Study of 2009 H1N1 Pandemic Influenza Virus as a Possible Causative Agent of Diabetes

Ilaria Capua,1 Alessia Mercalli,2 Aurora Romero-Tejeda,1 Matteo S. Pizzuto,1 Samantha Kasloff,1 Valeria Sordi,2 Ilaria Marzinotto,2 Vito Lampasona,2 Elisa Vicenzi,3 Cristiano De Battisti,1 Riccardo Bonfanti,2 Andrea Rigamonti,2 Calogero Terregino,1 Claudio Dogioni,4 Giovanni Cattoli,1 and Lorenzo Piemonti2,5

1Department of Comparative Biomedical Sciences, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padua 35020, Italy; 2San Raffaele Diabetes Research Institute, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy; 3Viral Pathogens and Biosafety Unit, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy; 4Unit of Pathology, San Raffaele Scientific Institute, Milan 20132, Italy; and Università Vita-Salute San Raffaele, Milan 20132, Italy

ORCiD numbers: 0000-0002-2172-2198 (L. Piemonti).

Context: Recent studies have suggested that influenza A virus (IAV) might be involved in the etiology of diabetes.

Objective and Methods: To address this question, we tested the ability of H1N1 pandemic IAV to infect, replicate, and damage human β cells/pancreatic islets in vitro and induce pancreatic damage and/or glucose metabolism alterations in chemical and autoimmune models of β cell damage in vivo. Moreover, we looked for direct and/or indirect evidence of correlation between IAV infection and autoimmunity/diabetes in humans.

Results: Human H1N1 A/California/2009-derived viruses infected human pancreatic islets in vitro, inducing a proinflammatory response associated with substantial increases of CXCL9 and CXCL10 release. In vivo, infected mice showed a clear susceptibility to the virus, with its localization also found in extrapulmonary organs, including the pancreas. Infection was able to induce mild modifications of glycemia in C57B6 mice after chemical damage of islets but did not modulate the autoimmune damage of islets in NOD mice. One of 69 nasopharyngeal swabs collected from patients at the onset of type 1 diabetes yielded positive results for IAV. Pancreas sections from 17 organ donors available from the Network for Pancreatic Organ Donors With Diabetes showed the persistence of CXCL10-positive cells in islet autoimmunity-positive subjects; however, extremely rare cells stained for viral RNA and not preferentially in autoimmune subjects.

Conclusion: Influenza H1N1 pdm strains are able to infect and replicate in mammalian pancreatic cells both in vitro and in vivo but did not cause any functional impairment consistent with diabetes. (U Clin Endocrinol Metab 103: 4343-4356, 2018)

The rapid worldwide incidence increase in type 1 diabetes (T1D) has suggested a major role for environmental factors in its etiology (1). According to cross-sectional and prospective studies of patients with T1D and/or individuals who are prediabetic, viral infections might be one of these factors (2, 3). The possible involvement of viral infections in the etiology of T1D has been suggested by several studies, implicating enteroviruses such as Coxsackievirus B, measles, congenital rubella, mumps, cytomegalovirus, and influenza B (4-15).

Abbreviations: AutoAb+, autoantibody-positive; Ctrl, control; HC, healthy control; IAV, influenza A virus; IN, intranasal; INF, infected with H1N1pdm mouse-adapted virus; MLD, multiple low dose; NCBI, National Center for Biotechnology Information; nPOD, Network for Pancreatic Organ Donors With Diabetes; pfu, plaque-forming units; (r)RT-PCR, real-time RT-PCR; STZ, streptozotocin; TCID50, 50% tissue culture infecting dose; T1D, type 1 diabetes; Vacc, vaccine.
Moreover, viruses such as Coxackie B4, rotavirus, and reovirus have been shown to be diabetogenic in mice (16-18). Recent studies have discussed the possibility that the influenza A virus (IAV) might also be involved in the etiology of T1D (19-21), and a number of studies have reported pancreatic damage in humans (acute pancreatitis or T1D onset) related to an H1N1 pandemic IAV infection (22-30). A very recent report of a large Norwegian cohort showed an increased risk of T1D after H1N1pdm infection (31). IAVs have their reservoir in wild birds and infect a variety of hosts that, in addition to wild and domestic birds, include a relevant number of mammals such as swine, equids, mustelids, sea mammals, canids, felids, and humans, in which they can become established. Observational studies and pathological findings from animals, naturally and experimentally infected with avian influenza viruses, have indicated a specific tropism and extensive replication of the virus in the pancreas (32). This is not unexpected, because influenza viruses are able to replicate only in the presence of trypsin or trypsin-like enzymes. Thus, their replication is restricted to the respiratory and enteric tract (32). Postmortem pancreatic lesions, ranging from inflammation to necrosis, have been observed in many species of birds, cats, and dogs after influenza infection (33-35), although the clinical and metabolic implications of this occurrence are unknown. Recently, we reported that two low pathogenicity avian influenza viruses, A/turkey/Italy/3675/1999 (H7N1) and A/turkey/Italy/2962/2003 (H7N3), are able to replicate in pancreatic cells of experimentally infected young turkeys and to cause diabetes, reflecting endocrine and exocrine damage (36). Moreover, in addition to the previously mentioned avian strains, at least two IAVs, which have circulated for extensive periods in humans, H1N1/A/New Caledonia/20/99 and H3N2/A/Wisconsin/67/05, were able to infect human pancreatic islet in vitro (36). No cytopathic effect or alteration in insulin secretion was evident, and a strong cytokine/chemokine expression was induced by infection with these two viruses. These results raise the question of whether influenza viruses might play a role in the etiopathogenesis of diabetes. In the present study, we selected two viruses derived from the 2009 pandemic precursor H1N1A/California/4/2009 (H1N1 pdm Cal) and a mouse-adapted derivative (H1N1pdm mouse-adapted), and we tested their ability in vitro to replicate, infect, and damage pancreatic islets and in vivo the ability to induce alterations of nonfasting glycemia or to promote autoimmune diabetes in mice. Finally, we looked for direct evidence of a correlation between influenza virus infection and autoimmunity/diabetes at onset in humans.

**Materials and Methods**

**Pancreatic islets and cell lines**

Human pancreatic islets were isolated and purified at San Raffaele Scientific Institute according to the method of Ricordi (37). The mouse insulinoma cell line Min6 was kindly provided by Dr. Maria Luisa Malosio. Mouse pancreatic islets were isolated from male C57BL/6j mice (age, 8 weeks; weight, 20 to 22 g; Charles River, Calco, Italy). More details have been provided in the Supplemental Materials and Methods section.

