

Insulin-dependent diabetes induced by pancreatic beta cell expression of IL-15 and IL-15R α

Jing Chen^a, Lionel Feigenbaum^b, Parirokh Awasthi^b, Donna O. Butcher^b, Miriam R. Anver^b, Yelena G. Golubeva^b, Richard Bamford^c, Xiaojie Zhang^d, Mark B. St. Claire^d, Craig J. Thomas^e, Valentina Discepolo^{f,g}, Bana Jabri^f, and Thomas A. Waldmann^{a,1}

^aMetabolism Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892-1374; ^bLaboratory Animal Sciences Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, National Cancer Institute, Frederick, MD 21702; ^cTransponics, Jacobus, PA 17407; ^dLaboratory Animal Science Section, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892; ^eNational Institutes of Health Chemical Genomics Center, National Human Genome Research Institute, Rockville, MD 20850; ^fDepartment of Pediatrics, University of Chicago, Chicago, IL 60637; and ^gDepartment of Pediatrics, University Federico II, 80131 Naples, Italy

Contributed by Thomas A. Waldmann, July 10, 2013 (sent for review February 25, 2013)

Increased serum levels of IL-15 are reported in type 1 diabetes (T1D). Here we report elevated serum soluble IL-15R α levels in human T1D. To investigate the role of IL-15/IL-15R α in the pathogenesis of T1D, we generated double transgenic mice with pancreatic β -cell expression of IL-15 and IL-15R α . The mice developed hyperglycemia, marked mononuclear cell infiltration, β -cell destruction, and anti-insulin autoantibodies that mimic early human T1D. The diabetes in this model was reversed by inhibiting IL-15 signaling with anti-IL2/IL15R β (anti-CD122), which blocks IL-15 transpresentation. Furthermore, the diabetes could be reversed by administration of the Janus kinase 2/3 inhibitor tofacitinib, which blocks IL-15 signaling. In an alternative diabetes model, nonobese diabetic mice, IL-15/IL-15R α expression was increased in islet cells in the prediabetic stage, and inhibition of IL-15 signaling with anti-CD122 at the prediabetic stage delayed diabetes development. In support of the view that these observations reflect the conditions in humans, we demonstrated pancreatic islet expression of both IL-15 and IL-15R α in human T1D. Taken together our data suggest that disordered IL-15 and IL-15R α may be involved in T1D pathogenesis and the IL-15/IL15R α system and its signaling pathway may be rational therapeutic targets for early T1D.

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-producing β cells in pancreatic islets are destroyed by autoreactive T cells. During prolonged lack of insulin, blood glucose increases (hyperglycemia) and tissue damage occurs. Studies in animal models and humans demonstrated that β -cell destruction is usually accompanied by inflammation of pancreatic islets (insulinitis), which suggests that activation of inflammatory T cells is important in the development of diabetes (1, 2). What triggers the T-cell infiltrate into the islets and subsequent β -cell destruction? What signaling pathways are important for this process? An understanding of the molecular events and signaling pathways that lead to T-cell activation and subsequent β -cell destruction would be useful in the development of new therapeutics for autoimmune T1D.

Interleukin-15 (IL-15) is a proinflammatory cytokine that promotes the activation and maintenance of natural killer (NK) and CD8 (+) T-effector memory (T-EM) cells (3, 4). IL-15R α (IL-15R α), the high affinity receptor for IL-15, stabilizes and chaperons IL-15 on dendritic cell membrane and activates neighboring NK and T cells via transpresentation (5–8). Therefore, IL-15 is not secreted; rather, it is a membrane-associated molecule that acts as part of an immunological synapse (5, 6, 8). During an immune response such as viral infection, IL-15 and its private receptor IL-15R α are coordinately induced (5, 8, 9). As related to T1D, it has been shown that exposure of human pancreatic islets to coxsackie virus, an enterovirus linked to T1D, or directly to IFN γ induced high gene expression of IL-15 and IL-15R α in the islets in vitro (10).

Abnormal expression of IL-15 has been reported in many autoimmune disorders including rheumatoid arthritis, celiac

disease, psoriasis, inflammatory bowel disease, and multiple sclerosis (11). In patients with T1D, elevated serum levels of IL-15 have been reported (12). Using a unique assay we developed for soluble IL-15R α (sIL-15R α) (13), we discovered elevated serum levels of sIL-15R α in T1D.

To investigate whether islet overexpression of IL-15 and IL-15R α could play a role in the pathogenesis of T1D, we generated double transgenic mice with β -cell-specific expression of both IL-15 and IL-15R α under a rat insulin promoter (RIP). The mice developed hyperglycemia, marked mononuclear cell infiltration, β -cell destruction, and anti-insulin autoantibodies that mimic the early events of human T1D. Inhibiting IL-15/IL-15R α signaling either by blocking IL-15 transpresentation using TM β 1, a monoclonal antibody that is directed to IL-2/IL-15R β (CD122) or by blocking IL-15 signaling by administration of the Janus kinase 2/3 (Jak2/3) inhibitor tofacitinib reversed the diabetes in the double transgenic mice. Moreover, in another diabetes mouse model, nonobese diabetic (NOD) mice, increased islet cell expression of IL-15 and IL-15R α were found at the prediabetic stage and the inhibition of IL-15 signaling delayed the diabetes development. Considering viral infection and interferons are often found in the pancreatic islets of patients with T1D (14–16), and they are potent inducers of IL-15/IL15R α (9, 17–19), we investigated whether IL-15 and IL-15R α were expressed in the islets of patients with T1D. Our data demonstrated increased expression of both IL-15 and IL-15R α in the islets of patients with T1D. Taken together, our data suggest that the disordered expression of IL-15/IL-15R α in islets may play a role in the pathogenesis of T1D and that the IL-15/IL15R α system and its signaling pathway may be rational therapeutic targets for early T1D.

