NLRP3 deficiency protects from type 1 diabetes through the regulation of chemotaxis into the pancreatic islets

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Studies in animal models and human subjects have shown that both innate and adaptive immunity contribute to the pathogenesis of type 1 diabetes (T1D). Whereas the role of TLR signaling pathways in T1D has been extensively studied, the contribution of the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein (NLRP) 3 inflammasome pathway remains to be explored. In this study, we report that NLRP3 plays an important role in the development of T1D in the nonobese diabetic (NOD) mouse model. NLRP3 deficiency not only affected T-cell activation and Th1 differentiation, but also modulated pathogenic T-cell migration to the pancreatic islet. The presence of NLRP3 is critical for the expression of the chemokine receptors CCR5 and CXCR3 on T cells. More importantly, NLRP3 ablation reduced the expression of chemokine genes *CCL5* and *CXCL10* on pancreatic islet cells in an IRF-1–dependent manner. Our results suggest that molecules involved in chemotaxis, accompanied by the activation of the NLRP3 inflammasome, may be effective targets for the treatment of T1D.

NLRP3 | islet | type 1 diabetes | chemokine | NOD mouse

Type 1 diabetes (T1D) is a T-cell–mediated autoimmune disease characterized by the destruction of insulin-producing pancreatic beta cells in genetically predisposed individuals. Studies in animal models and human subjects have shown that both innate and adaptive immunity play a role in disease pathogenesis. Strategies targeting either T or B cells have shown some efficacy in T1D in both animal and human studies (1–4). Recently, the role of innate immunity in T1D has been increasingly appreciated. We, and others, have demonstrated that Toll-like receptor (TLR) signaling pathways are essential for the development of T1D. Nonobese diabetic (NOD) mice deficient in TLR2, TLR9, or MyD88 showed delayed disease development or were protected from diabetes (5–9). However, the development of autoimmune diabetes was accelerated in TLR4$^{-/-}$ NOD mice (5–7, 10). Whereas the role of TLR signaling has been intensively studied, the contribution of the nucleotide binding domain-like receptor (NLR) signaling pathway to the pathogenesis of T1D remains to be explored.

Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein (NLRP) 3 is a NLR family member, together with ASC and caspase-1, forms protein complexes that are responsible for the innate immune response to pathogens and/or "danger" signals (11). Increasing evidence indicates that the NLRP3 inflammasome plays an important role in obesity and type 2 diabetes (12–14). However, little is known about the role of NLRP3 in autoimmune diabetes. Whereas the inflammasome has been extensively studied in the control of infection, only recently has the role of the NLRP inflammasome in autoimmune disease been recognized. Polymorphisms in inflammasome genes are involved in the predisposition to systemic lupus erythematosus (15). NLRP3 deficiency dramatically delayed the course and reduced severity of experimental autoimmune encephalomyelitis by suppression of Th1 and Th17 responses (16). Mice deficient in ASC, the adaptor protein of the NLRP3 inflammasome pathway, were also less susceptible to collagen-induced arthritis (17). Nevertheless, the role of the inflammasome pathway in the pathogenesis of T1D is unclear. Although caspase-1 or IL-1β deficiency did not protect NOD mice from T1D (18, 19), IL-1 blockade showed a synergistic protective effect when combined with anti-CD3 therapy for T1D in a mouse model (20). Interestingly, recent genetic association studies suggested that polymorphisms in inflammasome genes might be involved in the predisposition to T1D. A coding polymorphism in NLRP1 was demonstrated to confer susceptibility to T1D (21). Furthermore, two single-nucleotide polymorphisms in NLRP3 were identified in a separate association study as a predisposing factor for T1D (22).

Thus, we generated NLRP3-deficient (NLRP3$^{-/-}$ or KO) NOD mice to understand the role of NLRP3 in the pathogenesis of T1D. Here, we show that NOD mice deficient in NLRP3 were protected from T1D development. Mechanistic studies suggested that the expression of NLRP3, in both hematopoietic and nonhematopoietic cells, was important for diabetes development. Whereas NLRP3 deficiency in the hematopoietic compartment reduced the diabetogenicity of immune cells, its ablation in nonhematopoietic cells, particularly in the pancreatic islets, compromised the migration of immune cells into the target tissue. Destruction of beta cells was reduced via the down-regulation of chemokine gene expression in the pancreatic islets leading to protection from diabetes.

Significance

Our study demonstrated that the nucleotidebinding oligomerization domain, leucine-rich repeat and pyrin domaincontaining protein 3 (NLRP3) pathway plays an important role in type 1 diabetes (T1D) using a mouse model. NLRP3 is critical for chemokine receptors CCR5 and CXCR3 expression on T cells, influencing pathogenic T-cell migration to the islets. It also affects the chemokines CCL5 and CXCL10 expression in the islets, preventing pathogenic T cells from infiltration. Targeting this pathway may be useful in prevention and treatment of T1D as it affects both immune cells and pancreatic beta cells.