**Viruses**

Two IAV, namely H1N1/California/4/2009 (H1N1 pdm Cal) and H1N1/California/7/2009 mouse-adapted (H1N1 pdm mouse-adapted) have been used for the in vitro and in vivo studies. The H1N1pdm mouse-adapted (kindly supplied by Istituto Superiore Sanità, Rome, Italy) was previously adapted to the C57/BL 6j mouse model by 10 serial lung passages. All viruses were propagated in Madin Darby canine kidney cells, and the 50% tissue culture infecting dose (TCID50) was calculated using the Reed-Muench method. More details are provided in the Supplemental Materials and Methods section for regarding the Madin Darby canine kidney plaque assay, virus purification, TCID50 calculation method, and sequence analysis/nucleotide sequencing.

**Sialic acid receptor characterization**

The presence of α-2, 3- and α-2, 6-linked sialic acid residues was determined by flow cytometry, as previously described (36). α-2, 3 and α-2, 6 Sialic acid linkages were detected by incubating cells for 30 minutes with 100 μM of biotinylated Maackia amurensis lectin II (5 μg/mL; Vector Laboratories, Burlingame, CA), followed by 100 μM of phycocerythrin-streptavidin (10 μg/mL; BD Biosciences, San Jose, CA) for 30 minutes at 4°C in the dark or with 100 μM of fluorescein-conjugated Sambucus nigra lectin (5 μg/mL; Vector Laboratories). The samples were analyzed using a BD FACSCalibur or BD LSRII (BD Biosciences), and a minimum of 5000 events were recorded (Supplemental Materials and Methods).

**Influenza virus growth in human pancreatic islets**

Human pancreatic islets were infected with H1N1 pdm Cal and H1N1 pdm mouse-adapted influenza viruses by adding 10⁵ plaque-forming units (pfu) per well. Viral growth assays were performed with and without the addition of L-1-tosylamide-2-phenyl ethylchloromethyl ketone (TPCK)-trypsin (1 μg/mL; Sigma-Aldrich, St. Louis, MO). Uninfected islets were left as a negative control. Samples were collected every 48 hours from the day of infection until day 10. Each sample was centrifuged at 150g for 5 minutes. The pellets and supernatants were collected and stored at — 80°C for M gene quantitative real-time (r)RT-PCR, as previously described (36). More details are provided in the Supplemental Materials and Methods.

**In situ visualization of viral RNA**

Viral RNA was visualized using the Quantigene ViewRNA technique based on branched DNA signal amplification technology, according to the manufacturer’s instructions (36). A probe set containing multiple oligonucleotides was used, designed to hybridize to the H1N1 pdm Cal and H1N1 pdm mouse-adapted viruses [NP gene; National Center for
Biotechnology Information (NCBI) reference sequence AB538390). To identify the cell types within islets, the following Quantigene probes were used: insulin for β cells (INS gene; NCBI reference sequence NM_000207), α-amylase 1 for exocrine cells (AMY1A gene; NCBI reference sequence NM_004038), and cytokeratin 19 for duct cells (KRT19 gene; NCBI reference sequence NM_002276). Quantification of cells positive for each probe was performed within eight randomly chosen fields using the IN Cell Investigator software (GE Health Care United Kingdom Ltd.). More details are provided in the Supplemental Materials and Methods section.

Cytokine expression profile

The capability of influenza viruses to induce cytokine expression in human pancreatic cells was measured using multiplex bead-based assays and xMAP technology (Bio-Plex; Biorad Laboratories, Hercules, CA). The parallel wells of human islets (150 islets/well) were infected with pandemic H1N1 viruses at 10^6 and 10^7 pfu per well or were mock infected. The culture media supernatant was collected at six time points (0, 2, 4, 6, 8, and 10 days) after infection and assayed for 50 cytokines (Supplemental Materials and Methods).

Evaluation of cell death after infection
(live/dead assay)

The viability of islet cells after infection was measured using the live/dead cell assay kit (L-3224; Molecular Probes, Inc., Leiden, Netherlands), as previously described (36) (Supplemental Materials and Methods).

HINlpdm 2009 virus infection in C57Bl/6J mice

Six- to seven-week-old male C57/BL 6J mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). All experiments conducted using live viruses were performed in a biocontainment unit (Allentown, Inc.) under the biosafety level 3 containment facilities at the Istituto Zooprofilattico Sperimentale delle Venezie. All experiments were conducted in accordance with the local and national animal welfare bodies [Convention of European Council number 123 and National Guidelines (Legislative Decree 116/92)]. The experimental protocol was reviewed by the Committee on the ethics on animal experiments of the Istituto Zooprofilattico Sperimentale delle Venezie and approved by the Italian Ministry of Health (permit numbers 148/2012-B and 204/2013-B). Appropriate care was taken to ensure the animals’ welfare and the human endpoints throughout the experiment. More details are provided in the Supplemental Materials and Methods section on the determination of mouse infectious dose 50 and 50% minimal lethal dose serological assays, and tissue sample preparation. The experimental design used to evaluate the influence of HINlpdm 2009 virus infection on glucose tolerance is presented in Supplemental Fig. 1. Live groups of C57Bl/6J mice were formed and treated as follows:

1. Group 1 [control (Ctrl)]: mice were mock infected with 50 μl of sterile PBS-diluted negative allantoic fluid.
2. Group 2 [streptozotocin (STZ)]: mice were treated with multiple low doses (MLDs) of STZ (Sigma-Aldrich) by the IP route (40 mg/kg/d) for 3 or 4 consecutive days.
3. Group 3 [HINlpdm mouse-adapted virus (INF)]: mice were infected with 50 μl of viral suspension containing 10^25 TCID_{50}/50 μl of the HINlpdm mouse-adapted virus, under light anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg, IP).
4. Group 4 (STZ+INL): mice were treated with MLDs of STZ and subsequently INE (as described for group 3) 7 days after the first administration of STZ.
5. Group 5 [vaccine (Vacc)+STZ+INL]: mice were vaccinated according to the protocol as described in the Supplemental Materials and Methods section, with a boost given 21 days later. Eight days after booster immunization, the mice were treated as described for group 4.