Results

Generation of IL-15/IL-15R α Double Transgenic Mice. Viral infections and interferons are potent inducers of IL-15 and IL-15R α (9, 17–19). Both type I (alpha) (17) and type II (gamma) (18) IFN were shown to be able to induce the expression of IL-15/IL-15R α . In T1D, several reports demonstrated the presence of either enterovirus (14) or viral protein (20) and IFN (15, 16) in the islets. To investigate whether pancreatic islet expression of IL-15 and IL-15R α could play a role in the pathogenesis of T1D, we generated transgenic mice expressing IL-15 alone, IL-15R α alone, and both IL-15 and IL-15R α under the control of a rat insulin promoter

Author contributions: J.C. and T.A.W. designed research; J.C., P.A., D.O.B., M.R.A., Y.G.G., X.Z., and V.D. performed research; C.J.T. contributed new reagents/analytic tools; J.C., L.F., M.R.A., R.B., M.B.S.C., B.J., and T.A.W. analyzed data; and J.C. wrote the paper.

Conflict of interest statement: The National Cancer Institute (NCI) and T.A.W. hold patent 5,833,983 to monoclonal antibody to IL-2/IL-15R β . The sIL-15R α assay is under the patent applications 61/241,265 and 61/242,595 filed by NCI, National Institutes of Health. The authors have no additional financial interests.

¹To whom correspondence should be addressed. E-mail: tawald@helix.nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1312911110/-DCSupplemental.

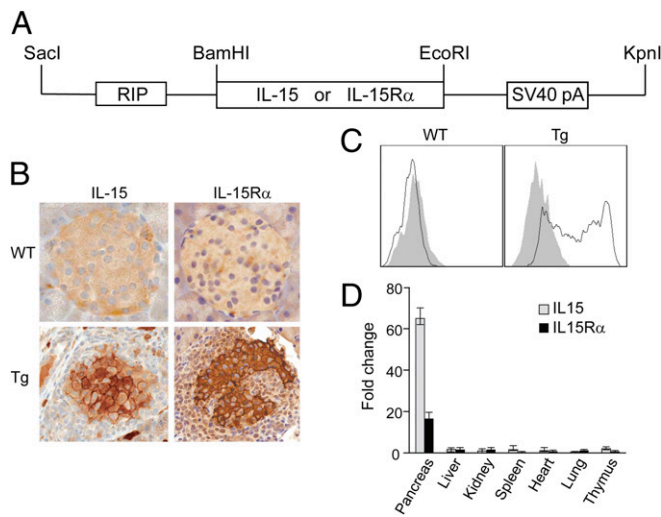


Fig. 1. Generation of IL-15 and IL-15R α double transgenic mice. (A) Schematic representation of the *IL-15* and *IL-15R α* transgene. (B) Immunohistochemistry analysis of IL-15 and IL-15R α expression in the pancreas of IL-15/IL-15R α double transgenic mice. (C) Surface expression of IL-15R α in purified islet cells. (D) Real-time RT-PCR analysis of *IL-15* and *IL-15R α* mRNA levels. The fold change was calculated compared with *IL-15* and *IL-15R α* mRNA levels in wild-type C57BL/6 mouse. Open bar, *IL-15*; solid bar, *IL-15R α* .

(RIP), which restricts IL-15 and/or IL-15R α expression to the β cells (21) (Fig. 1A). Fourteen different lines were obtained for each transgene and their combination. Four IL-15/IL-15R α double transgenic lines (J4, J8, BB1, and X5) were maintained either by in vitro fertilization (IVF) or by natural mating. Southern blot analysis

confirmed that all transgenic mice carried the transgene/s. IL-15 and IL-15R α expressions in the islets of double transgenic mice were confirmed by immunohistochemistry (IHC) (Fig. 1B), flow cytometry (Fig. 1C), and real-time RT-PCR (Fig. 1D).

IL-15/IL-15R α Double Transgenic Mice Developed Insulin-Dependent Diabetes at a Very Young Age. Eight IL-15/IL-15R α double transgenic mouse lines (I1, J4, BB1, E7, G10, J8, X5, and E1) developed hyperglycemia starting from 4 wk of age (both sexes) (Fig. 2A). The blood glucose levels in these mice continued to increase with time, suggesting continued loss of β cells (Fig. 2A). The diabetes onset age among offspring of the same line varied. Approximately 60% of transgene-positive J4 offspring mice developed hyperglycemia within 3 mo of age (Fig. 2B). None of the IL-15 single transgenic mice developed hyperglycemia when euthanized at 12 mo of age. Only one IL-15R α single transgenic mouse developed hyperglycemia. With this one exception, all of the single transgenic mice (both IL-15 and IL-15R α single transgenic) did not have any abnormal phenotype. This is probably due to the requirement of IL-15R α to stabilize and chaperon IL-15 on cell membrane and consequently, the necessity of both IL-15 and IL-15R α expression in the same cell for transpresentation as demonstrated by Mortier et al. (7) and Sandau et al. (8). As we will demonstrate later, IL-15 transpresentation was very important for the induction of diabetes in this model. None of the transgene-negative littermates developed hyperglycemia.

All IL-15/IL-15R α double transgenic mice with hyperglycemia demonstrated marked mononuclear cell infiltration in the islets and atrophy of islets (Fig. 2C). When stained with an anti-insulin antibody, the loss of insulin-producing β -islet cells was evident in the double transgenic mice, whereas glucagon-producing α cells and somatostatin-producing δ cells were intact at the early stage of the disease (Fig. 2C). The majority of the infiltrating mononuclear