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Results

NLRP3 Deficiency Prevented T1D in NOD Mice. To understand the role of NLRP3 in the pathogenesis of T1D, we backcrossed NLRP3$^{-/-}$ C57BL/6 mice with NOD mice for more than 10 generations. The purity of the NOD genetic background was further confirmed by verification of known *Idd* loci (type1diabetes.jax.org/gqc). To study the effect of NLRP3 ablation on the development of T1D, we set up a cohort of NLRP3$^{-/-}$ NOD mice, as well as a group of wild-type littermates, to observe the natural history of disease development. As shown in Fig. 1*A*, compared with WT littermates, diabetes development in NLRP3$^{-/-}$ NOD mice was significantly reduced. Moreover, to test whether pharmaceutical inhibition of NLRP3 had a similar effect, we treated a group of WT NOD mice with either the NLRP3 inhibitor, parthenolide, an extract of a natural herb, or vehicle, via oral gavage for 4 wk. Intriguingly, chemical inhibition of NLRP3 also significantly delayed and reduced disease development in NOD mice (Fig. 1*B*). To understand the effect of NLRP3 deficiency on the cellular infiltration in pancreatic islets, we randomly selected some pancreata from nondiabetic female NOD mice (NLRP3$^{-/-}$ NOD and NLRP3$^{+/+}$ littermates, n = 5–8 per group) and examined their insulitis after H&E staining. Consistent with the reduction in diabetes development, NLRP3$^{-/-}$ NOD mice showed considerably attenuated insulitis (Fig. S1*A*). There were significantly fewer T and B cells in the islet infiltrates (Fig. S1*B*).

The Expression of NLRP3 in Both Hematopoietic and Nonhematopoietic Cells Contributes to Diabetes Development. NLRP3 is expressed in both immune cells and tissue cells (23, 24). To further investigate whether deficiency of NLRP3 in the hematopoietic or nonhematopoietic cell compartment was responsible for the observed protective effect, we performed a set of bone marrow chimera (BMC) experiments. NLRP3$^{-/-}$ NOD or WT recipients (all females) were lethally irradiated (1,000 cGy) and transplanted with 10^7 bone marrow (BM) cells from NLRP3$^{-/-}$ NOD or WT mice (age-matched females). The BMC mice were observed for diabetes development. It is interesting that although WT mice that received NLRP3$^{-/-}$ BM cells showed modest protection from diabetes (solid triangles in Fig. S2) in comparison with WT mice that received WT BM cells (solid dots in Fig. S2), the development of diabetes was highly inhibited in NLRP3$^{-/-}$NOD recipients (squares and diamonds in Fig. S2) regardless of the genotype of the donor BM cells, which suggests that NLRP3 in nonhematopoietic cells plays a more important role in diabetes protection.

The Effect of NLRP3 Deficiency on the Hematopoietic Compartment. The BMC experiment showed that NOD WT recipients that received NLRP3$^{-/-}$ NOD BM cells exhibited delayed and reduced diabetes development, which suggested that NLRP3 expression on immune cells plays a role in promoting disease development. Indeed, when splenocytes from NLRP3$^{-/-}$ NOD mice were adoptively transferred into RAG1$^{-/-}$ NOD mice, diabetes onset was significantly delayed in recipients in comparison with RAG1$^{-/-}$ mice that received WT splenocytes (Fig. 2*A*). To understand which subsets of immune cells expressing NLRP3 were essential, we first characterized the NLRP3$^{-/-}$ NOD mice. Compared with WT NOD mice, NLRP3$^{-/-}$ NOD mice had reduced splenocyte numbers, including significantly fewer CD4$^+$ T cells, CD19$^+$ B cells, and CD11b$^+$ macrophages (Fig. S3*A*). There was also a reduced number of T and B cells, dendritic cells, and macrophages in pancreatic draining lymph nodes (Fig. S3*B*). However, we did not find any reduction of T cells in the thymus (CD4$^+$; Fig. S3*C*) or CD8$^+$ (Fig. S4*A*) or in B cells from the BM (Fig. S4*B*), but we did observe an increase in thymic B cells (Fig. S3*C*). Interestingly, the reduction of immune cells in the periphery of NLRP3$^{-/-}$ NOD mice appears to be genetic background-dependent because this reduction was not observed in NLRP3$^{-/-}$ C57BL/6 mice (Fig. S3*D*). It has been reported that T cells express NLRP3 (25), and deficiency of NLRP3 in our model system also led to less activated CD4$^+$ T cells in both pancreatic draining lymph nodes and islet infiltrates as shown by a reduction in CD69 (Fig. 2*B*). Moreover, there were fewer CD4$^+$ T cells producing IFN-γ in NLRP3$^{-/-}$ NOD mice in comparison with WT mice (Fig. 2*C*).

Furthermore, the deficiency of NLRP3 in T cells induced a change in T-cell function. Upon anti-CD3 stimulation, NLRP3$^{-/-}$ NOD T cells showed impaired proliferation in vitro (Fig. S5*A*). Interestingly, the reduced T-cell proliferation was not observed in NLRP3$^{-/-}$ C57BL/6 T cells (Fig. S5*B*). To test whether NLRP3 deficiency affected diabetogenic T-cell function in vivo, we labeled purified CD4$^+$ T cells from WT and NLRP3$^{-/-}$ BDC2.5 mice with CFSE and then transferred the cells into WT NOD recipients. We harvested pancreatic draining lymph nodes from the recipient mice 3 and 7 d later and analyzed CFSE dilution, i.e., the proliferation of BDC2.5 cells. The proliferation of diabetogenic NLRP3$^{-/-}$ BDC2.5 CD4$^+$ T cells was markedly reduced on day 3; however, the reduced proliferation was not obvious on day 7 (Fig. 2*D*) and diabetogenicity of BDC2.5 CD4$^+$ T cells was not affected by the deficiency of NLRP3, as the WT NOD recipients became diabetic on day 9 (Fig. S6).

