantification of CXCR5 (J) and PD-1 (K) on CCR9+ and CCR9−, CD44hiCD4+ T cells and Tfh cells by flow cytometry.

(L) Expression of ICOS, BTLA, and SLAM on pancreatic CD44hiCD4+ T cells; n = 3–6 from two experiments.

substantially reduced in *Ighm*$^{-/-}$ NOD mice compared with WT NOD mice (Figure S4), which may reflect the previous finding from our laboratory and others that ICOS:ICOSL interactions are important for optimal production of IL-21 or for the differentiation of IL-21-producing Th cells (Bauquet et al., 2009; Vogelzang et al., 2008).

Flow cytometric analyses of lymphocytes from the PLN and pancreas lesion confirmed a physical association between CD4$^+$ T cells and B cells, known as conjugates (Reinhardt et al., 2009). CCR9$^+$ Th cells from the PLN (Figure 5B) and pancreas (Figure 5C) were detected in conjugates with B cells, and chemical disruption of the conjugates ex vivo confirmed CCR9 expression on the bound CD4$^+$ T cells (Figure 5D). CCR9$^+$ Th cells from NOD mice facilitated this interaction as shown by the fact that adoptively transferred NOD CCR9$^+$ Th cells formed conjugates with NOD$^{B6.Idd3}$ B cells in pancreatic infiltrates (Figure 5E).

During the analyses, we noted with great interest a marked reduction in the percentage of CD8$^+$ T cells in the pancreas of NOD$^{B6.Idd3}$ mice compared with NOD mice (Figure 5F). Transfer of CCR9$^+$ Th cells from NOD mice also increased the fraction of CD8$^+$ T cells in the pancreas of NOD$^{B6.Idd3}$ recipients (Figure 5F). This effect was dependent on CCR9$^+$ Th cells as shown by the fact that donor CCR9$^-$ Th cells had no effect on the percentages of either T:B conjugates or CD8$^+$ T cells in NOD$^{B6.Idd3}$ recipients (Figures 5E and 5F). Taken together, these findings indicated that CCR9 Th cells interacted with B cells for optimal production of IL-21 and influenced the recruitment or maintenance of lymphocytes in the islet lesion.

CD8$^+$ T Cells Are the Downstream Targets for IL-21 in T1D

Despite the important role of IL-21 in T1D, the mechanism by which IL-21 imparts its effects remains unknown. Previous studies have shown that both CD4$^+$ Th cells and CD8$^+$ T cells are required for the adoptive transfer of T1D into immunodeficient NOD-SCID hosts (Christianson et al., 1993). We took advantage of this system to determine IL-21's influence on T cells by comparing the capacity of IL-21-producing versus IL-21-deficient CD4$^+$ Th cells to help diabetogenic CD8$^+$ T cells. The importance of IL-21 production from Th cells was demonstrated by the inability of IL-21-deficient Th cells to assist CD8$^+$ T cells to induce diabetes (Figure 6A). Furthermore, the transfer of diabetes by CD8$^+$ T cells or CD4$^+$ Th cells from NOD mice made genetically deficient in IL-21R (when mixed with WT Th and CD8$^+$ T cells) revealed that IL-21 competence by CD8$^+$ T cells was critical for diabetes development (Figure 6B).

IL-21 Promotes Expansion and Survival of CD8$^+$ T Cells

The requirement for IL-21 receptiveness by CD8$^+$ T cells prompted us to examine IL-21's role. Exploiting the available IL-21 in *Il21r*$^{-/-}$ NOD mice, polyclonal, CFSE-labeled, CD8$^+$ T cells purified from NOD mice were transferred into NOD and *Il21r*$^{-/-}$ NOD recipients. A small number of transferred, CFSE-labeled, memory phenotype CD8$^+$ T cells spontaneously proliferated in NOD recipients (Figure 6C). Remarkably, extensive proliferation was observed in *Il21r*$^{-/-}$ NOD recipients (Figure 6C). This spontaneous expansion of CD8$^+$ T cells was dependent upon IL-21 in the recipient mice because WT NOD CD8$^+$ T cells failed to proliferate spontaneously in NOD recipients made genetically deficient in both IL-21 and IL-21R (*Il21*$^{-/-}$*Il21r*$^{-/-}$ NOD mice) (Figure 6C). The increased IL-21-dependent proliferation was consistent with an increased recovery of CD8$^+$ T cells from the spleen, MLN, and PLN of recipient mice (Figure 6D) and was observed for both CD44^{hi} memory phenotype and CD44^{lo} naive donor CD8$^+$ T cells (Figure 6E). Interestingly, despite the broad influence of IL-21 on the proliferation of CD8$^+$ T cells, the increased recovery was attributed to the memory population (Figure 6F).