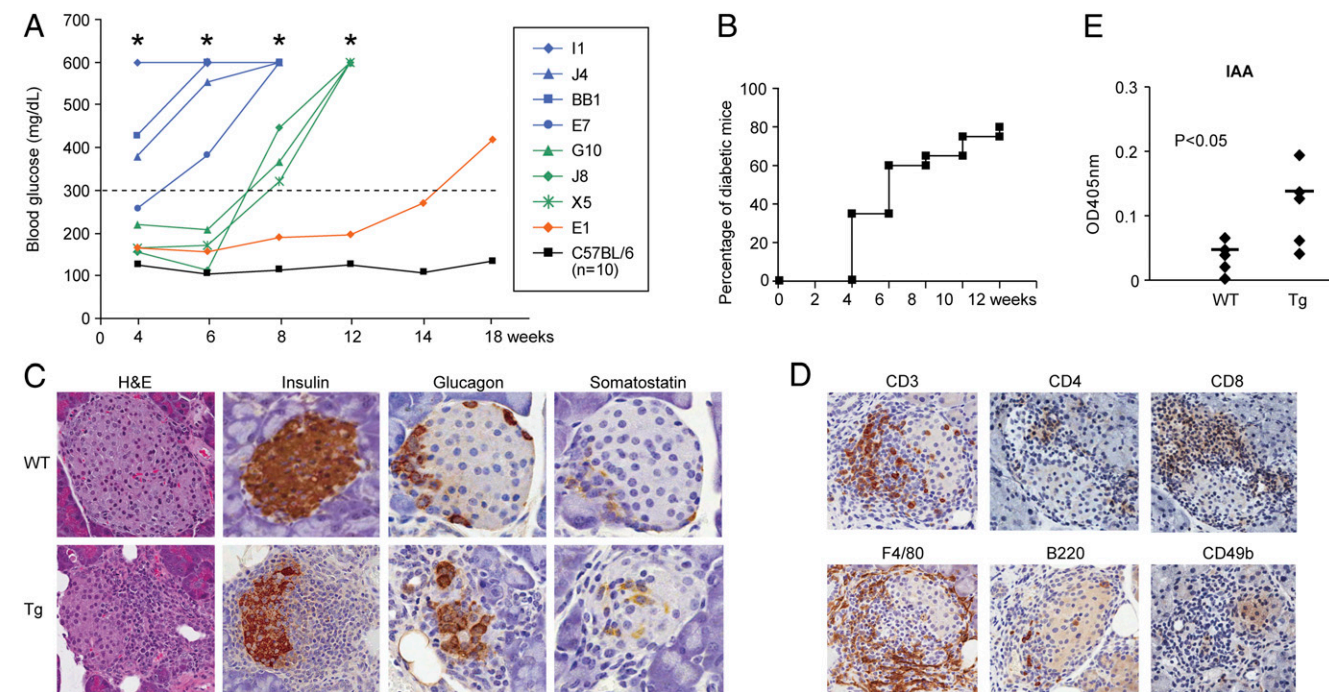


Fig. 2. IL-15/IL-15R α double transgenic mice developed insulin-dependent diabetes at a young age. (A) Blood glucose levels in the IL-15/IL-15R α double transgenic mouse lines (I1, J4, BB1, E7, G10, J8, X5, and E1) that developed diabetes from week 4 to week 14 compared with 10 controls indicated by black line. *Blood glucose levels >600 mg/dL. (B) The percentage of diabetic mice among the offspring of transgene-positive J4 line (10 female, 10 male) 3 mo after birth. (C) Histopathology analysis of pancreas from the IL-15/IL-15R α double transgenic mice. (D) Infiltrating mononuclear cells in islets of IL-15/IL-15R α double transgenic mice were visualized by anti-CD3, CD4, CD8a, F4/80, B220, and CD49b antibodies. (E) Increased serum levels of insulin autoantibody (IAA) in the double transgenic mice ($n = 5$, $P < 0.05$).

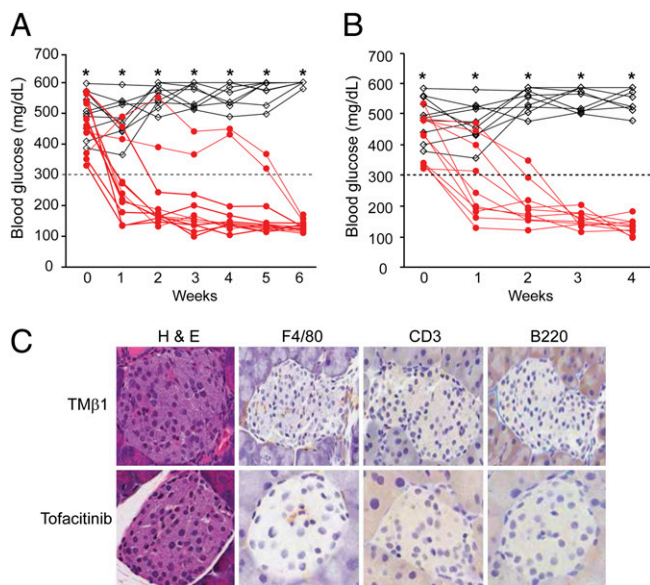


Fig. 3. Inhibiting IL-15 signaling reversed the diabetes in IL-15/IL-15R α double transgenic mice. (A) The blood glucose levels in the double transgenic mice with new onset of diabetes untreated (black, $n = 10$) or treated with monoclonal anti-IL-2/IL-15R β (anti-CD122) antibody TM β 1 for 6 wk (red, $n = 12$). (B) The blood glucose levels in the IL-15/IL-15R α transgenic mice with new onset of diabetes untreated (black, $n = 10$) or treated with the Jak2/3 inhibitor tofacitinib for 4 wk (red, $n = 10$). (C) Immunohistochemistry staining of F4/80 (macrophage), CD3 (T cells), and B220 (B cells) on the pancreas of mice treated with TM β 1 and the Jak2/3 inhibitor tofacitinib. The data represent five individual mice.

cells were T lymphocytes (CD3 positive) and macrophages (F4/80 positive) (Fig. 2D). Both CD4 and CD8 T cells were infiltrated into the islets (Fig. 2D). Few B cells (B220 positive) and much fewer NK cells (CD49b positive) infiltrated into the islets (Fig. 2D). The double transgenic mice had increased serum levels of insulin autoantibody (IAA) (Fig. 2E) and IgA/IgG at the onset of diabetes (Fig. S1). Moreover, increased expression of proinflammatory cytokine genes (TNF α , TNF β , IFN γ , IL18RAP, etc.) and chemokine genes (CCL3, CXCL9, and CXCL10, etc.) were found in the purified islet cells from the double transgenic mice before diabetes onset (Fig. S2, genes with fold induction ≥ 3). Increased cell surface expression of MHC class I, class II, and ICAM-1 were also found on the purified islet cells from the double transgenic mice before diabetes onset (Fig. S3).