Macrophages and dendritic cells are the major immune cells expressing NLRP3. To investigate the effect of NLRP3 deficiency on these professional antigen-presenting cells (APCs), we stimulated APCs with LPS, CpG, Pam3csk4, and Poly I:C. Both WT and knockout APCs showed comparable responses to these TLR ligands based on CD80 and MHC II molecule expression (Fig. S7*A*). Next, we studied their antigen processing and presenting functions. Neither purified splenic APCs nor BM-derived APCs from WT or knockout mice showed any difference in antigen processing and presentation (Fig. S7*B*). However, APCs from NLRP3$^{-/-}$ NOD mice suppressed the differentiation of naïve CD4 T cells into IFN-γ–producing Th1 cells (Fig. 2*E*).

NLRP3 Deficiency Impaired the Migration of Diabetogenic CD4 T Cells to Islets. NLRP3 deficiency impairs the migration of pathogenic cells into the brain in the EAE model (26, 27). Thus, we speculated that the improved insulitis in NLRP3$^{-/-}$ NOD mice could be a result of impaired immune cell migration to pancreatic islets. To understand whether NLRP3 deficiency impairs CD4$^+$ T-cell homing, we performed cell trafficking experiments. We labeled WT BDC2.5 CD4$^+$ T cells (Thy1.1$^+$) and NLRP3$^{-/-}$ BDC2.5 CD4$^+$ T cells (Thy1.2$^+$) with CFSE and mixed the cells at a 1:1

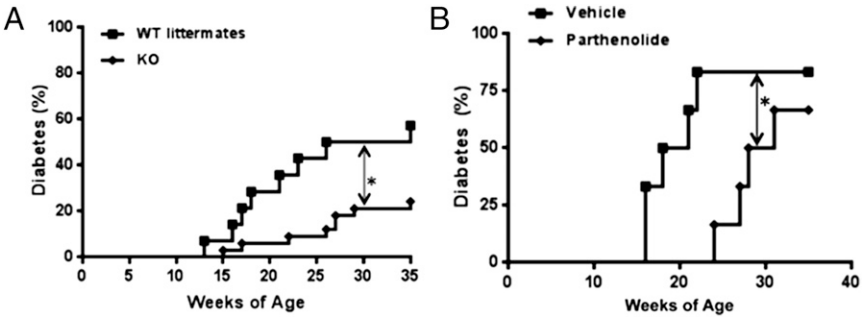

Fig. 1. Blockade of NLRP3 inflammasome delayed and reduced T1D. (*A*) Natural history of diabetes development in NLRP3$^{-/-}$ NOD mice (n = 33) and WT littermates (n = 16). (*B*) The NLRP3 inhibitor, parthenolide (10 mg/kg body weight, n = 6) or vehicle (n = 7) was administered to 10~12-wk-old prediabetic female NOD mice twice a week for 4 wk via oral gavage. Diabetes development was monitored by testing mice for glycosuria and confirmed by a blood glucose level over 250 mg/dL. *P < 0.05, log-rank test.

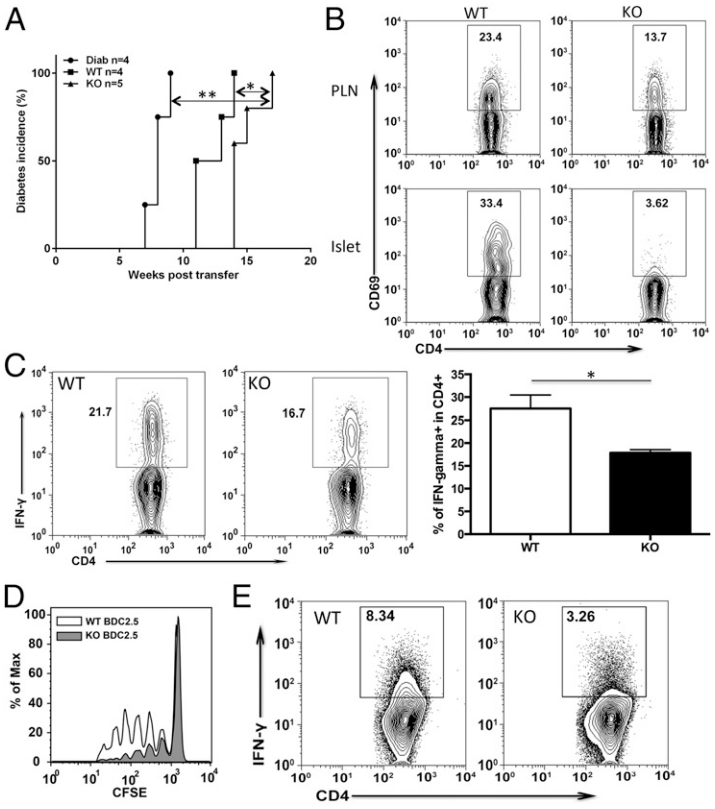

Fig. 2. Experiments to study the role of NLRP3 expression in hematopoietic cells for the development of T1D. (*A*) Diabetes induction by splenocytes of WT and NLRP3$^{-/-}$ NOD mice. Splenocytes (10^7) from 3-mo-old nondiabetic female WT ($n = 4$) or KO NOD ($n = 5$) were injected into RAG1$^{-/-}$ NOD mice i.v. As a positive control, 10^7 splenocytes from diabetic WT NOD mice were also injected into an additional group of RAG1$^{-/-}$ NOD recipients ($n = 4$). Diabetes development was monitored as described above. *$P < 0.05$, **$P < 0.01$, log-rank test. (*B*) Representative FACS plots showing CD69 expression in CD4$^+$ T cells from PLNs and islet infiltrates of WT or KO NOD mice ($n = 5$–6 per group). (*C*) Intracellular staining for IFN-γ in splenic CD4$^+$ T cells from WT and KO NOD mice. Data are shown as mean ± SEM ($n = 5$ per group). (*D*) CFSE-labeled CD4$^+$ T cells (3×10^6) from WT or KO BDC2.5 NOD mice were transferred into 6- to 8-wk-old female NOD mice. CD4$^+$ T-cell proliferation in PLN was determined 3 and 7 d after transfer. (*E*) FACS-sorted splenic naïve CD4$^+$ T cells (CD44$^{-/low}$CD62L^{high}) from NOD mice were cocultured with APCs (T-cell–depleted splenocytes) from WT or KO NOD mice at a 5:1 (T:APC) ratio in the presence of 1 μg of anti-CD3 at 37 °C, 5% CO_2. Three days later, IFN-γ–producing CD4$^+$ T cells were determined by intracellular cytokine staining. Data are representative of three independent experiments. *$P < 0.05$, unpaired Student's *t* test.