IL-21:IL-21R signaling on CD8$^+$ T cells had improved their recovery, suggesting that IL-21 supported the survival of CD8$^+$ T cells. In support of this notion, IL-21 induced an increased number of total CD8$^+$ T cells that expressed the prosurvival molecule Bcl-2 (Figure 6G), as well as a greater number of Bcl-2-expressing donor memory phenotype than donor naive CD8$^+$ T cells that had undergone cell division (Figure 6H). Taken together, these studies indicate that Th cells provide IL-21 to CD8$^+$ T cells, which facilitates CD8$^+$ T cell expansion and the subsequent destruction of islet β cells.

CCR9$^+$ Th Cells Provide IL-21-Dependent Help to CD8$^+$ T Cells to Induce Diabetes

To test directly whether CCR9$^+$ Th cells could help CD8$^+$ T cells to cause diabetes, we transferred CCR9$^+$ Th cells purified from the pancreas and PLN combined with WT CD8$^+$ T cells into *Il21r*$^{-/-}$ NOD recipients. Diabetes-resistant *Il21r*$^{-/-}$ NOD recipient mice were irradiated to provide room for the expansion of the small number of donor cells (Figure S4). Two weeks after transfer, *Il21r*$^{-/-}$ recipients of CCR9$^+$ Th cells plus CD8$^+$ T cells were exhibiting difficulty in regulating blood glucose after the administration of a bolus of glucose during a glucose tolerance test (Figure 7A). By contrast, all other groups, including *Il21r*$^{-/-}$ recipients of CCR9$^+$ Th cells plus CD8$^+$ T cells that had received IL-21RFc chimera, CCR9$^-$ Th cells plus CD8$^+$ T cells, CD8$^+$ T cells alone, or control *Il21r*$^{-/-}$ NOD mice that had received only PBS regulated blood glucose levels normally after an initial, expected, spike (Figure 7A).

Histological analyses of the pancreata of *Il21r*$^{-/-}$ NOD recipients of CCR9$^+$ Th cells plus CD8$^+$ T cells detected the presence of both CD8- and CCR9/CD4-expressing cells in and around the insulin-immunostained islets (Figure 7B). These mice developed rapid insulitis compared with recipients of CCR9$^-$ Th cells combined with WT CD8$^+$ T cells ($p < 0.001$), as well as CCR9$^+$ Th cells alone, WT CD8$^+$ T cells alone, and irradiated *Il21r*$^{-/-}$ NOD mice that did not receive cells, which were all free of

(M) Mean fluorescent intensity (MFI) of CCR9 expression from naive (CD44^{lo}) and Tfh cells after in vitro stimulation with mesenteric lymph node-derived dendritic cells and SRBC. Data shown as MFI increase above stimulation in the absence of dendritic cells (no-DC cultures); n = 3, representative of two experiments.
(N) Mean fluorescent intensity of CXCR5 expression from naive (CD44^{lo}), CCR9$^-$ and CCR9$^+$, CD44^{hi}CD4$^+$ T cells after in vitro stimulation under Tfh cell-polarizing conditions. Data shown as MFI increase above stimulation in the absence of Tfh cell-polarizing conditions (Th0); n = 3–4, representative of two experiments.