Body weight was recorded on days 0, 3, 5, and 7 after infection and then twice per week for 4 consecutive weeks and once per week until the end of the study. The mice were euthanized if they had lost 20% of their body weight. Blood samples from the tail vein were taken from all the mice before infection, daily for the first week after infection, twice a week during the next 4 consecutive weeks, and then once a week until the end of the experiment. The glucose levels were measured using the Glucose Meter Glucocard™ (A. Menarini Diagnostics S.r.l., Lirenze, Italy). Mice showing glycemia (>250 mg/mL) on two consecutive tests were considered to have diabetes. At different days after infection, organs and blood were collected from the dead mice and from mice that had been euthanized for ethical reasons during the experiment to be tested using (r)RT-PCR and virus isolation. Similarly, pancreas from the dead or euthanized mice were collected and fixed in 4% phosphate-buffered formalin for histopathological and immunohistochemistry examination.

HINlpdm 2009 virus infection in NOD mice

For studies of T1D prevention, female NOD/Ltj mice (Charles River Laboratories) were infected with 50 μl of viral suspension containing 10^25 TCID_{50}/50 μl of the HINlpdm mouse-adapted virus (under light anesthesia with ketamine 50 mg/kg and xylazine 5 mg/kg, IP) at 8 or 12 weeks of age. The blood glucose values of the mice were monitored twice a week. Diabetes was defined as two consecutive nonfasting blood glucose concentrations of >250 mg/dL separated by 24 hours.

Human sample collection and IAV detection

Nasopharyngeal swabs

A total of 69 nasopharyngeal swabs were collected from patients at the onset of T1D who had been admitted to the pediatric department of the IRCCS San Raffaele Scientific Institute (Milan, Italy) from October 2013 to February 2017. The institutional ethics committee of the IRCCS San Raffaele Scientific Institute approved the study, and all samples were collected after the patients or their legal guardians in the case of minors had provided written informed consent. Lor 45 patients, clinical data were available that included vaccination records, infection symptom assessment, and the use of antibiotics in the 3 months preceding the diagnosis of T1D. Nasopharyngeal swabs were collected in 3 to 4 mL viral transport media in accordance with the World Health Organization protocol, and viral RNA was extracted using the QIaAmp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The presence of the IAV genome was assessed using quantitative (r)RT-PCR using the Pandemic H1N1/09 Assay Set, version 2.0, kit (Thermo Fisher Scientific, Waltham, MA) and a StepOnePlus (r)RT-PCR System (Thermo Fisher Scientific) according to the manufacturer’s instructions. The kit includes primers and probes.
for specific reverse transcription, amplification, and detection of nucleic acid sequences from the influenza A, pandemic influenza A, and pandemic HI (based on an assay design for the H1N1 influenza virus licensed from the U.S. Centers for Disease Control and Prevention) viruses plus those of the Rnase P gene as an endogenous quality control of successful reverse transcription and amplification. In addition to the tested patient samples, in each run were included the positive controls from the Pandemic H1N1/09 Assay Controls, version 2.0, set (Thermo Fisher Scientific) specific for all three influenza virus genomes analyzed.

**Human pancreas**

Formalin-fixed, paraffin-embedded pancreas sections were available from the Network for Pancreatic Organ Donors with Diabetes (nPOD) collection of organ donor pancreases. Analyses were performed with five healthy control (HC), four nondiabetic autoantibody-positive (AutoAb+) subjects, and eight individuals with T1D for whom formalin-fixed, paraffin-embedded specimens were available. All tissue processing procedures were conducted by the nPOD Organ Processing and Pathology Core in accordance with federal guidelines for organ donation and the University of Florida institutional review board. The case identification number, disease condition, patient clinical parameters, tissue histopathological scoring, and serum immunological testing data provided by the nPOD are listed in Supplemental Table 1. The institutional review board of IRCCS San Raffaele Scientific Institute (Milan Italy) approved all work reported. In situ hybridization was performed as previously described (36) to visualize viral RNA and CXCL10 RNA using the Quantigene ViewRNA technique based on branched DNA signal amplification technology, according to the manufacturer’s instructions. A probe set containing multiple oligonucleotides was used, designed to hybridize to human CXCL10 (Quantigene probes, CXCL10 gene; NCBI reference sequence, NM_001565) Tissue sections from the pancreas head, body, tail, and duodenum were analyzed, depending on availability. Quantification of cells positive for each probe was performed within eight randomly chosen fields for section (magnification X20). The percentage of positive cells

![Figure 1](https://academic.oup.com/jcem/article-abstract/103/12/4343/5091458)

**Figure 1.** Viral RNA detection in human pancreatic Islets after in vitro infection with H1N1 pandemic human IAV. Islets were infected with H1N1 pdm Cal and H1N1 pdm mouse-adapted Influenza viruses using 10⁵ pfu per well in the presence or absence of TPCK-trypsln. (A) Viral RNA detection by (q)RT-PCR for M gene on pellets or supernatants derived from human pancreatic Islets infected. One experiment of two performed. (Lower) Viral RNA localization on Islets was performed by in situ hybridization using the Quantigene ViewRNA technique. The red signal corresponds to the presence of Influenza virus RNA, and the green signal to the presence of Insulin (magnification X63). (B,C) Viral RNA detection by in situ hybridization in human pancreatic Islets. Mock-Infected Islets were left as a negative control. Five d after infection, multiplex fluorescence-based in situ hybridization was performed. After disaggregation, Islet cells were cytocentrifuged onto glass slides. Viral RNA-, Insulin-, amylase-, and CK19-positive cells were assessed using a Carl Zeiss Axiovert 135TV fluorescence microscope. Quantification was performed using IN Cell Investigator software. Each dot represents the percentage of positive cells quantified on one systematically random field. Results from two experiments shown. A Mann-Whitney U test was used for statistical analysis. *P < 0.05 and **P < 0.01 vs mock infected.
examined was scored as 0 (negative), 1 (<20 cells per field), 2 (20 to 40 cells per field), and 3 (>40 cells per field). All the analyses were performed in blinded fashion.

For quantitative (r)RT-PCR of viral RNA, total RNA was extracted from frozen sections of the same pancreases using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) and then subjected to RT-PCR using the Pandemic H1N1/09 Assay Set, version 2.0, kit and the Pandemic H1N1/09 Assay Controls, version 2.0, set (Thermo Fisher Scientific), as previously described.

