Genetic Deficiency of Itgb2 or ItgaL Prevents Autoimmune Diabetes Through Distinctly Different Mechanisms in NOD/LtJ Mice

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OBJECTIVE—Insulitis is an important pathological feature of autoimmune diabetes; however, mechanisms governing the recruitment of diabetogenic T-cells into pancreatic islets are poorly understood. Here, we determined the importance of leukocyte integrins β_2 (Itgb2) and αL (ItgaL) in developing insulitis and frank diabetes.

RESEARCH DESIGN AND METHODS—Gene-targeted mutations of either Itgb2 or ItgaL were established on the NOD/LtJ mouse strain. Experiments were performed to measure insulitis and diabetes development. Studies were also performed measuring mutant T-cell adhesion to islet microvascular endothelial cells under hydrodynamic flow conditions. T-cell adhesion molecule profiles and adoptive transfer studies were also performed.

RESULTS—Genetic deficiency of either Itgb2 or ItgaL completely prevented the development of hyperglycemia and frank diabetes in NOD mice. Loss of Itgb2 or ItgaL prevented insulitis with Itgb2 deficiency conferring complete protection. In vitro hydrodynamic flow adhesion studies also showed that loss of Itgb2 completely abrogated T-cell adhesion. However, ItgaL deficiency did not alter NOD T-cell adhesion to or transmigration across islet endothelial cells. Adoptive transfer of ItgaL-deficient splenocytes into NOD/Rag-1 mice did not result in development of diabetes, suggesting a role for ItgaL in NOD/LtJ T-cell activation.

CONCLUSIONS—Together, these data demonstrate that genetic deficiency of Itgb2 or ItgaL confers protection against autoimmune diabetes through distinctly different mechanisms. ***Diabetes*** **58:1292–1301, 2009**

Autoimmune diabetes results from insulitis, the infiltration of T-cells into the pancreatic islets, leading to significant β-cell death. This instigates dysregulation of blood glucose levels, which has both genetic and environmental causes. The impetus for T-cell accumulation within pancreatic islets has been debated; however, activation of autoreactive $CD4^+$ Th1 (type 1 T-helper) cells (1,2), defective regulatory T-cell activity (3), local production of chemokines (4,5), and increased adhesion molecule expression are prime culprits (6–9). Although many different types of leukocytes are involved in insulitis, i.e. $CD4^+$ and $CD8^+$, T-cells are essential in the pathogenesis of diabetes and islet damage. Moreover, the specific molecules and pathways regulating T-cell recruitment into pancreatic islets remain largely unknown.

Regulation of T-cell recruitment is controlled by a host of cellular events within the microcirculation involving rolling, cellular activation, firm adhesion, and subsequent transmigration across endothelial cell monolayers. Several different adhesion molecules expressed by both T-cells and endothelial cells regulate these interactions with selectin proteins primarily governing rolling and integrin proteins regulating firm adhesion and transmigration (10,11). The multiple overlapping nature of these molecules helps to enable immune responses; however, certain adhesion molecules may play a more dominant role in the process, thereby controlling immune cell recruitment. Two classes of integrins, β_1 (Itgb1) and β_2 (Itgb2), have been identified in regulating T-cell adhesion and homing to various organs and may be important for homing of diabetogenic T-cells (12–15). Importantly, both clinical and animal studies demonstrate that several members of these integrin protein families are upregulated or differentially engaged during autoimmune diabetes (16–19). However, no specific information exists regarding which of the integrin proteins are critically necessary for development of autoimmune diabetes or the mechanism by which this occurs.

The Itgb2 protein family is exclusively expressed on leukocytes and forms heterodimeric molecules consisting of a common Itgb2 chain paired with a specific α chain of either αL (ItgaL), αM (ItgaM), αX (ItgaX), or αD (ItgaD). We previously reported that the Itgb2 chain is important for the development of murine autoimmune lupus and streptozotocin-induced diabetes (12,13). However, the importance of various heterodimer α chains and the molecular disease mechanisms involving Itgb2 proteins during the development of autoimmune diabetes is not known. Therefore, we generated NOD/LtJ mice containing gene-targeted null deletions of Itgb2 or ItgaL to determine whether these molecules play a role in the development of spontaneous autoimmune diabetes. Here, we report that genetic deficiency of either Itgb2 or ItgaL confers protection against the development of frank diabetes and insulitis in NOD mice. However, the protective mechanisms resulting from the loss of these proteins are distinctly different from one another. These findings also demonstrate that leukocyte Itgb2 predominate over other adhesion molecules during the development of diabetes, and

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they identify key molecular targets for possible therapeutic intervention.