Inhibiting IL-15 Signaling at the Onset of Diabetes Reversed the Diabetes in IL-15/IL-15R α Double Transgenic Mice. To investigate whether the diabetes in IL-15/IL-15R α double transgenic mice was IL-15/IL-15R α dependent, we inhibited IL-15-mediated signaling in the IL-15/IL-15R α double transgenic mice with new onset of diabetes. Inhibiting IL-15 signaling by blocking IL-15 transpresentation using a monoclonal antibody, TM β 1, which is directed to IL-2/IL-15R β (CD122), reversed the hyperglycemia in all 12 mice examined (Fig. 3A). Furthermore, a 4-wk continuous infusion of the Jak2/3 inhibitor tofacitinib (CP-690,550), which blocks the IL-15 signaling pathway, also reversed the hyperglycemia in all 10 mice examined (Fig. 3B). Mice treated with TM β 1 had minimal mononuclear cell infiltration in the pancreatic islets (Fig. 3C). The majority of the mice treated with tofacitinib had no mononuclear cell infiltration in the pancreas (Fig. 3C). These data suggested that blockade of IL-15 signaling in the double transgenic mice not only reversed the hyperglycemia but also eliminated mononuclear cell infiltration. Therefore, the

diabetes observed in the IL-15/IL-15R α double transgenic mice was IL-15/IL-15R α dependent.

Depletion of CD4 Cells at the Onset of Diabetes Reversed Hyperglycemia in IL-15/IL-15R α Double Transgenic Mice. Autoreactive T cells can play a major role in β -cell destruction (22, 23). However, recent studies suggested other cell types such as NK or B cells may also be involved in β -cell autoimmunity (24, 25). IL-15 activates both T and NK cells (26). To investigate what cells are required for the autoimmunity in the double transgenic mice, we depleted CD4, CD8, and NK cells from the double transgenic mice with new onset of diabetes. Depletion of CD4 T cells with a monoclonal anti-CD4-depleting antibody (clone GK1.5) slowly reversed the hyperglycemia in the double transgenic mice in all 10 mice examined (Fig. 4A). Histological examination of the mice with CD4 cell depletion showed persistent infiltration of CD3 cells, B cells, and macrophages (F4/80) (Fig. S4). However, CD8 cell depletion and NK cell depletion had no effect on the hyperglycemia in the double transgenic mice ($n = 10$, Fig. 4B and C). The depletion of CD4, CD8, and NK cells in the peripheral blood was confirmed by flow cytometry. These data suggested that CD4 cells were required for the development of the disease after diabetes onset in this model. Interestingly, CD4 cells (or peripheral blood mononuclear cells) from the double transgenic mice could respond to wild-type islets in vitro and wild-type islets were rejected when transplanted into the diabetic double transgenic mice in vivo (Fig. S5). This suggested that an immune response toward wild-type islets that have no exogenously induced IL-15/IL-15R α expression was generated in the double transgenic mice. However, transfer of CD4 or total T cells into congenic wild-type or Rag1-deficient mice did not induce diabetes in the recipient mice. This suggested that CD4 cells (or T cells) alone may not be sufficient to induce diabetes.

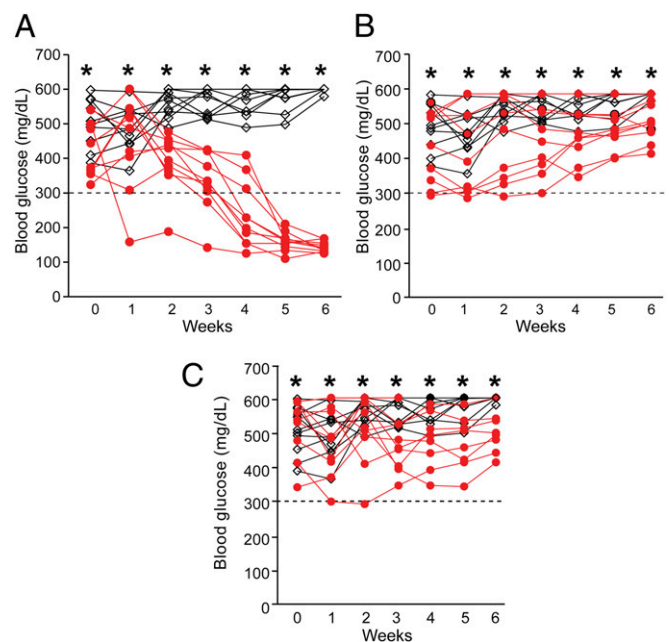


Fig. 4. Depletion of CD4 cells reversed hyperglycemia in IL-15/IL-15R α double transgenic mice. (A) The blood glucose levels in untreated (black, $n = 10$) or treated (with CD4 cell depletion antibody) mice (red, $n = 10$). (B) The blood glucose levels in untreated (black, $n = 10$) or treated (with CD8 cell depletion antibody) mice (red, $n = 10$). (C) The blood glucose levels in untreated (black, $n = 10$) or treated (with NK cell depletion antibody anti-asialo GM1) mice (red, $n = 10$).