Statistical analysis

Variables are summarized as the mean ± SD or median and interquartile range according to their distribution. Variables with a normal distribution were compared using a one-way unpaired Student t test (two groups). Variables with a non-normal distribution were compared using the Mann-Whitney U test. Categorical variables were compared using the χ² test or Fisher exact test, as appropriate. The cumulative diabetes incidence and overall survival was evaluated using Kaplan-Meier analysis, and the significance was estimated using the log-rank test. Statistical analysis of nonfasting glycemia during the follow-up period was performed using the general linear model for repeated measures. All statistical analyses were performed using SPSS statistical software, version 13.0 (SPSS Inc., Chicago, IL).

Results

Human pancreatic islets are susceptible to H1N1 pandemic human IAV

Human pancreatic islets were infected with human influenza H1N1 pdm Cal and H1N1 pdm mouse-adapted viruses using 1000 pfu per well. The examination of viral RNA replication by (r)RT-PCR, tested in both pellets and supernatant specimens in the presence or absence of TPCK-trypsin, showed a continued increase in viral replication up to day 4 after infection. The H1N1 pdm mouse-adapted virus grew faster than did the H1N1 pdm Cal in pancreatic islets. In situ hybridization was performed to visualize viral RNA localized within islet cells. The results clearly demonstrated the presence of viral RNA after infection with either virus (Fig. 1A). Because human islet primary cultures contain both endocrine and exocrine cells, a fluorescence-based multiplex in situ hybridization strategy was applied to determine which and how many cells were infected in the islets. Distinctly labeled probes were combined to analyze simultaneously viral RNA and insulin, amylase, or

![Figure 2](https://academic.oup.com/jcem/article-abstract/103/12/4343/5091458)Cytokine/chemokine expression profile modification induced by pandemic IAV infection Islets were infected with H1N1 pdm Cal and H1N1 pdm mouse-adapted viruses. Mock-Infected Islets were left as a negative control. (A) Islet survival after infection with human IAV. The viability of pancreatic islets was evaluated 5 d after infection. The viability was assessed using live/dead assay. Quantification was performed using the IN Cell Investigator software. Each dot represents the percentage of dead cells quantified on one random field. Results from islet of two donors (HP 1039 and HP 1035; 10 fields each) shown. *P < 0.05 and **P < 0.01 vs mock infected. (B) Virus-induced modification in islet cytokine/chemokine profile. Samples were collected every 48 h from the d of infection until d 10. The supernatant was collected and assayed for 50 cytokines. Data presented as mean fold increase for each factor detected during the culture compared with mock-infected islets (n = 2). Dotted line indicates fivefold Increase threshold.
cytokeratin in 19 transcripts. After probe hybridization, human islets were disaggregated, and cell positivity was quantified. Five days after infection, 0%, 0% (range, 0% to 3%) and 10.5% (range, 6.2% to 13.9%) of total cells were positive for viral RNA in mock, H1N1 pdm Cal, and H1N1 pdm mouse-adapted infected islets, respectively (P < 0.001; Fig. 1B). Of the insulin-positive cells, 0% (range, 0% to 1.3%) and 19% (range, 2.6% to 26.8%) were positive for viral RNA 5 days after H1N1 pdm Cal and H1N1 pdm mouse-adapted infection, respectively. Of the amylase-positive cells, 0% (range, 0% to 22%) and 36% (range, 2.5% to 48%) were positive for viral RNA after H1N1 pdm Cal and H1N1 pdm mouse-adapted infection, respectively. Of the CK19-positive cells, 0% (range, 0% to 0%) and 0% (range, 0% to 0%) were positive for viral RNA after H1N1 pdm Cal and H1N1 pdm mouse-adapted infection, respectively (Fig. 1C).

Modulation of survival and islet cytokine/chemokine profile in human pancreatic islets infected with H1N1 pandemic human IAV in vitro

Visual examination of the infected islets using light microscopy and live/dead assay revealed a modestly substantial cytopathic effect only for the H1N1 pdm mouse-adapted virus (Fig. 2A). Five days after infection, overall mortality was 12% (range, 5% to 16%) in the mock-infected, 11% (range, 7% to 19%) in H1N1 pdm Cal-infected cells, and 17% (range, 11% to 22%) in H1N1 pdm mouse-adapted infected cells (P = 0.29 and P = 0.004 vs. mock-infected cells, respectively). The capability of H1N1 pdm Cal and H1N1 pdm mouse-adapted viruses to induce cytokine/chemokines expression in human pancreatic islet

Figure 3. Susceptibility of mouse to H1N1 pdm mouse-adapted virus. (A) Expression of α-2,3 and α-2,6, 6-linked sialic acid receptors on MIN6 and primary mouse islet cells. Dotted lines represent unlabeled control cells. One experiment of two performed. (B) Cells were INF by adding 100 µL of viral suspension containing a viral dilution of 4.8 × 10^3 pfu per well. Four d after infection, viral RNA localization cell recovery and viability were evaluated. (C) Viral RNA localization was performed by in situ hybridization using the Quantigene ViewRNA technique. The red signal corresponds to the presence of Influenza virus RNA, and the green signal to the presence of Insulin (magnification, X63). (C) The viability was assessed using live/dead assay (one experiment of two performed). (D) Two groups of nine mice each were Infected with 10^6 TCID_50/L of virus H1N1 pdm mouse-adapted by IN (under light anesthesia) and IP route, respectively. A third group of six mice (IPIn) was inoculated by IP route with inactivated H1N1 pdm mouse-adapted virus to evaluate the absence of virus replication. (E) RT-PCR for M gene on lung and pancreas tissues of infected mice was performed. Three mice from each group (IN and IP) were euthanized on d 3 (white bars), 4 (gray bars), and 5 (black bars), respectively. In contrast, three animals of the IPIn group were euthanized only on d 3 and 4. Viral RNA localization on tissue (Right) was performed by in situ hybridization using the Quantigene ViewRNA technique. (Upper) The red signal corresponds to the presence of Insulin, and the green signal to the presence Influenza virus RNA. (Lower) The green signal corresponds to the presence Influenza virus RNA (magnification X63).
was measured using multiplex bead-based assays and xMAP technology (Fig. 2B). With the exception of IL-1/3, IL-5, IL-15, and TNF-3, all cytokines showed detectable expression. In the mock-infected mice, the greatest concentrations were detected for CCL2/MCP1 (maximum, 24,920 pg/mL on day 10), CXCL8/IL-8 (maximum, 26,658 pg/mL on day 8); IL-6 (12,699 pg/mL on day 10), CXCL1/GRO-a (maximum, 5571 pg/mL on day 10), vascular endothelial growth factor (maximum, 8552 pg/mL on day 10), stem cell growth factor-3 (maximum, 1071 pg/mL on day 6). MIF (maximum, 378 pg/mL on day 6), G-CSF (maximum, 649 pg/mL on day 10), IL-12p70 (maximum, 184 pg/mL on day 10), CCL11/Eotaxin (maximum, 216 pg/mL on day 10), CXCL9/MIG (maximum, 221 pg/mL on day 6), and CXCL4/SDF1 (maximum, 178 pg/mL on day 10) showed lower but consistent expression. IL-1Ra, CXCL10/IP-10, CCL5/RANTES, INF-α2, CCL27/CTAK, LIF, M-CSF, TRAIL, and HGF showed low but consistent expression (maximum, 20 to 100 pg/mL). Very low (maximum <20 pg/mL), but detectable, expression was present for PDGF-BB, IL-2, IL-4, IL-7, IL-9, IL-10, IL-13, FGF-3, GM-CSF, CCL5/RANTES, GGL/11P-10, INF-α, IL-17, IL-15, IL-2Rα, IL-3, IL-12p40, IL-16, SCF, and INFα2. CXCL10/IP-10, CCL5/RANTES, IFN-α2, CCL27/CTAK, IL-2Ra, and /3-NGF showed a greater than fivefold increase during culture in H1N1 pdm mouse-adapted infected cell supernatants compared with mock-infected controls (Fig. 2B). CXCL10/IP-10, CXCL9/MIG INF-α2 showed a greater than fivefold increase during culture in H1N1pdm Ca-infected cell supernatants compared with mock-infected controls. With both viruses, CXCL10/IP-10 showed the strongest response to viral infection (> 100-fold increase) that peaked 6 to 8 days after infection and showed a dose response with the viruses’ multiplicity of infection.