RESEARCH DESIGN AND METHODS

Mice used in this study were bred and housed at the Louisiana State University Health Sciences Center Shreveport animal resource facility, an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility, and maintained according to the National Research Council's *Guide for Care and Use of Laboratory Animals*. All experiments reported in this study were approved by our institutional care and use committee. Gene-targeted null mutations of either Itgb2 or ItgaL were backcrossed onto the NOD/LtJ strain for at least nine generations. Map pair microsatellite marker analysis was performed for *Idd* loci analysis in mutant and littermate control mice. All known *Idd* loci were present in all of the different strains of mice. Results from littermate control wild-type alleles for either mutation were similar to NOD/LtJ mice; therefore, controls are referred to as NOD/LtJ throughout the study. Mice were maintained on acidified water, sterile caging and bedding, and sterile chow (diet 7012; Harlan Teklad, Madison, WI). The glucose levels of female wild-type control, Itgb2$^{-/-}$, and ItgaL$^{-/-}$ NOD/LtJ mice were followed starting at 12 weeks of age. Blood glucose measurements were taken every week using Ascensia Elite blood glucose test strips and a Bayer Glucometer Elite until either there were two consecutive hyperglycemic (≥250 mg/dl) measurements or the mice reached 34 weeks of age.

Cell culture. Pancreatic islet microvascular endothelial cells were cultured as we have previously reported (20,21). Cells were grown in high-glucose DMEM obtained from VWR, supplemented with L-glutamine-penicillin-streptomycin (Sigma) and 5% fetal bovine serum (Atlanta Biologicals). Endothelial cells cultured in T-75 flasks were seeded into either 35-mm culture dishes (for use with the parallel plate flow chamber) or Fluoroblock inserts (for the transmigration assay).

Histology. Pancreata were harvested from wild-type control, Itgb2$^{-/-}$ null NOD/LtJ, and ItgaL$^{-/-}$ null NOD/LtJ mice as follows. The pancreas was removed, placed into cassettes, and fixed in 10% buffered neutral formalin. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were scored by a blinded observer on a scale of 0 to 4, with 0 representing no insulitis, 1 peri-insulitis, 2 <25% of islet area being leukocytes, 3 >25% of islet area being leukocytes, and 4 being pseudoatrophic islets without β-cells (22). Insulitis scores were generated by evaluating four serial sections of pancreata per animal. A minimum of 20 islets were scored per animal to obtain an aggregate insulitis score, which was then combined with other aggregate scores to obtain the overall insulitis score per genotype.

T-cell isolation. Spleens from different NOD/LtJ mice were removed and ground between two frosted slides over a petri dish containing buffer (PBS with 2% fetal bovine serum). Splenocytes were then twice-filtered through a 70-μm pore size cell strainer and red cells lysed with 5 ml ACK lysis buffer (0.15 mmol/l ammonium chloride, 1.0 mmol/l potassium bicarbonate, and 0.1 mmol/l sodium EDTA, pH 7.2) per spleen. The resulting splenocyte population was then resuspended in buffer at a concentration of 5×10^7 cells/ml. Specific T-cell populations were isolated from mouse spleens as we have previously reported, using a SpinSep mouse T-cell enrichment kit from Stem Cell Technologies (20).

Flow cytometry. Splenocytes from different NOD/LtJ mice were prepared as described above and diluted to 1×10^7 cells/ml of buffer. Cells were blocked with an equal volume of Fc block (anti-mouse CD16/32) and rocked on ice for 15 min. Cells were then either dual- or triple-stained. Cells for two-color flow cytometry were stained with antibodies against CD3 and one of the following integrins: ItgaL (CD11a), Itgb2 (CD18), Itgb1 (CD29), Itga4 (CD49d), ItgaM (CD11b), ItgaX (CD11c), or lymphocyte Peyer's patch adhesion molecule (LPAM-1; Itga4/Itgb7). Cells for three-color flow cytometry were stained with CD3, CD4, and CD8. Specimens were gated on CD3 staining and subsequently analyzed for CD4/CD8 positivity. Samples were analyzed with a FACS Calibur flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson) by the Research Core Facility at the Louisiana State University Health Sciences Center. Cells were gated using forward versus side scatter, with 10,000 events collected. All cell staining analyses were performed in triplicate.