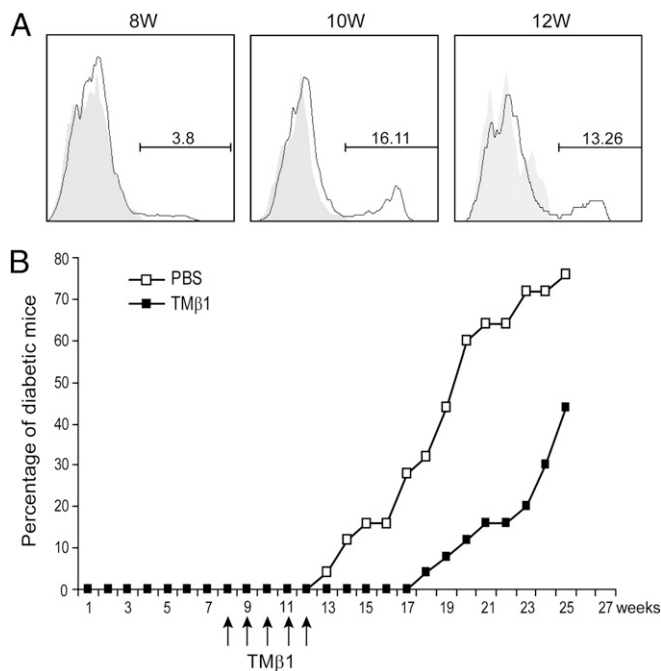


Fig. 5. Inhibiting IL-15 signaling with TM β 1 delayed the diabetes development in NOD mice. (A) IL-15R α expression on the purified islet cells from NOD mice. The histograms were gated on CD45⁺ population. Gray, isotype control; solid, IL-15R α . (B) The percentage of diabetic mice in NOD mice treated with PBS ($n = 25$) or monoclonal anti-CD122 antibody TM β 1 ($n = 25$) ($P < 0.0001$).

Inhibiting IL-15 Signaling at the Prediabetic Stage Delayed the Diabetes Development in NOD Mice. NOD mice are widely used for the study of T1D where multiple gene loci have been shown to participate in the diabetes development. In NOD mice, IL-15 expression was increased in purified islet cells at the prediabetic stage (27). We investigated whether IL-15R α was also induced at this stage. Flow cytometry showed an increase of IL-15R α expression in purified islet cells (CD45 negative population) at week 10 (16.11%) and week 12 (13.26%) compared with week 8 (3.8%) (Fig. 5A). We inhibited IL-15 signaling at the prediabetic stage (from week 8 to week 12) using the monoclonal antibody TM β 1, which blocks IL-15 transpresentation. TM β 1 treatment at the prediabetic stage delayed diabetes development in NOD mice (Fig. 5B, $n = 25$, $P < 0.001$). This suggested that IL-15 signaling was also involved in the diabetes development in NOD mice.

Increased Expression of IL-15 and IL-15R α in the Islets of Patients with T1D. We next investigated whether IL-15 and IL-15R α were expressed in the islets of patients with T1D. The pancreatic tissue samples were obtained from Network for Pancreatic Organ Donors (nPOD) (www.jdrfnpod.org). Immunohistochemistry demonstrated IL-15 expression in the islets in all T1D patients examined (four out of four, nPOD-6038, nPOD-6046, nPOD-6050, and nPOD-6084), whereas IL-15 was detected only in the pancreatic islets in one out of four normal donors and two out of five autoantibody positive donors (Fig. 6A). Interestingly, in two cases where IL-15 was positive, we also detected islet expression of MxA, a GTPase induced by type 1 IFN (α/β), that has been reported to interfere with virus multiplication and spread (28) (Fig. S6). Furthermore, we analyzed *IL-15* and *IL-15R α* mRNA levels in the islets from T1D patients, autoantibody positive, and normal donors. The islets were microdissected based on insulin positivity using laser capture microscopy (LCM) (29). Consistent with the immunohistochemistry results, we found higher *IL-15* (Fig. 6B) and *IL-15R α* (Fig. 6C) mRNA levels in T1D compared

with normal or autoantibody positive donors (T1D vs. normal: $P < 0.01$ *IL-15*; $P = 0.07$ *IL-15R α*). T1D vs. autoantibody positive donors: $P < 0.01$ *IL-15*; $P = 0.06$ *IL-15R α*). Moreover, because IL-15 and IL-15R α are usually coordinately induced and elevated serum levels of IL-15 have been found in T1D (14), we measured the serum levels of soluble IL-15R α in T1D using a unique assay we developed (13). The serum sIL-15R α levels were significantly higher in patients with T1D compared with those of normal controls (Fig. 6D, $P < 0.001$). The average sIL-15R α level in patients with T1D was 6.18 pM compared with 1.7 pM in normal donors (Fig. 6D). Taken together, our data suggest that disordered IL-15 and IL-15R α may be involved in the pathogenesis of T1D.

Discussion

In the present study, we demonstrated islet expression of both IL-15 and IL-15R α and elevated serum levels of sIL-15R α in patients with T1D. Furthermore, we demonstrated β -cell expression of IL-15- and IL-15R α -induced insulin-dependent diabetes in mice with many characteristics similar to human T1D. Inhibiting IL-15 signaling by blocking IL-15 transpresentation with TM β 1 or by Jak2/3 inhibitor tofacitinib completely reversed the diabetes in the mice. Moreover, in NOD mice, IL-15 and IL-15R α up-regulations were demonstrated in the islets at the prediabetic stage and the inhibition of IL-15 signaling at the prediabetic stage delayed diabetes development. Collectively, these data suggest islet expression of IL-15 and IL-15R α may be involved in the pathogenesis of human T1D.

The pathogenesis of T1D remains elusive even after decades of study. Increasing evidence suggests that both genetic susceptibility and environmental influences are likely to contribute to the disease development. Although the precise etiology of T1D is still unknown, viruses have long been suggested as a potential environmental trigger for the disease. A recent meta-analysis by Yeung et al. showed a clinically significant association between enterovirus infection and T1D (30). Furthermore, rare variants of IFIH1, a gene implicated in antiviral responses, were shown to protect against T1D (31). Moreover, recent studies suggested a causative role for enteroviruses in T1D (30, 32, 33). Oikarinen et al. showed that enterovirus RNA positivity during the 6-mo period preceding