**In vitro** susceptibility of mouse islet cells to **H1N1 pdm** mouse-adapted virus and its detection in the lung and pancreas of experimentally infected C57BL/6J mice

The susceptibility of primary mouse islet cells and the MIN6 insulinoma cell line to **H1N1 pdm** mouse-adapted infection was investigated. A preliminary experiment using...
lectin staining for receptor detection on both cell sources was performed and revealed high levels of a-2,6 sialic acid-linked sialic acid molecules (required by human tropic viruses) and a-2,3-linked residues (used by avian-tropic viruses; Fig. 3A). Both cell types were then INF at a multiplicity of infection of 0.001. In situ hybridization clearly demonstrated the presence within cells of viral RNA 4 days after virus infection (Fig. 3B). In dispersed mouse islet cells, the colocalization between viral and insulin RNAs demonstrated that primary mouse β cells are susceptible to infection. Visual examination of the infected cells by light microscopy, cell recovery, and live/dead assay revealed no substantial cytopathic effect at any point after infection (days 0 to 4; Fig. 3C). C57B1/6J mice were infected by intranasal (IN; natural route of infection) or IP routes using a previously calculated standard dose of H1N1 pdm mouse-adapted virus (Supplemental Materials and Methods; Fig. 3C). After 3 to 5 days, RNA for the viral M gene was detected by (r)RT-PCR in the lung and pancreas of nine of nine mice and three of nine mice infected by the IN route, respectively. Moreover, eight of nine mice infected by the IP route had positive results in both pancreas and lung for the viral M gene. Histologically, mice infected by the IN route showed lung lesions typical of IAV infection with inflammatory changes in the bronchioles and peribronchial areas, exudation of bronchiole cavity and alveoli, histolytic alveolitis, and lung consolidation. In situ hybridization confirmed the presence of many virus-positive epithelial cells in the bronchioles and positively stained cells diffusely scattered in the lung parenchyma. Pancreatic virus-positive cells were not identified by in situ hybridization.

Nonfasting glycemia in experimentally infected C57Bl/6J mice

To investigate whether the infection with the H1N1 pdm influenza virus was able to impair glucose tolerance, two sets of experiments were performed (Supplemental Fig. 1). For the first set of experiments, MLDs of STZ were administered IP to C57BL/6J mice for 3 consecutive days, and then the mice were infected by the IN route with the H1N1 pdm mouse-adapted virus (STZ+INF; n = 22). To establish the protective efficacy of an influenza vaccine on any alteration of nonfasting glycemia due to the virus infection, a group of naïve mice was vaccinated against the influenza before the treatment with a MLDs of STZ and virus infection (Vacc+STZ+INF; n = 20). Three additional groups were monitored in parallel: mice receiving only the virus infection (INF; n = 24), only treatment with MLD-STZ (STZ; n = 12), or mock infection plus vehicle (Ctrl; n = 12). Nonfasting glucose concentrations were monitored at different points after infection for 4 months. After 4 months, mice were fed ad libitum with a high-fat diet for a second period of 4 months. All mice belonging to the Ctrl, STZ, and Vacc+STZ+INF groups showed a normal behavior and did not show any clinical signs throughout the duration of the experiment. As expected, severe clinical signs were observed in the INF and STZ+INF groups. Starting from day 6, most of the mice started to show a ruffled coat and mild depression. Clinical signs became