ratio before transferring into WT NOD.SCID mice. Interestingly, 16 h after adoptive transfer, more labeled NLRP3$^{-/-}$ BDC2.5 CD4$^+$ T cells than WT BDC2.5 CD4$^+$ T cells resided in the spleen and pancreatic draining lymph nodes (Fig. 3*A*, *Left*). In contrast to WT BDC2.5 CD4$^+$ T cells, significantly fewer NLRP3$^{-/-}$ BDC2.5 CD4$^+$ T cells migrated into islets (Fig. 3*A*, *Left*). To clarify whether NLRP3 deficiency in the tissue organ prevents the recruitment of diabetogenic CD4$^+$ T cells, we transferred the same CFSE-labeled mixture of BDC2.5 CD4$^+$ T cells into NLRP3$^{-/-}$ NOD.SCID mice. Compared with WT NOD.SCID recipients, WT BDC2.5 CD4$^+$ T cells also showed reduced recruitment (8 vs. 4 cells) to NLRP3$^{-/-}$ NOD.SCID islets 16 h after the transfer (Fig. 3*A*, *Right*). Furthermore, we found that the proliferation of CFSE-labeled WT BDC2.5 CD4$^+$ T cells was impaired in NLRP3$^{-/-}$ NOD.SCID recipients compared with proliferation that occurred in the WT NOD.SCID recipients (Fig. 3*B*). More importantly, when we transferred WT BDC2.5 CD4$^+$ T cells into WT or NLRP3$^{-/-}$ female NOD.SCID mice, diabetes development was significantly delayed and reduced in NLRP3$^{-/-}$ NOD.SCID recipients in comparison with WT NOD.SCID recipients (Fig. 3*C*).

NLRP3 Deficiency Affects Chemotaxis of Immune Cells Both Intrinsically and Extrinsically. There were two possible reasons for the impaired T-cell proliferation and function in NLRP3$^{-/-}$ NOD islets. One was that there were more regulatory T cells in NLRP3$^{-/-}$ NOD islets, which suppressed T-cell function. However, we did not find a significant difference in the numbers of regulatory T cells in spleens and pancreatic draining lymph nodes between WT and NLRP3$^{-/-}$ NOD mice (Fig. S8). The other explanation was that NLRP3 deficiency impaired the chemotaxis of T cells to pancreatic islets, which was more likely, as evidenced by fewer BDC2.5 CD4$^+$ T cells migrating to and proliferating in NLRP3$^{-/-}$ NOD islets in comparison with the WT islets. To further dissect the molecular mechanisms, we tested the gene expression of chemokines and chemokine receptors in islet cells and purified T cells, respectively.

As shown earlier in Fig. 3*A*, more NLRP3$^{-/-}$ NOD T cells were homing to, or staying in, the spleen and pancreatic draining lymph nodes. To clarify whether NLRP3 deficiency affected chemokine receptor gene expression on CD4$^+$ T cells, we sorted CD4$^+$ T cells from spleen, pancreatic draining lymph nodes, and islets of NLRP3$^{-/-}$ NOD and WT NOD mice and investigated CCR5, CCR7 and CXCR3 [chemokine (C-X-C motif) receptor 3] expression. Consistent with the alteration in cell migration, chemokine receptor CXCR3 gene expression was up-regulated in CD4$^+$ T cells from NLRP3$^{-/-}$ NOD spleen and pancreatic draining lymph node in comparison with WT CD4 T cells (Fig. 4*A*). We also found enhanced expression of CCR5 in splenic CD4$^+$ and CCR7 in pancreatic draining lymph node CD4$^+$ T cells of NLRP3$^{-/-}$ mice (Fig. S9). However, the expression of CCR5, CCR7, and CXCR3 genes in islet-derived CD4$^+$ T cells was all down-regulated in NLRP3$^{-/-}$ NOD mice compared with WT mice (Fig. 4*B*).

Adoptive transfer experiments indicated that fewer diabetogenic BDC2.5 CD4$^+$ T cells migrated to NLRP3$^{-/-}$ NOD.SCID islets, in comparison with WT NOD.SCID islets (Fig. 3*A*), which suggested that NLRP3 deficiency also affected homing of diabetogenic cells to islets. An array of chemokine genes is expressed in islet cells (28). Thus, we speculated that the impaired migration of CD4$^+$ T cells to NLRP3$^{-/-}$ NOD islets could be due to suppressed chemokine gene expression by NLRP3$^{-/-}$ NOD pancreatic islets. We isolated islets from 3-wk-old WT and knockout NOD mice and performed quantitative PCR (qPCR) (see Table S1 for primer sequences) to analyze the expression of chemokine genes including *CCL3* [chemokine (C-C motif) ligand 3], *CCL5, CCL21, CXCL9* [chemokine (C-X-C motif) ligand 9], and *CXCL10*. As expected, there was a significant down-regulation of all of the chemokine genes tested in NLRP3$^{-/-}$ NOD pancreatic islets compared with WT islets (Fig. 4*C*). Moreover, the NLRP3 inhibitor, parthenolide, also inhibited LPS-induced chemokine gene expression in WT NOD islets (Fig. 4*D*). To further confirm the impaired chemoattractant expression by NLRP3$^{-/-}$ NOD islets at the protein level, we performed in vitro transwell migration experiments.