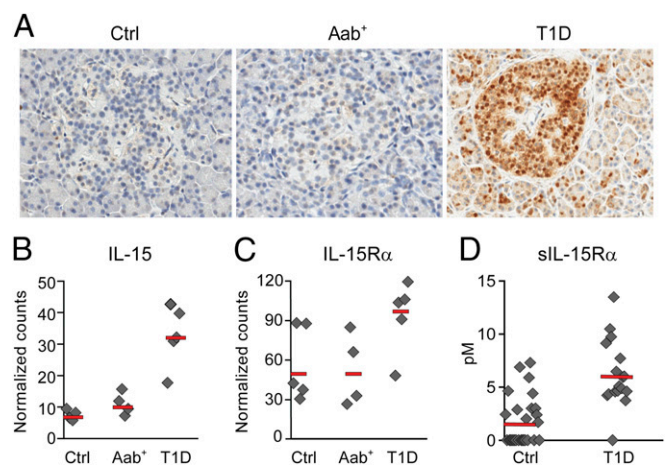


Fig. 6. Increased expression of IL-15 and IL-15R α in patients with T1D. (A) Immunohistochemistry staining of IL-15 in pancreatic tissues from T1D, autoantibody positive (Aab⁺), and normal donors (Ctrl). (B) *IL-15* mRNA levels in microdissected islets analyzed by Nanostring. The counts were normalized to three housekeeping genes. T1D vs. ctrl: $P < 0.01$. (C) *IL-15R α* mRNA levels in microdissected islets analyzed by Nanostring. The counts were normalized to three housekeeping genes. T1D vs. ctrl: $P = 0.07$. (D) sIL-15R α levels in the serum from 29 controls and 20 T1D patients ($P < 0.001$).

the first autoantibody-positive sample was associated with increased risk for the development of islet autoimmunity (33). The study published by Diabetes and Autoimmunity Study in the Young (DAISY) consortium concluded that the rate of progression from islet autoimmunity (islet autoantibody positive) to T1D was significantly increased following detection of enterovirus RNA in the serum in genetically susceptible children (32). Together, these studies suggested enteroviruses may be involved in both the initiation and progression of T1D. In support of these association studies, clinically, coxsackie B4 virus, an enterovirus that belongs to Picornaviridae family, was found in the β cells in 3 out of 6 recent onset T1D patients (9). Furthermore, enteroviral capsid protein VP1 was found in islets from 44 out of 72 recent onset patients versus 3 out of 50 controls (20). As indirect evidence, viral signatures such as IFN, MHC hyperexpression, and CD8 infiltrations were found in T1D (15, 34–36). All of these studies suggested an important role for viral infection in T1D. Interestingly, viral infection induced the expression of interferon, which in turn acts on the upstream promoters of IL-15 and IL-15R α and induces their expressions (17). Exposure of human pancreatic islets to coxsackie virus, an enterovirus linked to T1D or directly to IFN γ , induced the expression of both IL-15 and IL-15R α in vitro (10). Based on these observations, we hypothesized that IL-15 and IL-15R α may be expressed in the islets in patients with T1D. As predicted by this hypothesis, we detected islet expression of both IL-15 and IL-15R α in 4 out of 4 patients with T1D (Fig. 6 A–C). Moreover, higher levels of serum IL-15 and IL-15R α (Fig. 6D) were also demonstrated in T1D. Interestingly, in 2 cases where IL-15 was positive, we also detected islet expression of MxA (Fig. S6), a GTPase that is induced during viral infection exclusively and in a dose-dependent manner by IFN α and IFN β , but not by IFN γ , IL-1, TNF α , or other cytokines. MxA has been used clinically as a marker for viral infection and has antiviral activity by interfering with virus multiplication and spread (28).

To investigate if islet expression of IL-15 and IL-15R α could play a role in the pathogenesis of T1D, we generated double transgenic mice with beta cell expression of IL-15 and IL-15R α . The mice developed insulin-dependent diabetes with many characteristics similar to the human disease (Fig. 2). Furthermore, we demonstrated that the diabetes in the mice was specifically IL-15/IL-15R α dependent because inhibiting IL-15 signaling with TM β 1, which blocks IL-15 transpresentation or by addition of the Jak2/3 inhibitor tofacitinib, which blocks IL-15 signaling, reversed the diabetes in the mice (Fig. 3). IL-15 is a proinflammatory cytokine that has been described as being at the apex of a proinflammatory cytokine cascade (37). It has been suggested that IL-15 expression might precede expression of tumor necrosis factor and the downstream cytokines IL-1, IL-6, and granulocyte/macrophage colony-stimulating factor, as well as the proinflammatory chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CXCL8 (IL-8). We tried to define the molecular mechanisms that underlie the IL-15/IL-15R α -induced autoimmune diabetes in the double transgenic mice. We found increased gene expression of both proinflammatory cytokines and proinflammatory chemokines in the islets before the diabetes onset, which suggested ongoing inflammation long before the disease onset (Fig. S2). Moreover, there were hyperexpressions of both MHC class I, class II, and the adhesion molecule ICAM-1 on the islet cells, which could greatly enhance islet antigen presentation to the infiltrating T cells (Fig. S3). These events could eventually lead to autoimmune diabetes. Collectively, our data suggested a causative role of islet expression of IL-15 and IL-15R α and T1D development in mice. Although what exactly induced the islet expression of IL-15 and IL-15R α is still unknown in human T1D, the murine data would suggest that IL-15 and IL-15R α may act as downstream mediators to induce diabetes development in humans.