Parallel plate flow chamber. CD3 T-cells were prepared for the parallel plate flow chamber by resuspending the cells in Hanks' balanced salt solution (HBSS) as we have previously reported (21). Cells were labeled with CellTracker Green at a concentration of 6.25 μg/ml for 45 min on ice. Cells were rinsed twice and resuspended at a concentration of 2×10^5 cells/ml of HBSS and placed in a beaker kept at 37°C and stirred at 60 r/min. A flow chamber insert and gasket (Glycotech) were used with Corning 35-mm cell culture dishes to form a parallel plate flow chamber. Cells were pulled across either unstimulated or tumor necrosis factor-α (TNF-α)-stimulated (10 ng/ml) islet microvascular endothelial monolayers in the cell culture dish at a physiological shear stress of 1.5 dyn/cm^2. A Nikon Eclipse TE-2000 epifluorescence microscope and Hamamatsu Digital Camera were used to acquire real-time digital video of the cells as they were flowed over islet endothelium. We used Simple PCI software (Compix) to analyze the video and extract rolling velocities of the T-cells. Cells were considered to be firmly adherent if they remained stationary for 10 s within the field of view.

T-cell transmigration assays. Pancreatic islet endothelial cells were grown to confluence on Falcon Fluoroblok tissue culture inserts (8-μm pore size; BD, Franklin Lakes, NJ). T-cells were harvested and labeled as described above. Labeled CD3$^+$ T-cells at 5×10^5 cells per insert in 750 μl HBSS were added to the luminal compartment of inserts in triplicate, and regulated upon activation, normal T-cell expressed and secreted (RANTES; 100 ng/ml) or control HBSS was placed into the abluminal compartment. In some experiments, TNF-α stimulation (10 ng/ml) was used to activate endothelial monolayers before performing transmigration studies. Plates were incubated at 37°C in a 5% CO_2 atmosphere, and fluorescence measurements were taken at 0, 15, 30, 60, and 120 min using a Tecan GENios Plus plate reader. Wells with 0 and 50,000 cells in the abluminal compartment served to provide control settings for the software.

Diabetes adoptive transfer. Whole splenocytes were isolated from diabetic NOD/LtJ wild-type or NOD/LtJ ItgaL null mice and injected retro-orbitally (2×10^7 per mouse) into 8-week-old NOD Rag-1 mutant mice. Blood glucose was measured weekly until either two consecutive glucose measurements in excess of 250 mg/dl occurred or until 9 weeks posttransfer, whichever came first. At that point, pancreata were removed, formalin-fixed, and processed from histological analysis of insulitis. Splenocytes were also obtained; triple-stained for CD3, CD4, and CD8; and analyzed by flow cytometry.

Statistical analysis. Changes in blood glucose, diabetes incidence, and insulitis were compared by one-way ANOVA with Bonferroni's post-test versus NOD/LtJ controls. Comparison of T-cell biophysical interactions between unstimulated versus TNF-α–stimulated islet endothelial cells was performed with an unpaired Student's *t* test. Changes in T-cell transmigration across islet endothelial cells was compared against transmigration rates across unstimulated control islet endothelial monolayers using one-way ANOVA with Bonferroni's post-test versus control at each specified time point. A P value of <0.05 was required for significance among all analyses performed with experimental n values reported in the figure legends.

RESULTS

Lack of Itgb2 or ItgaL is protective against diabetes and insulitis. Blood glucose data obtained from NOD/LtJ wild-type, Itgb2$^{-/-}$ null NOD/LtJ, and ItgaL$^{-/-}$ null NOD/LtJ mice demonstrate that loss of either Itgb2 or ItgaL offers protection from diabetes. Figure 1*A* reports that Itgb2 and ItgaL knockout mice maintained normal blood glucose levels throughout the study, whereas wild-type NOD/LtJ mice showed a marked increase between weeks 14 and 16, plateauing at 400 mg/dl by week 24. Only 20% of NOD/LtJ wild-type mice remained normoglycemic by week 30, whereas ItgaL$^{-/-}$ and Itgb2$^{-/-}$ mice remained normoglycemic for the entire study period (Fig. 1*B*). These results clearly reveal an important pathophysiological role for Itgb2 and ItgaL during the onset of diabetes.