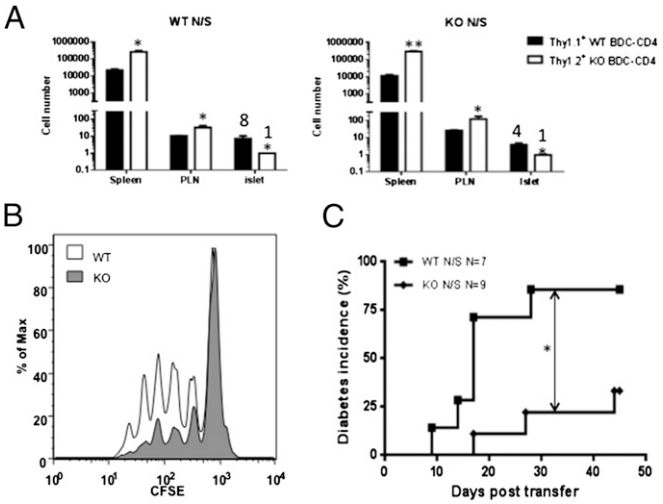

Fig. 3. Experiments to study the role of NLRP3 deficiency in the nonhematopoietic compartment in the development of T1D in NOD mice. (*A*) Purified $CD4^+$ T cells from Thy1.1 WT BDC2.5 or Thy1.2 KO BDC2.5 NOD mice were labeled with CFSE and mixed at 1:1 ratio (10^6:10^6). The cells were i.v. injected into NOD.SCID (WT N/S, $n = 3$) or NLRP3$^{-/-}$ NOD.SCID (KO N/S, $n = 4$) mice. Sixteen hours later, the number of migrated CFSE-labeled $CD4^+$ cells into spleen, PLN, and islets was analyzed by FACS. $*P < 0.05$, $**P < 0.01$, unpaired Student's *t* test. Data are shown from one of the two experiments. (*B*) Purified BDC2.5 $CD4^+$ T cells (3×10^6) were labeled with CFSE and transferred into WT or KO NOD mice. Three days later, the proliferation of BDC2.5 CD4 T cells in PLN of the recipients was determined by FACS. Data are shown from one of the two experiments. (*C*) Purified WT BDC2.5 $CD4^+$ T cells (2×10^6) were injected i.v. into WT N/S ($n = 7$) or KO N/S ($n = 9$) mice. Diabetes development was monitored as described above. Data were pooled from two experiments. $*P < 0.05$, log-rank test.

We cultured isolated WT or NLRP3$^{-/-}$ NOD islets overnight at 37 °C. The islet culture supernatant was used as the source of chemokine(s). Consistent with qPCR results, the supernatants from NLRP3$^{-/-}$ NOD islets had reduced capacity to recruit WT $CD4^+$ T cells and the addition of CCL5 and CXCL10 to the supernatants completely restored the migration of $CD4^+$ T cells (Fig. 4*E*). Moreover, the protein levels of CCL5 and CXCL10 in the supernatants from the islets were also reduced (Fig. S10). However, in comparison with WT islet supernatants, NLRP3$^{-/-}$ NOD islet supernatant showed similar ability to recruit WT macrophages (Fig. 4*F*), and the addition of CCL5 and CXCL10 enhanced macrophage migration (Fig. 4*F*). Our results suggest that the migration of immune cells to islets is regulated by chemokine gradients from islet cells. To understand whether NLRP3 deficiency also affected the expression of chemokine receptor gene(s) on immune cells, we sorted splenic $CD4^+$ T cells and macrophages from WT and NLRP3$^{-/-}$ NOD mice to determine their capability to migrate in a transwell migration experiment. As shown in Fig. 4 *G* and *H*, in the presence of WT islet culture supernatants, both CD4 T^+ cells and macrophages from NLRP3-deficient NOD mice exhibited impaired migration in comparison with WT $CD4^+$ T cells and macrophages. Thus, our data indicate that NLRP3 acts on both immune cells and pancreatic islets through chemokines and chemokine receptors in regulating immune cell migration to islets.

IFN-Regulatory Factor 1 Pathway Is Involved in the Regulation of Chemokine Gene Expression. IFN-regulatory factor 1 (IRF-1) deficiency leads to reduction in antigen-specific autoimmune diseases including collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE) (29). More recently, Harikumar et al. reported that the transcription factor IRF1 is essential for IL-1β–induced production of the chemokines CCL5 and CXCL10 through its formation of a signaling complex with TRAF6, SphK1, and cIAP2 (30). To understand whether this signaling pathway is involved in controlling the production of CCL5 and CXCL10 in pancreatic islets, we isolated islets from NOD mice and analyzed the expression of IRF1, TRAF6, Sph1, and cIAP2 by real-time PCR (Table S1). Interestingly, all of the four genes were detected in WT NOD islet cells (Fig. 5*A*). Moreover, the expression of these genes was up-regulated in NOD islets upon stimulation by LPS (Fig. 5*A*). However, NLRP3 ablation repressed their up-regulation induced by LPS (Fig. 5*A*). Consistent with published data (30), the expression of the chemokine genes *CCL5* and *CXCL10* also correlated with the expression of IRF1 signaling complex genes (Fig. 5*B*), which suggested that the IRF1 signaling pathway is most likely involved in the impaired homing of diabetogenic T cells in to pancreatic islets by regulating chemokine gene expression in islets of NLRP3$^{-/-}$ NOD mice.