Table 1. Nonfasting Glycemia After Influenza Virus Infection

<table>
<thead>
<tr>
<th>First Set, MLD-STZ</th>
<th>Time, d</th>
<th>Ctrl</th>
<th>STZ</th>
<th>INF</th>
<th>STZ+INF</th>
<th>Vacc+STZ+INF</th>
<th>P Value5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 d; Infection by IN route</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute Infection</td>
<td>0-30</td>
<td>12 (119-130)</td>
<td>121 (126-136)</td>
<td>124 (120-129)</td>
<td>137b (132-142)</td>
<td>132 (128-136)</td>
<td>0.002</td>
</tr>
<tr>
<td>Post infection normal diet</td>
<td>30-120</td>
<td>122 (118-127)</td>
<td>134b (129-138)</td>
<td>131c (127-135)</td>
<td>141b (136-146)</td>
<td>139b (145-132)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Overall normal diet</td>
<td>0-120</td>
<td>123 (119-128)</td>
<td>132b (128-136)</td>
<td>127 (123-131)</td>
<td>139b (134-143)</td>
<td>135b (131-138)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>High fat diet</td>
<td>120-240</td>
<td>148 (143-153)</td>
<td>158b (153-163)</td>
<td>156b (152-160)</td>
<td>163b (158-168)</td>
<td>164b (160-167)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Overall experiment</td>
<td>0-240</td>
<td>134 (130-138)</td>
<td>143b (139-147)</td>
<td>139 (136-143)</td>
<td>149b (145-153)</td>
<td>147b (144-150)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>4 d; Infection by IP route</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute Infection</td>
<td>0-30</td>
<td>151 (143-159)</td>
<td>166 (159-175)</td>
<td>148 (140-156)</td>
<td>156 (148-165)</td>
<td>170b (163-178)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Post infection</td>
<td>30-60</td>
<td>138 (129-146)</td>
<td>164b (155-172)</td>
<td>141 (133-149)</td>
<td>159b (150-168)</td>
<td>162b (155-169)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Overall experiment</td>
<td>0-60</td>
<td>147 (139-154)</td>
<td>166b (158-173)</td>
<td>145 (139-153)</td>
<td>157 (149-166)</td>
<td>168b (161-174)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data presented as mean (95% CI).

5General linear mixed model for repeated measures.

P < 0.01 vs control at post hoc test (Dunnett).

P < 0.05 vs control at post hoc test (Dunnett).
more severe within the first 20 days after infection and were characterized by severe depression, reluctance to move, dyspnea, dehydration, anorexia, and severe body weight loss: 25% (6 of 24) and 45% (10 of 22) of the infected mice died in the INF and STZ+INF groups, respectively (P < 0.001; Fig. 4A). These mice were excluded from any subsequent analysis. Complete clinical recovery of the surviving mice in both groups was observed by day 30. No mouse in any group developed diabetes for the entire follow-up duration. Nonfasting glycemia and weight profiles are shown in Fig. 4A. Nonfasting glycemia resulted in statistically significant difference, both considering the overall time of the experiment and the specific intervals (Table 1). The post hoc analysis revealed, as expected, a statistically significant increase related to STZ treatment but also, in part, to the infection. Vaccination apparently was unable to prevent the effects of infection. For the second set of experiments, the MLDs of STZ were administered for 4 consecutive days, and the mice were infected by the IP route with the H1N1 pdm mouse-adapted virus. The same groups were monitored in parallel: Ctrl (n = 14), STZ (n = 14), INF (n = 24), STZ+INF (n = 25), and Vacc+STZ+INF (n = 18). As expected, severe clinical signs were observed in the INF and STZ+INF group, and mortality was greater than in the first experiment. The mortality rate was 40% (10 of 25) and 50% (12 of 24) in the INF and STZ+INF groups, respectively. Nonfasting glycemia and weight profiles are presented in Fig. 4B. Only one mouse in the STZ group developed diabetes during the follow-up. As expected, the post hoc analysis (Table 1) revealed a substantial increase of fasting glycemia related to STZ treatment; however, neither infection nor vaccination appeared relevant. Organs and blood were collected at different days after infection from 11 dead or euthanized mice of both groups during the experiment (Supplemental Table 2). Viral RNA was detected by (r)RT-PCR of the M gene in all the lungs collected (Ct range, 17 to 37), 10 of 11 blood samples (90.9%; Ct range, 20 to 31), in eight of 11 spleen samples (72.7%), and five of 11 intestine samples (45.45%). Viral RNA was detected in two of 11 pancreas samples (18.18%) collected on days 9 and 10.

To determine the presence of viable challenge virus in the collected pancreas, tissue homogenates were inoculated into the allantoic cavity of embryonated eggs, and the presence of the H1N1 influenza virus was confirmed in both pancreas samples.

H1N1 influenza virus infection does not promote diabetes in NOD mice

To determine whether experimental infection with the influenza virus promotes the development of spontaneous diabetes in NOD mice, female mice were randomized to receive H1N1 pdm mouse-adapted or vehicle (mock infection) at the early (8 weeks; IN or oral route) or late (12 weeks; IN) preclinical stage of the disease. On day 4, most of the infected mice started to show a ruffled coat and mild depression. Clinical signs became more severe within the first 10 days after infection, characterized by severe depression, reluctance to move, dyspnea, dehydration, anorexia, and severe body weight loss. Clinical signs were less severe in the 12-week-old mice than in the 8-week-old infected mice. With the exception of the 8-week-old mice infected orally, viral infection did not affect survival (Fig. 5). In any condition studied, IAV neither promoted nor prevented diabetes onset (Fig. 5).
Specifically, 68% (13 of 19) of the mice IN infected at 12 weeks of age developed diabetes during the follow-up period (i.e., 30 weeks of age; mean time to diabetes, 19.8±1.4 weeks), and 75% (nine of 12) of the mock-infected mice developed diabetes during the same period (mean time to diabetes, 18.7±1.9 weeks; P = 0.94). Similarly, 65% (11 of 17) of the mice IN infected at 8 weeks of age developed diabetes (follow-up, 24 weeks old; mean time to diabetes, 20 ± 2.45 weeks) compared with 57% (eight of 14) of the mock-infected mice (mean time to diabetes, 18.1±1.8 weeks; P = 0.74). Finally, 47% (nine of 19) of the mice orally infected at 8 weeks of age developed diabetes (mean time to diabetes, 20.8 ± 1.1 weeks) compared with 60% (nine of 15) of the mock-infected mice (mean time to diabetes, 19.1 ± 2.3 weeks; P = 0.94).

Prevalence of IAV in patients with T1D at onset
To evaluate whether a natural infection with IAV could be clinically associated with T1D, from April 2014 through February 2017, 69 nasopharyngeal swabs were collected from patients at T1D onset. The mean age of the study population was 11.1 ± 3.6 years, and 58% of them were boys. Of the 69 cases of diabetes, 39 (56.5%) were diagnosed within the period of regular seasonal epidemics of influenza in Italy [weeks 42 to 17 (October to April)]. Reliable clinical records available from 45 of the 69 patients showed that all the children had received the mandatory vaccinations within the recommended period; however, only 7% (three of 45) had been previously vaccinated against influenza. Of the 45 patients, 21 (47%) had presented in the previous 3 months with symptoms related to respiratory tract infection (cough, sore throat, or stuffy nose) that had been sometimes associated with gastrointestinal symptoms (diarrhea, abdominal pain) and/or fever (six of 21; 29%), and seven of 21 required antibiotic treatment (amoxicillin, n = 4; amoxicillin + clavulanic acid, n = 2; azithromycin, n = 1). No seasonality was found in the occurrence of symptoms of respiratory tract infection. Of the 69 nasopharyngeal swab samples tested, one yielded positive results for influenza virus A RNA using an (r)RT-PCR test.