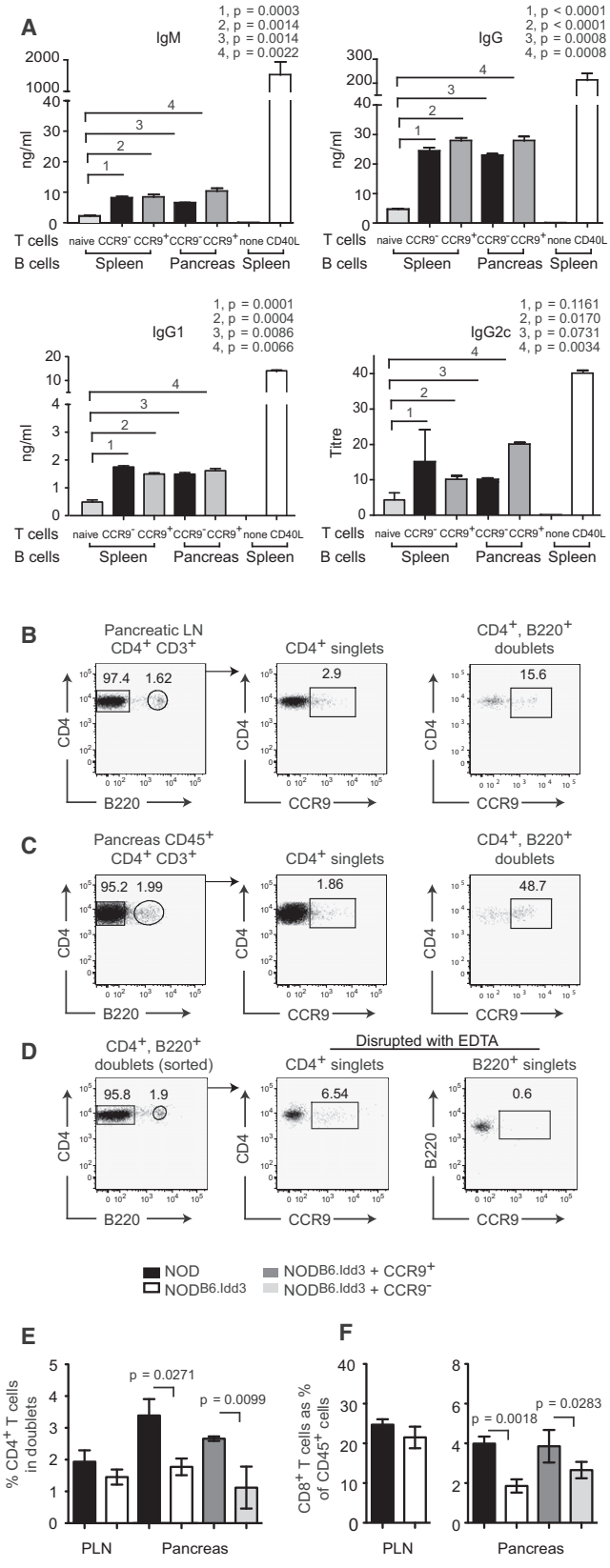

Figure 5. CCR9⁺CD4⁺ T Cells Interact with B Cells and Influence CD8⁺ T Cells

(A) IgM, IgG, IgG1, and IgG2c production from sorted splenic or pancreatic B220⁺ B cells cultured with plate-bound CD3 mAb and soluble CD28 mAb and equal numbers of splenic naive CD44^{lo}CD4⁺ T cells (naive), CCR9⁻ICOS⁺ CD44^{hi}CD4⁺ T cells (CCR9⁻) and pancreatic CCR9⁺CD44^{hi}CD4⁺ T cells (CCR9⁺), or CD40 mAb. Antibody isotypes measured in supernatant by ELISA on day 4, data presented as mean ± SEM; n = 3–5 from two experiments.

(B and C) Representative flow cytometric plots of CD4 and B220, and CCR9 expression, gated on CD4⁺ T cells from pancreatic lymph node (B) and pancreas (C).

(D) Pancreatic lymph node CD4⁺ T cells as shown in (B) sorted on CD4⁺B220⁺ doublets, subsequently disrupted with EDTA containing buffer and immunostained for CCR9. 5 × 10^5 CCR9⁺ and CCR9⁻CD44^{hi}CD4⁺ T cells sorted from pancreatic lymph nodes and pancreas were transferred into NOD$^{B6.ldd3}$ mice.

(E and F) Pancreatic lymph nodes and pancreas were analyzed on day 16 for CD4⁺B220⁺ doublets (E) and CD8⁺ T cells (F).

Data are shown as mean ± SEM; n = 3–6 from three experiments.

insulitis (Figures 7B and 7C). In addition, infiltration of the submandibular salivary glands was observed only in the recipients of CCR9⁺ Th cells plus CD8⁺ T cells, complementing the observations in the pancreas (Figure 7B).

Recipient mice treated with neutralizing IL-21RFc exhibited relatively mild peri-insulitis, indicating that IL-21 production by CCR9⁺ Th cells was critical for the observed islet inflammation (Figure 7C). In accordance with increased islet infiltration, approximately 70% of the *Il21r*$^{-/-}$ NOD recipients of CCR9⁺ Th cells combined with CD8⁺ T cells had developed rapid diabetes within 8 weeks of transfer compared with 0% of *Il21r*$^{-/-}$ NOD recipients of CCR9⁻ Th cells and WT CD8⁺ T cells or CCR9⁺ Th cells alone (Figure 7D). Neutralization of IL-21 with IL-21RFc confirmed that the ability of CCR9⁺ Th cells to induce diabetes was dependent upon IL-21 (Figure 7D). Thus, CCR9⁺ Th cells have an enhanced potential to provide IL-21-mediated help to CD8⁺ T cells for the transfer of diabetes.