Insulitis histopathology of pancreatic islets from age-matched 18-week-old wild-type, Itgb2$^{-/-}$, and ItgaL$^{-/-}$ NOD/LtJ mice are shown in Fig. 1*C–E*. Wild-type mice show a large number of cell infiltrates (Fig. 1*C*), with Itgb2$^{-/-}$ mice showing no signs of insulitis (Fig. 1*D*). ItgaL$^{-/-}$ mouse pancreatic islets also appear essentially normal; however, some lymphocyte infiltrates can be observed (Fig. 1*E*). Histopathology sections were scored for the degree of insulitis (Fig. 1*F*), with wild-type NOD/LtJ mice showing a significantly higher score than either the Itgb2$^{-/-}$ or ItgaL$^{-/-}$ mice. Together, these data demonstrate that Itgb2 or ItgaL expression is necessary for the development of frank diabetes in the NOD mouse model.

Adhesion molecule expression of NOD/LtJ CD3 T-cells. Figure 2 reports the percent of CD3 T-cells that express various surface adhesion molecules between wild-type, Itgb2 null, and ItgaL null NOD/LtJ mice. Adhesion molecule expression analysis was performed using pre-

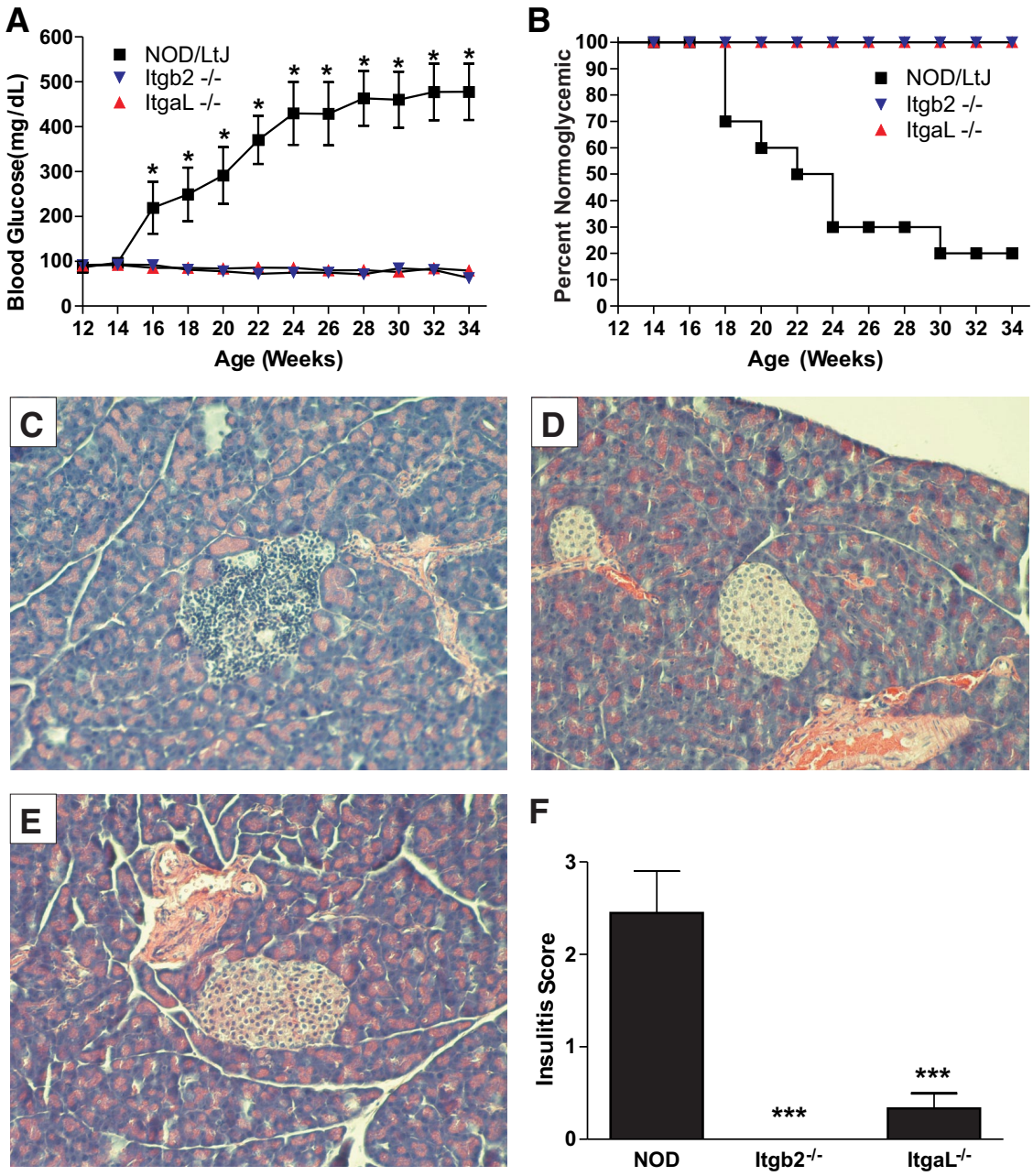

FIG. 1. Genetic deficiency of Itgb2 or ItgaL confers protection against autoimmune diabetes in NOD/LtJ mice. *A*: Blood glucose measurements from wild-type, Itgb2$^{-/-}$ null, and ItgaL$^{-/-}$ null NOD/LtJ mice over time. *B*: The percent of mice remaining normoglycemic over time between wild-type, Itgb2$^{-/-}$ null, and ItgaL$^{-/-}$ null NOD/LtJ mice. *C–E*: Representative hematoxylin and eosin stains of islet histopathology from wild-type, Itgb2$^{-/-}$ null, and ItgaL$^{-/-}$ null NOD/LtJ mice, respectively. *F*: The insulitis score between the different genotypes of NOD/LtJ mice. *$P < 0.01$ vs. mutant NOD/LtJ strains, $n = 20$ mice per genotype. ***$P < 0.001$, $n = 15$ mice per genotype. (A high-quality digital representation of this figure is available in the online issue.)