CXCL10 expression and IAV in human pancreas donors
To evaluate whether IAV RNA could be visualized within the pancreas of patients with T1D and its eventual association with CXCL10 expression, we performed in situ hybridization for CXCL10 and influenza viral RNA in human donor pancreas specimens obtained by nPOD. Cells positive for CXCL10 RNA were found in two of five, four of four, and eight of eight of HC, AutoAb+, and T1D pancreas donors, respectively (P = 0.013; Table 2), confirming previous reports of CXCL10 overexpression in the T1D pancreas (38). Moreover, the percentage of CXCL10 RNA-positive cells in each section was greater in T1D donors than in AutoAb+ and HC donors (P = 0.008; Fig. 6). Cells positive for viral RNA were found in two of five, one

Table 2. FISH for Influenza Viral RNA and CXCL10 RNA in Human Pancreas

<table>
<thead>
<tr>
<th>ID No.</th>
<th>Disease Status</th>
<th>FISH Viral RNA</th>
<th>FISH CXCL10 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>B</td>
<td>T</td>
</tr>
<tr>
<td>6113</td>
<td>T1D</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6087</td>
<td>T1D</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6089</td>
<td>T1D</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>6052</td>
<td>T1D</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>6024</td>
<td>T1D</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>6198</td>
<td>T1D</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>6209</td>
<td>T1D</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>6211</td>
<td>T1D</td>
<td>0</td>
<td>1?</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0/6</td>
<td>1?/6</td>
</tr>
</tbody>
</table>

Abbreviations: B, body; FISH, fluorescence in situ hybridization; H, head; ID No., Identification number; NA, not available; T, tail
Score: 0, none; 1, <20 cells per field; 2, 20-40 cells per field; 3, >40 cells per field.
of four, and one of eight of HC, AutoAb+, and T1D pancreas donors, respectively (P = 0.66; Fig. 7 and Table 2). Three of four pancreas specimens from donors presented with a single virus RNA-positive cell (ID6211, T1D; ID6197, AutoAb+; ID6174, HC). One donor pancreas (GO6178, HC) presented with more than one cell positive for viral RNA colocalized with CXCL10 RNA (Fig. 7). All samples were negative for IAV RNA using (r)RT-PCR.

Discussion

We previously generated data showing that infection of an avian model with two low pathogenicity avian influenza isolates cause pancreatic damage, resulting in hyperlipasemia in >50% of subjects, which evolved into hyperglycemia and, subsequently, diabetes (36). Moreover, at least two human seasonal IAV, H1N1/A/New Caledonia/20/99 and H3N2/A/Wisconsin/67/05, were shown to be able to infect in vitro human pancreatic islets (36). These results raised the question of whether influenza viruses might play a role in the etiopathogenesis of diabetes in mammals, including humans. This concept, if demonstrated, could be of major relevance for T1D prevention, because it could lead to the possible use of vaccination against influenza for the prevention of glucose metabolism alterations and the onset of diabetes. In the present study, we addressed this question by testing, in vitro, the ability of IAV to infect, replicate, and damage β cells/pancreatic islets and, in vivo, the ability of IAV to induce pancreatic damage and/or glucose metabolism alterations in chemical and autoimmune models of β-cell damage. Furthermore, we looked for direct and/or indirect evidence of a correlation between a potential persistent influenza virus infection and autoimmunity/diabetes onset in humans. To perform the in vitro and in vivo experiments, we selected two viruses derived from the 2009 pandemic precursor. Our choice was driven by two factors. First, the number of human patients showing influenza-related pancreatic damage, including cases of acute pancreatitis and the establishment of T1D, significantly increased after the H1N1 pandemic (22-30). Second, a mouse-adapted H1N1 pdm strain was available for use in experimental models. The latter contained mutations that have been associated with disease severity in humans (39). More specifically the D222G (H1 numbering) hemagglutinin mutation within the receptor-binding site was detected with greater frequencies in severe cases of 2009 pandemic H1N1 infection but was virtually absent among clinically mild cases. In addition, studies in animal models demonstrated that the D222G hemagglutinin mutation alters the receptor binding specificity, inducing greater lung viral titers and alveolar inflammation in mice. These findings appeared to correlate with the greater case fatality rates reported in humans (40). Both H1N1 pdm 2009 viruses were able to grow in human and mouse pancreatic islets in vitro. As previously described for H1N1/A/New Caledonia/20/99 and H3N2/A/Wisconsin/67/05, H1N1 pdm 2009 viruses showed a low ability to induce pancreatic cell death during infection. This suggests that although replication occurs in pancreatic tissue, it is unlikely that human T1D could be a direct consequence of a single influenza infection. However, recurrent or possibly persistent infections in human pancreatic tissue without causing immediate damage might indirectly cause islet destruction and might lead to T1D over long periods, such as has been reported for other viruses (41).

Moreover, the cytokine expression profile showed that these viruses (and, in particular, H1N1 pdm mouse-adapted) were able to induce a proinflammatory response in pancreatic islets. The IFN-γ-inducible chemokines IP-10/CXCL10 and MIG/CXCL9 showed the greatest...
increase after infection. Previously, IP10/CXCL10 was identified as the dominant chemokine expressed in vivo in the islet environment of prediabetic animals and patients with T1D (42), and islet-specific expression of CXCL10 in a mouse model of autoimmune diabetes accelerated disease development by enhancing the migration of antigen-specific lymphocytes (43).

In vivo, C57BL/6J infected mice showed a clear susceptibility to both H1N1 pdm strains mouse-adapted virus, with more evident clinical manifestations in the group infected with the mouse-adapted virus. These results are similar to those previously reported in other studies, which showed the susceptibility of C57BL/6J mice to pandemic H1N1 influenza isolates (44, 45), although mice are not among the natural hosts of influenza viruses. Virus positivity found in extrapulmonary organs using (r)RT-PCR in infected mice was an important finding. This has confirmed the systemic infection and viral replication in organs other than the lungs (target) and are similar to those previously reported by Otte and Gabriel (44) and Otte et al. (44, 45), who described the detection of substantial virus titers in extrapulmonary organs [i.e., intestine] of C57 mice infected with the pandemic H1N1 virus. In our experiments, the H1N1 pandemic mouse-adapted strain was able to reach the pancreas of infected mice. IAV could have reached this organ after replication in target organs (lung) and subsequently through viremia, or through a reflux from the gut through the pancreatic duct. The detected Ct values reported in the pancreas indicate the presence of low levels of viral RNA, in agreement with the absence of substantial histological changes and virus-positive cells by in situ hybridization. Despite this, a major finding was the recovery of viable virus from pancreatic samples, as demonstrated by the successful expansion of the virus in embryonated eggs, thus confirming the real positivity of the tissue.