DISCUSSION

Region-specific Th cell subsets, such as Th2 cells in the respiratory mucosa or the newly identified Th22 cells in the skin (Duhen et al., 2009; Trifari et al., 2009), express their unique effector functions during immune defense. In this study we have identified a Th cell subset based upon expression of the chemokine receptor CCR9 that contributes to the regional specification of organ-specific autoimmune diseases. CCR9⁺ Th cells constituted only a small fraction of CD4⁺ T cells in the lymphoid tissues and circulation of healthy mice and humans but exhibited an inappropriate accumulation in the autoimmune lesions of the nonlymphoid accessory organs of the digestive system in NOD mice and were abundant in the peripheral blood of most Sjögren's syndrome patients.

Antigen specificity is a prerequisite for the accumulation of T cells in the islet lesion (Lennon et al., 2009), but the site of priming of diabetogenic T cells remains unknown. The finding that CCR9 marked the IL-21-producing Th cell subset in islet lesions, PLN, and mesenteric LN support the notion that diabetes-causing T cells are primed in the gut and the previous finding that lymphocytes from gut-associated lymph nodes are highly effective at inducing diabetes (Jaakkola et al., 2003). In

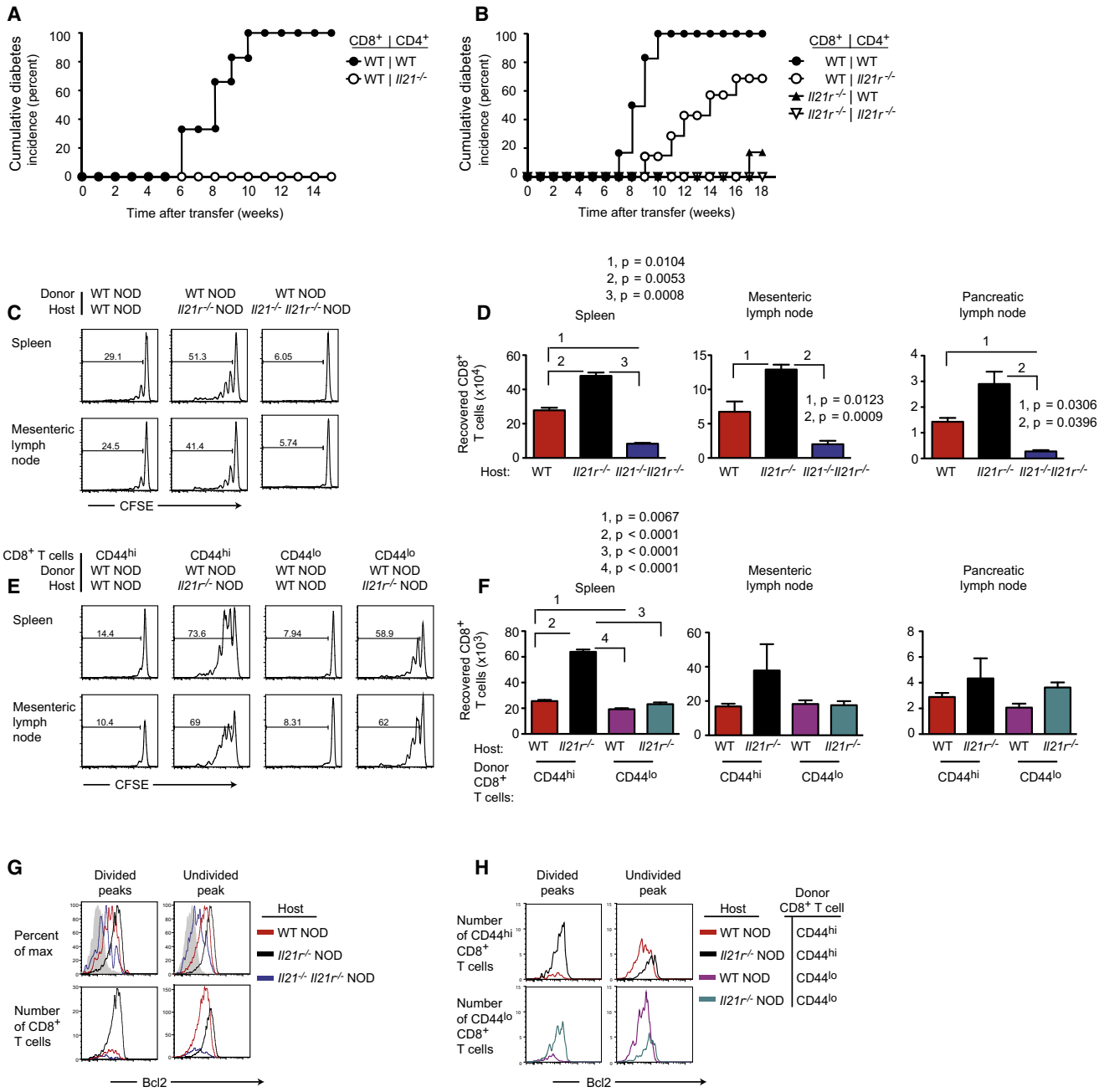

Figure 6. IL-21 Supports Survival of CD8+ T Cells