To verify the expression of NLRP3 and IL-1β transcripts in pancreatic islet cells, we stained dispersed islet cells with CD45 and sorted $CD45^+$ (hematopoietic) and $CD45^-$ (nonhematopoietic) cells and examined the expression of NLRP3 and IL-1β transcripts in these two cell populations. We found that nonhematopoietic cells in WT pancreatic islets clearly expressed NLRP3 and IL-1β transcripts (Fig. 5*C*). To further confirm the finding, we sorted (*i*) beta cells ($CD45^-$FluoZin-3-AM^+); (*ii*) nonbeta cells and

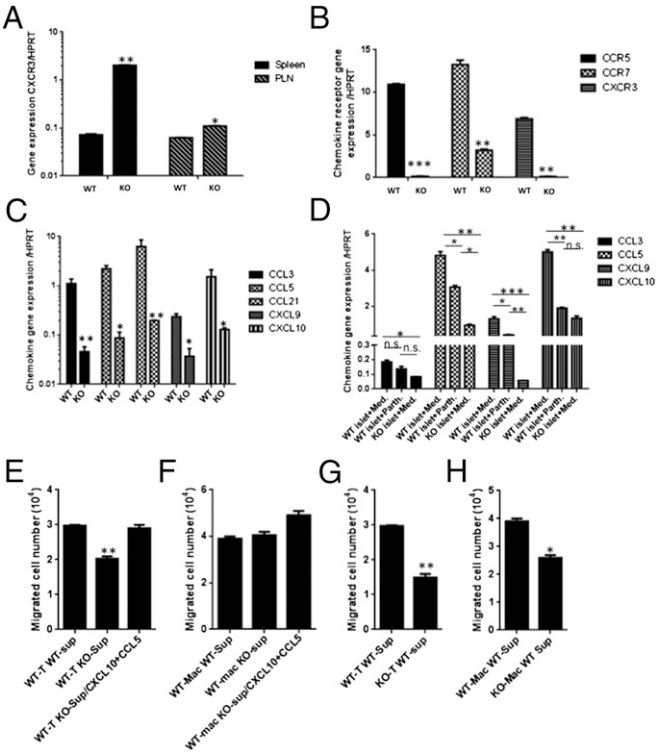

Fig. 4. NLRP3 deficiency impairs the migration of immune cells to islet via inhibition of chemotaxis. (*A*) Chemokine receptor *CXCR3* gene expression in $CD4^+$ T cells isolated from spleen and PLNs of WT or KO NOD mice was detected by qPCR. (*B*) Chemokine receptor *CCR5, CCR7*, and *CXCR3* gene expression in $CD4^+$ T cells isolated from islet of WT and KO NOD mice were detected by qPCR. (*C*) Chemokine *CCL3, CCL5, CCL21, CXCL9*, and *CXCL10* gene expression in islet cells of WT or NLRP3$^{-/-}$ NOD mice were determined by qPCR. (*D*) Islets were isolated from 3-wk-old female WT or KO NOD mice and stimulated with 100 ng/mL LPS in the presence or absence of 5 μM/mL parthenolide (Parth) for 3 h. Chemokine *CCL3, CCL5, CXCL9*, and *CXCL10* gene expression in treated islet cells was detected by qPCR. (*E–H*) Transwell migration assay was performed by using overnight culture medium of WT or KO islets (100 islets per mL) as source of chemokines, which was added into the transwell culture system, for the migration of purified $CD4^+$ T cells or macrophages from WT or KO NOD mice. The migrated cells in the lower chamber were counted under a microscope (*SI Materials and Methods*). Data are shown as mean ± SEM; $*P < 0.05$, $**P < 0.01$, unpaired Student's *t* test. The experiments were repeated twice with similar results.

nonimmune cells ($CD45^-FluoZin\text{-}3\text{-}AM^-$) and (*iii*) immune cells ($CD45^+$, which includes macrophage) from pancreatic islets of wild-type NOD mice and detected IL-1β and NLRP3 expression by qPCR in the three subsets of cells. As shown in Fig. 5*D*, compared with $CD45^+$ immune cells, beta cells produced significant amounts of IL-1β and increased expression of NLRP3, and even nonbeta, nonimmune cells ($CD45^-FluoZin\text{-}3\text{-}AM^-$) expressed IL-1β and NLRP3. Thus, our results suggest that the NLRP3 and IL-1β, expressed in nonhematopoietic cells in islets, are involved in the reduced incidence of diabetes in $NLRP3^{-/-}$ NOD mice. Together with the data from BMC experiments (Fig. S2), our results support the notion that expression NLRP3 gene expression in nonhematopoietic cells plays a more important role in diabetes development in the NOD mouse than NLRP3 gene expression in hematopoietic cells. To verify the expression of chemokine genes in nonhematopoietic cells in pancreatic islets, we further tested the gene expression of *CCL2* [chemokine (C-C motif) ligand 2], *CCL5, CXCL9*, and *CXCL10* in $CD45^-$ islet cells from WT or $NLRP3^{-/-}$ NOD islets. All of the chemokine genes tested could be readily detected in $CD45^-$ islet cells; however, except for *CCL5,* the expression levels were significantly lower in $CD45^-$ islet cells from $NLRP3^{-/-}$ NOD mice (Fig. 5*E*). In line with the chemokine gene expression, not only was the expression of *IRF1, TRAF6, Sph1,* and *cIAP2* genes detected in sorted $CD45^-$ islets cells, but the expression of the genes was also significantly down-regulated in $NLRP3^{-/-}$ NOD $CD45^-$ islets cells compared with their counterparts in WT nonhematopoietic islet cells (Fig. 5*F*).