diabetic wild-type mice at 12 weeks of age and diabetic wild-type and mutant mice at 18 weeks of age. As expected, genetic deficiency of Itgb2 eliminates CD18 and CD11a surface expression (Fig. 2*A* and *B*). Interestingly, genetic deficiency of ItgaL does not completely abolish CD18 surface expression, indicating the presence of other β_2 integrins on the T-cell surface (Fig. 2*B*). This is an important observation because previous studies have assumed that the only relevant Itgb2 expressed on the T-cell surface is CD18/CD11a and that genetic deficiency of CD18 serves as a surrogate for knockout of CD11a (23,24). Our data demonstrate that this is clearly not the case and that genetic disruption of ItgaL still results in a Itgb2 phenotype in NOD/LtJ mice, highlighting that these molecules are not interchangeable. Deficiency of ItgaL or Itgb2 did not significantly alter CD49d positivity (Fig. 2*C*); however, deficiency of Itgb2 did significantly increase CD29 (β_1 integrin) positivity (Fig. 2*D*). Interestingly, genetic deficiency of Itgb2 significantly decreased the number of CD3 T-cells positive for CD62L (Fig. 2*E*), suggesting differential regulation of adhesion molecule expression between ItgaL and Itgb2.

Figure 3 reports the mean fluorescence intensity of adhesion molecule expression on CD3 T-cells from wild-type and mutant NOD/LtJ mice. Figure 3*A* shows that genetic deficiency of either ItgaL or Itgb2 significantly