(A and B) Cumulative incidence of diabetes in NOD-SCID recipients of (A) 5 × 10^6 WT NOD or *Il21*$^{-/-}$ NOD CD4$^+$ T cells combined with 5 × 10^6 WT CD8$^+$ T cells, n = 8 and (B) 5 × 10^6 CD4$^+$ T cells and 5 × 10^6 CD8$^+$ T cell from WT and *Il21r*$^{-/-}$ NOD mice, CD4$^+$ T cell and CD8$^+$ T cell combined transfers as indicated; n = 6.

(C and D) Total CFSE-labeled NOD CD8$^+$ T cells (10 × 10^6) recovered from WT NOD, *Il21r*$^{-/-}$ NOD, and *Il21*$^{-/-}$*Il21r*$^{-/-}$ NOD mice shown as (C) representative histograms of CFSE dilution and (D) numbers of recovered transferred CD8$^+$ T cells.

(E and F) CFSE-labeled CD44^{hi} and CD44^{lo}CD8$^+$ T cells (2 × 10^6) recovered from WT NOD, *Il21r*$^{-/-}$ NOD, and *Il21*$^{-/-}$*Il21r*$^{-/-}$ NOD mice shown as (E) representative histograms of CFSE dilution and (F) numbers of recovered CD8$^+$ T cells.

(G and H) Bcl2 expression of recovered CD8$^+$ T cells as shown in (C) and (E), with isotype control reference (gray filled histogram). Data are shown on day 7 after transfer; n = 3–8, representative of four experiments.

healthy humans and mice, CCR9 is expressed predominately by a subset of T cells that migrate selectively to the gut (Papadakis et al., 2003) and local dendritic cells can imprint CCR9 on mature T cells (Mora et al., 2003). CCR9$^+$ T cells play a role in several gastrointestinal tract inflammatory disorders (Koenecke and Förster, 2009). However, the relatedness to the CCR9$^+$ Th cells described herein awaits confirmation. CCL25 is found in the small intestine and thymus (Wurbel et al., 2000), and the