Studies in human and in animal models suggest that T cells are the primary effector cells in T1D (22, 23). Recently, a number of studies suggested that B cells and NK cells might also contribute to the beta cell loss (24, 25, 38). IL-15 is a growth factor for both T cells and NK cells. In vivo administration of IL-15 induced expansion of circulating NK and CD8 central and effector-memory T cells (39). It would be logical to suggest that both NK and T cells might contribute to the beta cell autoimmunity in the IL-15/IL-15R α double transgenic mice. However, we did not observe NK cell expansion in the peripheral and pancreatic lymph nodes in the double transgenic mice. Only very few NK cells were found in the infiltrating lymphocytes in the islets (Fig. 2D). Moreover, depletion of NK cells with asialo-GM1, which impacted both NK and CD8 T cells, had no effect on the diabetes development in the double transgenic mice (Fig. 4C). Therefore, NK cells were not likely to play a major role in the beta-cell autoimmunity in the double transgenic mice. On the other hand, T cells and macrophages were the dominant cells that infiltrated into the pancreas in the double transgenic mice (Fig. 2D). To determine which cell types are required for the disease development after the diabetes onset, we depleted CD4, CD8, and macrophages in the double transgenic mice with new onset of diabetes. CD8 T-cell depletion did not reverse the hyperglycemia in these mice (Fig. 4B). Interestingly, we noticed that there was an increase of CD8CD44^{high} effector memory T cells in the pancreatic lymph node in the double transgenic mice at the onset of diabetes (Fig. S7). Therefore, it is possible that CD8 T cells may be needed for the disease development before the diabetes onset and became dispensable after the diabetes onset. Macrophage depletion with clodronate liposomes resulted in a 50% reversal of the diabetes in treated mice. However, further analysis suggested that clodronate liposomes treatment not only depleted macrophages, but also significantly reduced the number of CD4 and CD8 cells. Therefore, it remains elusive whether macrophages were required for the disease development after the diabetes onset. CD4 T-cell depletion slowly reversed the hyperglycemia in all treated mice (Fig. 4A). Persistent mononuclear cell infiltration in the islets was found in the mice depleted of CD4 T cells (Fig. S4). The reversal of diabetes would suggest that those infiltrating cells were unable to mount further beta-cell destruction. These data suggested that CD4 T cells were very important for the disease development after diabetes onset. Interestingly, we noticed the double transgenic mice had lower CD4 cell percentages (and total cell numbers) both in the spleen and in the pancreatic lymph node (PLN) at the diabetes onset (Fig. S7). This may be due to the increased B-cell number in circulation that dilute out CD4 cells as we noticed higher B-cell percentages (and total cell numbers) were observed both in the spleen and in the PLN in the mice (Fig. S7). Furthermore, the CD4 cells from the double transgenic mice were more prone to Th1 priming in vitro (Fig. S8). However, transfer of CD4 or total T cells into congenic wild-type or Rag1-deficient mice did not induce diabetes in the recipient mice. This suggested that CD4 (or T cells) alone may not be sufficient to induce diabetes. It will be interesting to investigate whether B cells and their antibodies are also required in diabetes development in the double transgenic mice because higher numbers of B cells were observed both in the spleen and in the pancreatic lymph nodes at the diabetes onset.

In summary, we demonstrated islet expression of both IL-15 and IL-15R α in patients with T1D. Coexpression of IL-15 and IL-15R α on beta islet cells induced insulin-dependent diabetes in mice. Inhibiting IL-15 signaling with TM β 1 or the Jak2/3 inhibitor tofacitinib completely reversed the diabetes in the mice. These preclinical data in combination with human data suggested that disordered IL-15 and IL-15R α expression may be involved in the pathogenesis of type I diabetes in humans and

that the IL-15/IL-15Ra pathway may be a rational therapeutic target for the prevention of the development of T1D.

Materials and Methods

Pancreatic Tissues and Sera. Pancreatic tissue samples from patients with T1D, autoantibody positive donors, and normal donors were obtained from the Network for Pancreatic Organ Donors (nPOD) (www.jdrfnpod.org). Islets from pancreatic tissues were microdissected on Arcturus PixCell ILC instrument (Arcturus Engineering) using a series of cut slides that were stained with insulin as a reference. Sera from 20 patients with T1D were acquired from Promeddx, Inc. Sera from 29 normal donors were collected from the National Institutes of Health blood bank. Patient informed consent was obtained. The study was approved by the institutional review board of the National Cancer Institute (NCI), Bethesda, MD.

Mice. The transgenic mice were generated in NCI-Frederick (accredited by American Association for the Accreditation of Laboratory Animal Care International). C57BL/6, B6.Rag1^{-/-}, and NOD/ShiLtJ mice were purchased from The Jackson Laboratory. The animal study was approved by the Animal Care and Use Committee of the National Institutes of Health, Bethesda, MD.

Plasmid Construction and Generation of Transgenic Mice. RIP-IL15 and RIP-IL15R α transgene constructs were generated by cloning mouse IL-15 and IL-15R α coding regions into BamHI and EcoRI sites of pKS-RIP (provided by Jun-Li Liu, McGill University Health Center, Montreal). Mouse oocytes from inbred C57BL/6 mice were used to establish transgenic mice. RIP-IL15/IL15R α double transgenic mice were established by coinjection of RIP-IL15 and RIP-IL15R α gene fragments. Genotyping was performed on genomic DNA extracted from tail-tip biopsies by Southern blotting analysis.

- Delovitch TL, Singh B (1997) The nonobese diabetic mouse as a model of autoimmune diabetes: Immune dysregulation gets the NOD. *Immunity* 7(6):727–738.
- Kikutani H, Makino S (1992) The murine autoimmune diabetes model: NOD and related strains. *Adv Immunol* 51:285–322.
- Lodolce JP, et al. (1998) IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 9(5):669–676.
- Schluns KS, Williams K, Ma A, Zheng XX, Lefrançois L (2002) Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol* 168(10):4827–4831.
- Burkett PR, et al. (2004) Coordinate expression and trans presentation of interleukin (IL)-15R α and IL-15 supports natural killer cell and memory CD8⁺ T cell homeostasis. *J Exp Med* 200(7):825–834.
- Dubois S, Mariner J, Waldmann TA, Tagaya Y (2002) IL-15R α recycles and presents IL-15 in trans to neighboring cells. *Immunity* 17(5):537–547.
- Mortier E, Woo T, Advincula R, Gozalo S, Ma A (2008) IL-15R α chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *J Exp Med* 205(5):1213–1225.
- Sandau MM, Schluns KS, Lefrançois L, Jameson SC (2004) Cutting edge: Transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R α by the same cells. *J Immunol* 173(11):6537–6541.
- Verbist KC, Rose DL, Cole CJ, Field MB, Klonowski KD (2012) IL-15 participates in the respiratory innate immune response to influenza virus infection. *PLoS ONE* 7(5):e37539.
- Ylipaasto P, et al. (2005) Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. *Diabetologia* 48(8):1510–1522.
- Waldmann TA (2004) Targeting the interleukin-15/interleukin-15 receptor system in inflammatory autoimmune diseases. *Arthritis Res Ther* 6(4):174–177.
- Kuczyński S, et al. (2005) IL-15 is elevated in serum patients with type 1 diabetes mellitus. *Diabetes Res Clin Pract* 69(3):231–236.
- Chen J, et al. (2011) Increased serum soluble interleukin-15 receptor alpha (sIL-15R α) levels in T cell large granular lymphocyte leukemia. *Blood* 119(1):137–143.
- Dotta F, et al. (2007) Coxsackie B4 virus infection of beta cells and natural killer cell insulinitis in recent-onset type 1 diabetic patients. *Proc Natl Acad Sci USA* 104(12):5115–5120.
- Foulis AK, Farquharson MA, Meager A (1987) Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus. *Lancet* 2(8573):1423–1427.
- Huang X, et al. (1995) Interferon expression in the pancreases of patients with type 1 diabetes. *Diabetes* 44(6):658–664.
- Dubois SP, Waldmann TA, Müller JR (2005) Survival adjustment of mature dendritic cells by IL-15. *Proc Natl Acad Sci USA* 102(24):8662–8667.
- Zhang X, Sun S, Hwang I, Tough DF, Sprent J (1998) Potent and selective stimulation of memory-phenotype CD8⁺ T cells in vivo by IL-15. *Immunity* 8(5):591–599.
- Azimi N, Shiramizu KM, Tagaya Y, Mariner J, Waldmann TA (2000) Viral activation of interleukin-15 (IL-15): Characterization of a virus-inducible element in the IL-15 promoter region. *J Virol* 74(16):7338–7348.
- Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG (2009) The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia* 52(6):1143–1151.
- Smith SJ, et al. (2006) In vivo monitoring of pancreatic beta-cells in a transgenic mouse model. *Mol Imaging* 5(2):65–75.