Discussion

It is known that the NLRP3 inflammasome plays a role in the development of insulin resistance and T2D (12–14, 31). However, the role of the NLRP3 inflammasome in autoimmune T1D remained to be explored. Here, we show that NLRP3 is critical for the development of autoimmune diabetes in the NOD mouse model. The genetic ablation or pharmaceutical inhibition of NLRP3 delayed and reduced the development of T1D in the NOD mouse. Mechanistic studies indicated that the expression of NLRP3 is required in both hematopoietic and nonhematopoietic cells for disease development, although its expression in the nonhematopoietic compartment of pancreatic islet cells exerts a stronger influence than expression in hematopoietic cells. Our results demonstrated that NLRP3 deficiency not only led to suppressed T-cell activation and Th1 cell differentiation, but also impaired the migration of diabetogenic cells into pancreatic islets through down-regulation of chemotaxis-related gene expression on both T cells and nonhematopoietic cells from islets. The down-regulation of chemokine gene expression in nonhematopoietic cells from $NLRP3^{-/-}$ NOD islets is likely to be mediated through the IRF1 signaling pathway.

In addition to the delayed and reduced spontaneous diabetes development in $NLRP3^{-/-}$ NOD mice, in two different adoptive transfer model systems, we found that NLRP3-deficient immature BM cells or mature splenocytes could not transfer diabetes and their wild-type counterparts. This result clearly implies that the expression of NLRP3 in hematopoietic cells plays an important role in the pathogenesis of T1D. Our study shows that NLRP3 deficiency suppresses Th1 responses in $NLRP3^{-/-}$ NOD mice, which is consistent with recent work demonstrating that $NLRP3^{-/-}$ mice exhibited significantly milder EAE through the reduction in IFN-γ–expressing T helper cells (16, 26). Chemokine receptors such as CCR5 and CXCR3 are preferentially expressed by Th1 cells (32, 33). Interestingly, we found that $CD4^+$ T cells in $NLRP3^{-/-}$ NOD islets expressed significantly less CCR5 and/or CXCR3 in comparison with $CD4^+$ T cells in WT islets, suggesting that fewer Th1 cells were recruited to the pancreatic islets. In contrast to the study by Gris et al. in the EAE mouse model (16), we did not detect suppressed Th17 responses in $NLRP3^{-/-}$ NOD mice.

Unlike tissue resident cells such as macrophages and dendritic cells, T cells are rarely found in healthy islets. Thus, the recruitment of diabetogenic effector T cells to islets is a critical step for the islet inflammation and beta cell destruction in T1D. The expression of certain chemokines in the hematopoietic and

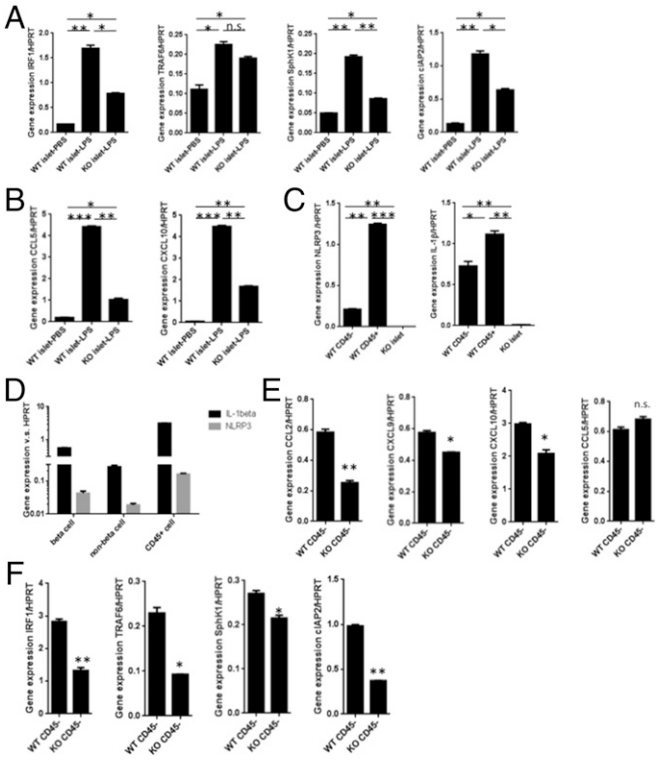

Fig. 5. The IRF-1 pathway is involved in regulation of chemokine gene expression. LPS stimulation induced *IRF1, TRAF6, SphK1*, and *cIAP2* gene expression (*A*) and the chemokine genes *CCL5* and *CXCL10* (*B*). NLRP3 deficiency down-regulated *IRF1, TRAF6, SphK1*, and *cIAP2* expression (*A*), as well as *CCL5* and *CXCL10* (*B*), detected by qPCR. NLRP3 and IL-1β expressed in both purified nonhematopoietic $CD45^-$ islet cells and hematopoietic $CD45^+$ islet-infiltrating immune cells (*C*). IL-1β and NLRP3 expression in sorted beta cells ($CD45^-FluoZin\text{-}3\text{-}AM^+$); nonbeta cells ($CD45^-FluoZin\text{-}3\text{-}AM^-$) and immune cells ($CD45^+FluoZin\text{-}3\text{-}AM^-$) from islets of WT and KO mice (*D*). NLRP3 deficiency also down-regulated *CCL2, CXCL9*, and *CXCL10* but not *CCL5* expression in $CD45^-$ islet cells (*E*). Deficiency of NLRP3 in $CD45^-$ islet cells suppressed *IRF1, TRAF6, SphK1*, and *cIAP2* expression (*F*). Data are shown as mean ± SEM. $n = 3$ mice per group, and the qPCR was performed in triplicate for each experiment. The results shown are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired Student's *t* test.