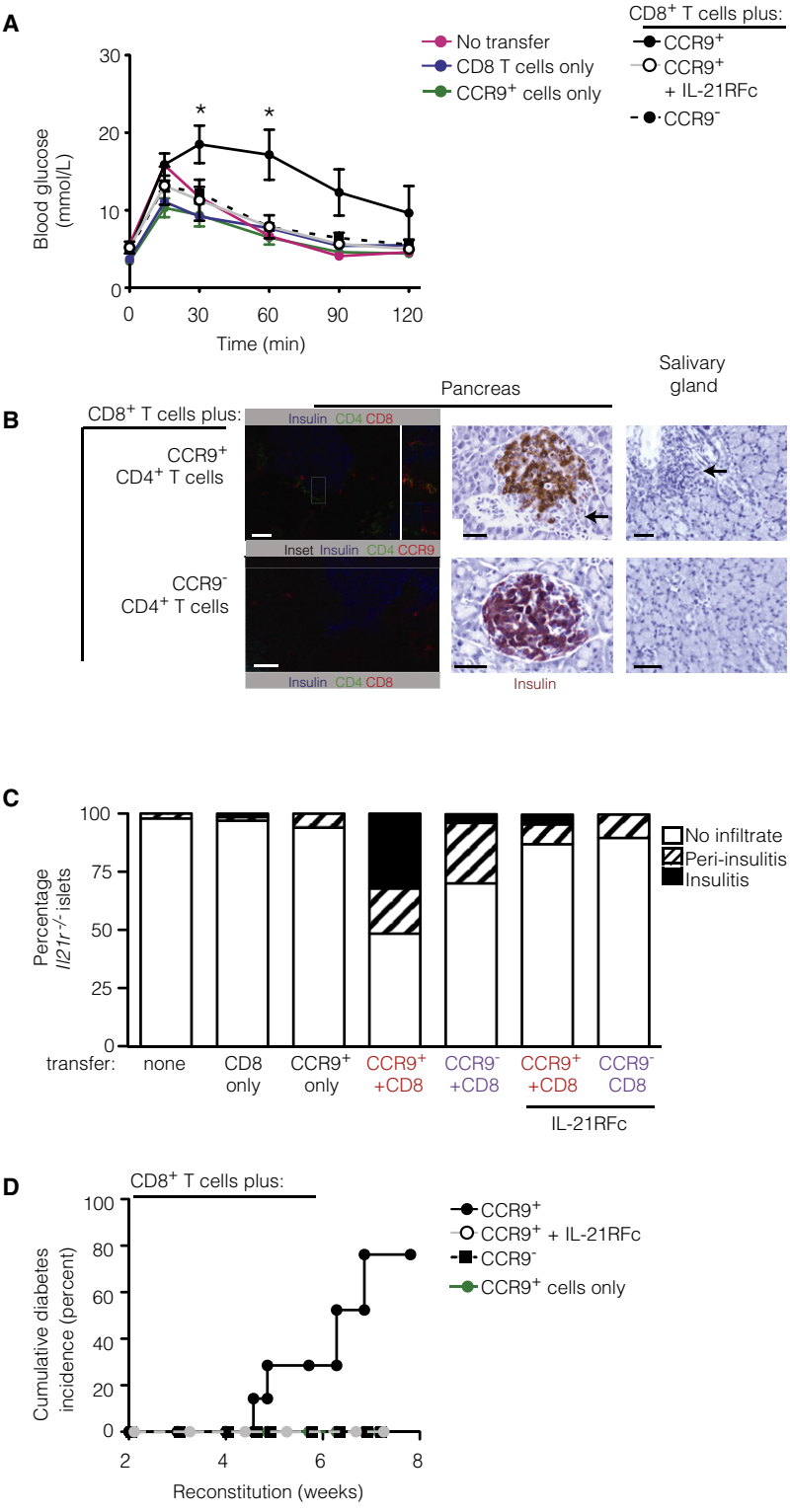

Figure 7. IL-21 Is Critical for Diabetes Caused by CCR9+ Th Cells

Il21r$^{-/-}$ NOD mice received 5 × 10^5 CCR9$^+$CD44^{hi}CD4$^+$ T cells or CCR9$^-$CD44^{hi}CD4$^+$ T cells either alone or combined with CD8$^+$ T cells, with or without IL-21RFc 10 μg/mouse every other day for 12 days.

(A and B) Two weeks after transfer, (A) glucose tolerance tests were conducted at 30 and 60 min, with significant difference (*) between CCR9$^+$ + CD8$^+$ T cells ± IL-21R/FC; (B) pancreas and salivary glands were assessed by immunofluorescence showing insulin (blue), CD8 (green), and CCR9 (red) and immunohistochemistry, showing insulin stained brown, with hematoxylin. Scale bars represent 50 μm.

(C and D) Pancreata from *Il21r*$^{-/-}$ NOD recipients were scored for insulitis on day 14 (C) and incidence of diabetes (D); n = 5 from three experiments.

detection of CCL25 in the pancreas offered a plausible link between CCR9$^+$ Th cells, the intestine, and pancreas and that pancreatic CCL25 may aid CCR9$^+$ Th cell access to, or retention in, the islet lesion. The ability of CCL25 blockade to reduce insulitis and inhibit diabetes was of therapeutic importance, but whether this reflected a singular effect on CD4$^+$ T cells remains unknown.

Phenotypic characterization indicated that CCR9$^+$ Th cells were neither Th1, Th2, nor Th17 cells. Instead, they exhibited high amounts of the Tfh cell transcription factor Bcl-6 and a cytokine profile that was largely restricted to IL-21, which is produced by Tfh cells, but under strong epigenetic repression in both Th1 and Th2 cell subsets (Wei et al., 2009). Bcl6 represses IL-4, IFN-γ, and IL-17 expression (but not IL-21 expression) during Tfh cell differentiation (Kusam et al., 2003; Nurieva et al., 2009; Yu et al., 2009), indicating that the high expression of Bcl6 in CCR9$^+$ Th cells could explain their restricted cytokine profile.

A question that warrants closer inspection is how CCR9$^+$ Th cells acquire Tfh cell-like features in unmanipulated mice housed under SPF conditions. CCR9$^+$ Th cells could be distinguished from Tfh cells by their lack of CXCR5, SAP, and reduced expression of PD-1, but may be selectively recruited from a CCR9$^+$ precursor population in the follicular environment of gut-associated lymphoid tissue. In support of this, approximately 30% of CCR9$^+$ Th cells in the Peyer's patches coexpressed CXCR5. However, pancreatic CCR9$^+$ Th cells and Tfh cells resisted the in vitro modulation of CXCR5 and CCR9, respectively. Alternatively, CCR9$^+$ Th cells may emerge from the extrafollicular foci of lymphoid organs. IL-21-producing extrafollicular Th (Tefh) cells, which also lack expression of CXCR5, have been described in the MLR^{lpr} mouse model of lupus, where they regulate plasma cell differentiation (Odegard et al., 2008).