Assessment of Hyperglycemia and Diabetes. Blood glucose levels were measured using the test strips and the Contour monitoring system (Bayer). Mice were considered hyperglycemic when their blood glucose constantly rose above 300 mg/dL.

Antibody Treatment and Jak2/3 Inhibitor Tofacitinib Treatment. The double transgenic mice with blood glucose higher than 300 mg/dL in two consecutive weeks were treated with (i) i.p. 200 μ g of TM β 1 twice per week; (ii) s.c. 4-wk pump infusion of Jak2/3 inhibitor tofacitinib (CP-690,550) 30 mg/kg per d; (iii) i.p. anti-CD4 (clone GK1.5) 200 μ g on days 1, 2, and 3, then twice per week; (iv) i.p. anti-CD8 (clone 2.43) 200 μ g on days 1, 2, and 3, then twice per week; (v) i.p. anti-asialo GM1 50 μ L on days 1, 2, and 3, then twice per week; and (vi) i.p. clodronate liposome 100 μ L every 2 d. TM β -1, anti-CD4, and anti-CD8 monoclonal antibodies were obtained from Bio X Cell and were administered for 6 wk. Anti-asialo GM1 was obtained from Wako Pure Chemical Industries and was administered for 6 wk. Clodronate liposomes were obtained from www.clodronateliposomes.org and were administered for 2 wk.

Statistical Analyses. Statistical significance between two means was determined by unpaired *t* tests. Statistical significance was two-tailed and *P* < 0.05 was considered significant.

ACKNOWLEDGMENTS. We thank Dr. Oksana Gavrilova (National Institute of Diabetes and Digestive and Kidney Diseases) for the technical support of islet isolation and Dr. Jun-Li Liu (McGill University) for kindly providing us the rat insulin promoter construct. This work was supported by the intramural research program of the Center for Cancer Research, National Cancer Institute (NCI), National Institutes of Health (NIH) and in part by federal funds from NCI, NIH, under Contract HHSN261200800001E.

- Miller BJ, Appel MC, O'Neil JJ, Wicker LS (1988) Both the Lyt-2⁺ and L3T4⁺ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. *J Immunol* 140(1):52–58.
- Herold KC, et al. (2002) Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* 346(22):1692–1698.
- Gur C, et al. (2010) The activating receptor NKp46 is essential for the development of type 1 diabetes. *Nat Immunol* 11(2):121–128.
- Pescovitz MD, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group (2009) Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med* 361(22):2143–2152.
- Bamford RN, et al. (1994) The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc Natl Acad Sci USA* 91(11):4940–4944.
- Cardozo AK, et al. (2003) IL-1 β and IFN- γ induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. *Diabetologia* 46(2):255–266.
- Kochs G, Haller O (1999) GTP-bound human MxA protein interacts with the nucleocapsid of Thogoto virus (Orthomyxoviridae). *J Biol Chem* 274(7):4370–4376.
- Golubeva Y, Salcedo R, Mueller C, Liotta LA, Espina V (2013) Laser capture microdissection for protein and NanoString RNA analysis. *Methods Mol Biol* 931:213–257.
- Yeung WC, Rawlinson WD, Craig ME (2011) Enterovirus infection and type 1 diabetes mellitus: Systematic review and meta-analysis of observational molecular studies. *BMJ* 342:d35.
- Nejentsev S, Walker N, Riches D, Egholm M, Todd JA (2009) Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* 324(5925):387–389.
- Stene LC, et al. (2010) Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: The Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes* 59(12):3174–3180.
- Oikarinen S, et al. (2011) Enterovirus RNA in blood is linked to the development of type 1 diabetes. *Diabetes* 60(1):276–279.
- Itoh N, et al. (1993) Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J Clin Invest* 92(5):2313–2322.
- Bottazzo GF, et al. (1985) In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulinitis. *N Engl J Med* 313(6):353–360.
- Coppieters KT, et al. (2012) Demonstration of islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients. *J Exp Med* 209(1):51–60.
- Waldmann TA (2006) The biology of interleukin-2 and interleukin-15: Implications for cancer therapy and vaccine design. *Nat Rev Immunol* 6(8):595–601.
- Flodström M, Shi FD, Sarvetnick N, Ljunggren HG (2002) The natural killer cell—friend or foe in autoimmune disease? *Scand J Immunol* 55(5):432–441.
- Waldmann TA, et al. (2011) Safety (toxicity), pharmacokinetics, immunogenicity, and impact on elements of the normal immune system of recombinant human IL-15 in rhesus macaques. *Blood* 117(18):4787–4795.