Although it was evident that IAV can reach the pancreas of infected mice, the consequences in terms of pancreatic damage and/or glucose metabolism were less clear. We found no evidence that IAV infection is able to modulate the autoimmune damage of islets in the NOD mouse. However, mild modifications, although not always consistent, of nonfasting glycemia were present in the chemical damage models.

The main objectives of these experiments were to investigate the synergy between the administration of low doses of STZ for the induction of a mild to moderate pancreatic chemical insult and subsequent infection with an H1N1 pdm influenza virus. The analysis of the first set of experiments revealed, as expected, a substantial increase in nonfasting glycemia related to the mild STZ treatment but also, in part, to the infection. This observation was also confirmed when mice were fed ad libitum with a high-fat diet, which could unmask any functional β-cell damage caused by the chemical insult or the subsequent infection. Vaccination apparently did not prevent the metabolic effect of infection, although, as expected, it was able to prevent the respiratory clinical syndrome. The substantial increase in nonfasting glycemia was not confirmed when the virus was administered using the IP route instead of the natural IN route and when moderate, instead of mild, chemical damage was induced by STZ. Among the reasons for these discordant results include the different route of administration, the higher dose of STZ, and the effect of infection on eating behavior, as demonstrated by the weight loss, which was the most evident clinical sign observed in the infected mice. This last factor could have masked an increase in the nonfasting glycemia, in particular, in the acute phase of the infection. Certain infections can lead to long-term consequences, and we could not exclude that a prolonged period of observation would have been more appropriate for detecting altered glucose homeostasis. Finally, the development of virus-induced diabetes will be influenced by the mouse strain (41, 46, 47), which could
have played a role in the results obtained. Susceptibility has been attributed to viral receptors in the pancreas itself; however, extrapancreatic host factors such as sex, infiltration of macrophages, production of cytokines/chemokines, and genetic background of the host could certainly play a role in the establishment of the diabetic condition. In our experiment, we used male C57/BL 6J mice after the demonstration that islets can be infected by the H1N1 pdm mouse-adapted virus in vitro. The C57BL/6J mouse strain represents the standard model for MLDs of STZ and the high-fat diet and is believed to be more appropriate for in vitro studies than other strains of mice such as BALB/c mice (45). In contrast, the C57BL/6J mouse strain was described as resistant to the diabetogenic action of Coxsackie B4 virus (48) and encephalomyocarditis virus (49). Although we were not able to identify diabetogenic activity for the H1N1 influenza virus, we do not believe that the possible link between influenza A infection and pancreatic function should be ruled out.

Altogether, our analyses of human samples demonstrated that influenza virus does not persist in the pancreas or nasopharyngeal mucosa of patients with T1D, a pathophysiological mechanism previously suggested for other viruses such as enterovirus (50). In the pancreas, we were able to confirm the presence of CXCL10-positive cells in islet autoimmunity-positive subjects (38). Also, extremely rare cells stained for pancreatic cells replicate but do not kill or substantially damage mammalian islets can be infected by the H1N1 pdm mouse-adapted virus in vitro. The C57BL/6J mouse strain represents the standard model for MLDs of STZ and the high-fat diet and is believed to be more appropriate for in vitro studies than other strains of mice such as BALB/c mice (45). In contrast, the C57BL/6J mouse strain was described as resistant to the diabetogenic action of Coxsackie B4 virus (48) and encephalomyocarditis virus (49). Although we were not able to identify diabetogenic activity for the H1N1 influenza virus, we do not believe that the possible link between influenza A infection and pancreatic function should be ruled out.

In conclusion, and in line with previous studies, the present study has shown that selected HIN1pdm influenza strains replicate but do not kill or substantially damage mammalian pancreatic cells in vitro. Findings in keeping with the in vitro results were obtained in our in vivo experiments, indicating that viral replication occurs in the mammalian pancreas and elicits an inflammatory response but does not cause diabetes in our experimental setting. However, it would seem reasonable to investigate the interaction between influenza infection and pancreatic function further, especially given the clear evidence for pancreatic tropism of IAVs, the extensive variability of influenza strains, and the occurrence of repeated infections in humans during life.

Acknowledgments

This research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative T1D research project sponsored by the JDRF. Organ Procurement Organizations partnering with nPOD to provide research resources are listed at www.jdrfnpod.org/for-partners/npod-partners/. We thank Maria Rita Castrucci (Istituto Superiore di Sanità, Rome, Italy) for providing the H1N1 pdm mouse-adapted influenza virus.

Financial Support: This work was supported by the Italian Ministry of Health (grant RF-2010-2318512) and a sponsored research agreement between Novartis Vaccines and Diagnostics s.r.l. and Fondazione San Raffaele del Monte Tabor and Istituto Zooprofilattico Sperimentale delle Venezie. This research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative T1D research project sponsored by JDRF.

Current Affiliation: I. Capua’s current affiliation is the One Health Center of Excellence, IFAS-Department of Animal Sciences, University of Florida, Gainesville, Florida 32611. G. Cattoli’s current affiliation is Animal Production and Health Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Seibersdorf 2444, Austria.

Correspondence and Reprint Requests: Lorenzo Piemonti, MD, Diabetes Research Institute, San Raffaele Scientific Institute, Via Olgettina 60, Milan 20132, Italy. E-mail: piemonti.lorenzo@hsr.it.

Disclosure Summary: I.C. and L.P. are inventors on the U.K. patent application no. 1218195.4. The remaining authors have nothing to disclose.

References

29. Watanabe N. Conversion to type 1 diabetes after H1N1 influenza.


22. Habib A, Jain A, Singh B, Jamshed N. H1N1 influenza presenting...


...with acute encephalomyocarditis? Encephalomyocarditis virus infection in gut mucosa.