Another possibility is that CCR9$^+$ Th cells acquire their Tfh cell-like characteristics in the islet lesion. Evidence for lymphoid

neogenesis in NOD mice, with ectopic GC in the pancreas, salivary glands, and thyroid (Astorri et al., 2010; Jansen et al., 1993; Kendall et al., 2007), indicates that a suitable environment may exist for the maintenance and/or generation of a Tfh cell-like transcriptome in CCR9+ Th cells. Indeed, the association of CCR9+ Th cells and B cells in the islet lesion may have supported CCR9+ Th cell differentiation and/or optimal production of IL-21 (Vogelzang et al., 2008). Dysregulated Tfh cells that provide help to B cells have been associated with antibody-mediated autoimmune diseases (Linterman et al., 2009) and therefore are not typically associated with T1D. Yet NOD mice carry a high expressing IL-21 allele (McGuire et al., 2009), and both ICOS:ICOSL interactions and IL-21:IL-21R interactions are necessary for the development of T1D (Datta and Sarvetnick, 2008; Hawiger et al., 2008; Spolski et al., 2008), highlighting the importance of pathways that influence antigen receptor signals in autoimmune disease.

Despite their close association with B cells, CCR9+ Th cells were ultimately helpers of CD8+ T cells. The development of diabetes in immunodeficient hosts was dependent upon CD4+ T cells that produced IL-21 and CD8+ T cells that responded to IL-21. Furthermore, the ability of CCR9+ Th cells combined with CD8+ T cells to induce both salivary gland and islet infiltration as well as clinical diabetes in otherwise protected IL-21R-deficient NOD mice was dependent upon IL-21. Recent studies have demonstrated that IL-21 is critical for the maintenance of CD8+ T cell responses during chronic infection (Søndergaard and Skak, 2009). In this study, we demonstrate that IL-21 is similarly important for the survival of diabetogenic CD8+ T cells in nonlymphoid tissues. In contrast to the spontaneous or homeostatic expansion of CD4+ T cells in NOD mice that was dependent upon the unique NOD MHC class II molecule IAg7 alone (D'Alise et al., 2008), IL-21 was critical for the spontaneous proliferation of CD8+ T cells that we have previously observed in NOD mice (King et al., 2004). The inappropriate expansion and survival of CD8+ T cells may have reflected responses to self or environmental antigen that were facilitated by IL-21 either alone or in conjunction with other endogenous cytokine growth factors. In this manner, IL-21 formed a nexus between B cells, CD4+ Th cells, and CD8+ T cells in the dynamic islet lesion and united their collective roles in the pathogenesis of T1D.

EXPERIMENTAL PROCEDURES

Mice

Female NOD Ltj and C7BL/6 mice were purchased from ARC (Perth, WA), *Ighm*$^{-/-}$ NOD mice from JAX (Serreze et al., 1996), and Congenic C57BL/6 (R450).NOD (NOD$^{B6.Idd3}$) mice from Taconic Farms. *Il21*$^{-/-}$ mice created on the 129 background (Lexicon and Deltagen) were backcrossed to NOD N10 by selecting for known NOD Idd loci (versus B6 regions) by PCR of genomic DNA (speed congenics) (Serreze et al., 1996). *Il21r*$^{-/-}$ mice (Ozaki et al., 2002) were kindly provided by M. Smyth (Peter Mac Callum Cancer Centre, Melbourne) and backcrossed to NOD N10 by speed congenics. *Il21*$^{-/-}$ and *Il21r*$^{-/-}$ NOD mice underwent a genome scan of >600 markers including detailed analyses of construct flanking regions (Australian Genome Research Facility Ltd [AGRF]). Animals were housed under specific-pathogen-free conditions and handled in accordance with the Garvan Institute of Medical Research and St. Vincent's Hospital Animal Experimentation and Ethics Committee, which comply with the Australian code of practice for the care and use of animals for scientific purposes. Blood glucose values (BGV) were determined with Accu-chek Advantage blood glucose strips (Roche).