nonhematopoietic cells in islets is essential for the migration of T cells into pancreatic islets. It has been shown that, in both humans and rodents, pancreatic islet cells express many chemokine genes including *CCL2, CCL3, CCL5*, and *CXCL10*, and among them, *CXCL10* is the most abundant chemokine gene expressed by the pancreatic islet (28). Consistent with a recent study in human islets (28), we also found that CXCL10 was the predominant chemokine expressed by WT NOD islets. However, its expression was significantly down-regulated in $NLRP3^{-/-}$ NOD islets (Fig. 4*C* and Fig. S10). Both CCL5 and CXCL10 are important for the recruitment of Th1 cells, and the expression of CCL5 was also suppressed in NLRP3-deficient NOD pancreatic islets (Fig. 4*C*). CCR5 and CXCR3 are the chemokine receptors for CCL5 and CXCL10, respectively. Interestingly, the expression pattern of Th1-targeting chemokine genes in $NLRP3^{-/-}$ NOD islets correlated with the chemokine receptor gene expression profile of the islet-infiltrating $CD4^+$ T cells. Down-regulated *CCR5* and *CXCR3* expression was observed in $CD4^+$ T cells from NLRP3-deficient NOD islets (Fig. 4*B*). In line with the chemokine gene expression results, there was impaired recruitment of polyclonal $CD4^+$ T cells (Fig. 4*E*) and monoclonal BDC2.5 $CD4^+$ T cells (Fig. 3) into $NLRP3^{-/-}$ NOD islets. Thus, it is likely that the genetic ablation of NLRP3 leads to the down-regulation of chemokine gene expression in pancreatic islets (Fig. 4*A*) and

the reduced insulitis (Fig. S1), thus protecting NOD mice from diabetes development (Fig. 1).

There are two possible explanations for the chemokine gene down-regulation in NLRP3$^{-/-}$ NOD islets: (*i*) suppressed Th1 responses and (*ii*) impaired IL-1β production. It is known that IFN-γ can induce the production of CCL5 and CXCL10 (34, 35). Together with IL-1β and TNF-α, IFN-γ also induces significant amount of CXCL10, CXCL9, and CCL5 in human or mouse islets and the NIT-1 insulinoma cell line (28, 36). However, it is likely that the down-regulation of *CCL5* and *CXCL10* gene expression observed in NLRP3$^{-/-}$ NOD pancreatic islets (Fig. 4 *C* and *D*) may only be partially due to the suppressed Th1 responses, because the level of circulating IFN-γ between WT and NLRP3$^{-/-}$ NOD mice was similar at the time points measured (Fig. S11). Regardless, IL-1β alone can also induce significant amounts of chemokine gene expression in islets (28). IRF-1 is essential for IL-1β–induced secretion of the chemokines CCL5 and CXCL10 through the formation of signaling complexes with TRAF6, cIAP2, and SphK1 (30). Thus, it is likely that islet cell-derived IL-1β (Fig. 5*D*) can up-regulate chemokine gene expression in an IRF-1 signaling pathway-dependent manner. Indeed, we have found *IRF-1, TRAF6, cIAP2*, and *SphK1* expression in islet cells (Fig. 5*A*), and the expression could be up-regulated by LPS in WT islets but suppressed in NLRP3$^{-/-}$ NOD islets. The low expression level of IRF-1 including the secreted IRF-1 (Fig. S10) is correlated with the low expression of the chemokines CCL5 and CXCL10 in NLRP3$^{-/-}$ NOD islets (Fig. 5 *A* and *B*). Importantly, the expression of *IRF1, TRAF6, cIAP2*, and *SphK1* is not only found in CD45^{+} cells in islets, but also in CD45^{-} islet cells (Fig. 5*F*). Interestingly, the expression of *IRF-1, TRAF6, cIAP2*, and *SphK1* was also down-regulated in NLRP3$^{-/-}$ CD45^{-} islet cells (Fig. 5*F*), which correlated with the expression of *CXCL10* gene in CD45^{-} islet cells (Fig. 5*E*).

NLRP3 is an important inflammasome family member that plays a key role in various types of sterile autoinflammation. In addition to IL-1β, NLRP3 also activates IL-18. The role of IL-18 in T1D is controversial; some studies suggest that IL-18 promotes T1D development (37), possibly through inducing diabetogenic T-cell expansion in NOD mice (38), whereas other studies, including our own, indicate that IL-18 is not required for T1D development (18, 19). However, the role of IL-18 in diabetes protection in the NLRP3-deficient NOD mice is not clear.

In summary, our study showed that inhibition of NLRP3 protected from T1D development in NOD mice. The ablation of NLRP3 led to suppressed Th1 responses and impaired T-cell migration to pancreatic islets through the down-regulation of chemokine expression in islets. Investigating the role of the inflammasome in the development of autoimmunity adds a further dimension to our understanding of the multifactorial nature of the immunopathology that leads to the development of T1D but also opens a new area of research for potential therapy.

Materials and Methods

For additional information regarding mice, antibodies and reagents, real-time PCR, BMC, transwell migration assay, adoptive transfer, islet beta cell isolation, insulitis score, and statistical analysis, see *SI Materials and Methods*.

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