Human Samples

Human tonsils were obtained from routine tonsillectomy at the St Vincent's Hospital (Darlinghurst, NSW, Australia). PB buffy coats were obtained from the Australian Red Cross Blood Service. Blood samples were collected from 15 patients with Sjögren's syndrome recruited via immunology clinics at Westmead Hospital, Sydney. All patients met accepted diagnostic criteria for this condition (Vitali et al., 2002). Clinical and laboratory data were collected on all patients; clinical data included the presence of symptomatic dry eyes, mouth, skin, or vagina; gastrointestinal, renal, and respiratory symptoms; lachrymal or parotid swelling; Schirmer's test result; Raynaud's; presence of other autoimmune diseases; recurrent oral candidiasis; the state of dentition; and the use of medication. Laboratory data included the results of testing for antinuclear antibodies, antibodies to extractable nuclear antigens, cryoglobulins, hypergammaglobulinaemia, paraproteinaemia, rheumatoid factor, and anemia. Institutional Human Research Ethics Committees approved all studies.

Flow Cytometric Analyses

Isolation of lymphocytes from the pancreas and salivary gland were performed as follows. Mice were euthanized by i.p. injection of ketamine and perfused with ice-cold PBS for 10 min and isolated as described (Faveeuw et al., 1995). After ex vivo staining of surface markers (Supplemental Experimental Procedures), intracellular immunostaining (BD Biosciences) was performed with biotin-labeled IL-21RFc (R&D Systems) as described (Suto et al., 2008). Cytokines were detected ex vivo or after stimulation with CD3 (2.5 μg/ml) and CD28 (2.5 μg/ml) mAb in culture medium containing monensin (GolgiStop 1 μl/ml, BD Biosciences) at 37°C for 4 hr. For analysis of CD4+ T cell conjugates, EDTA was omitted from sample preparation buffers. Sorted CD4+B220+ conjugates were dissociated with 2 mM EDTA and vigorous vortexing, as previously described (Reinhardt et al., 2009). Data were collected on a Canto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.).

Immunohistochemistry

Serial sections of paraffin-embedded sections of pancreata were stained for insulin (DakoCytomation) and glucagon (Chemicon) or PNA (Sigma). For immunofluorescence, histological sections (5 μM) of frozen pancreata were examined with a Leica laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany), DM IRE2 TCS SP2 AOBS, with 20×/0.70 (HCX PL APO) and 63×/1.40 (HCX PL APO) objectives. Excitation of fluorchromes was via 488 nm (Ar), 543 nm (HeNe), or 633 nm (HeNe) lasers and emission was recorded at 510–560 nm, 565–620 nm, or 650–720 nm. Images were average projections of three optical sections and processed with the Leica confocal software (Leica Microsystems, Wetzlar, Germany), ImageJ (http://rsbweb.nih.gov/ij/), and Adobe Photoshop CS3 (San José, CA). The antibodies used for immunofluorescence were CCR9 (MAB.2435, Abnova), CD4 (RM4-4, eBioscience), CD8 (53-6.7, eBioscience), and B220 (RA3-6B2, eBioscience).

Chemotaxis Assay

Migration of lymphocytes from pancreatic lymph nodes and pancreatic infiltrate toward rCCL25 was assessed with Transwell tissue culture inserts with a 3 μM pore size polycarbonate filter (Millipore). Lymphocytes were resuspended at 1 × 10^7 cells/ml in RPMI 1640 medium supplemented with 0.5% BSA, and 75 μl aliquots were loaded into the upper inserts. Buffer with or without rCCL25 (10 μg/ml) (R&D Systems), with or without CCL25 Ab (4 μg/ml) (R&D Systems), prepared in the same medium, were placed in the lower wells. Chambers were incubated for 2 hr, and cell migration was quantified by counting the number of cells acquired by flow cytometry in an equivalent time period.

In Vivo Antibody Treatments

Mice received 50 μg/mouse total recombinant mouse IL-21RFc chimera administered intravenously every other day for 12 days. IL-21RFc chimera was made in house as previously described (Herber et al., 2007) and described in Supplemental Experimental Procedures. For diabetes incidence, 20 μg of CCL25 Ab (89818 R&D Systems) or rat IgG2b isotype control antibody (A95-1; PharMingen) per mouse was given intravenously three times per week.

Statistical Analysis

p values between data sets were determined by two-tailed Student's t test assuming equal variance. Human data were compared with a nonparametric t test. Data are reported as the mean ± SEM, along with the calculated p values. The frequency of diabetes was compared between treatment groups with the Kaplan-Meier log-rank test with Prism software (Graphpad).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures (RNA and qRT-PCR, CFSE proliferation, immunoblot, B cell help assay, and sublethal irradiation), four figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.01.021.

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