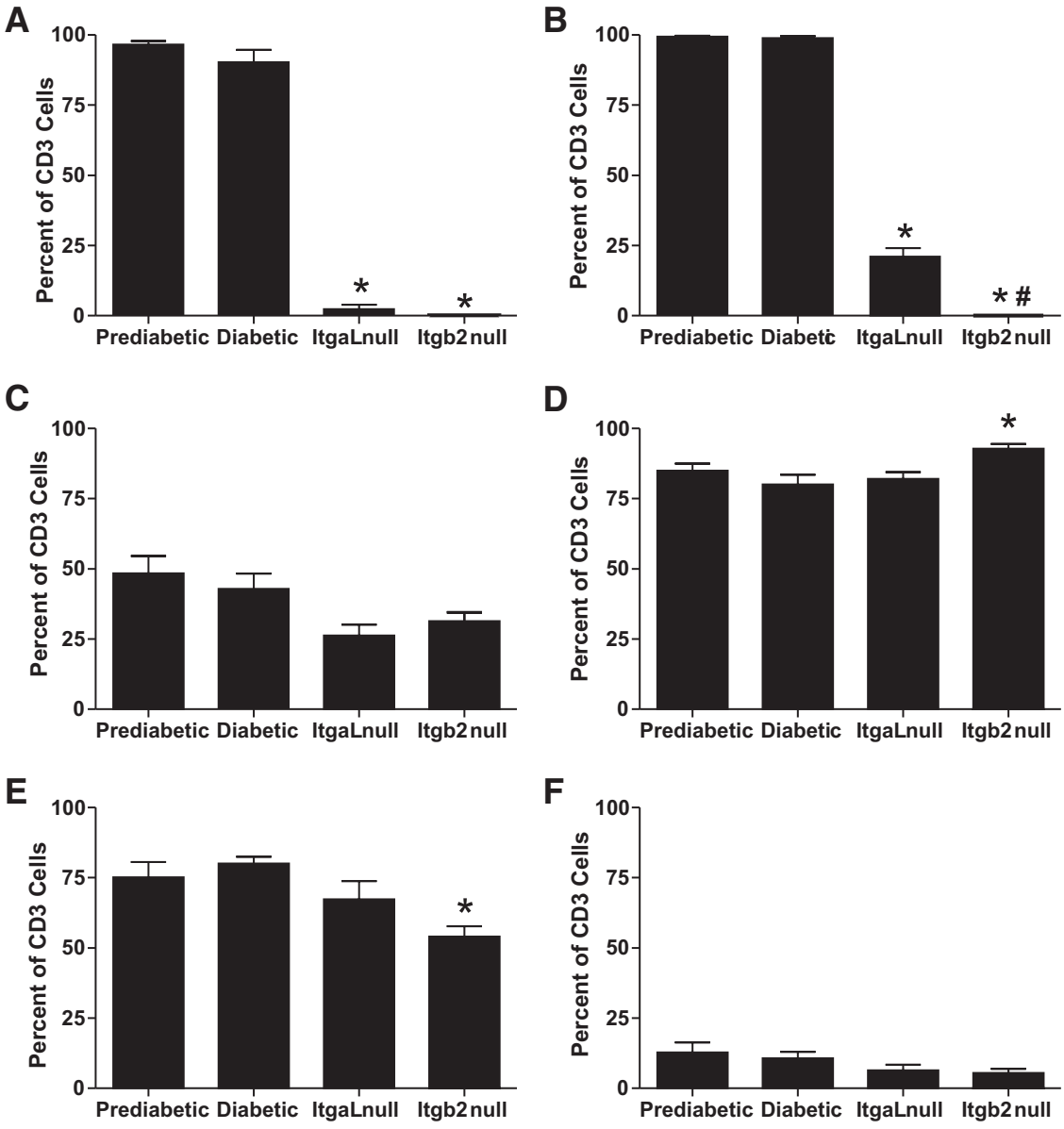

FIG. 2. Genetic deficiency of Itgb2 or ItgaL alters the phenotype of NOD/LtJ CD3 T-cells. Whole splenocytes were isolated from wild-type pre-diabetic (12 weeks old), wild-type diabetic (18 weeks old), Itgb2$^{-/-}$ null (18 weeks old), and ItgaL$^{-/-}$ null (18 weeks old) NOD/LtJ mice and stained for CD3 and individual adhesion molecules to determine the percentage of cells expressing various molecules. *A*: The percent of CD3 T-cells that are positive for CD11a expression. *B*: The percent of CD3 T-cells that are positive for CD18 expression. *C*: The percent of CD3 T-cells that are positive for CD49d expression. *D*: The percent of CD3 T-cells that are positive for CD29 expression. *E*: The percent of CD3 T-cells that are positive for CD62L expression. *F*: The percent of CD3 T-cells that are positive for LPAM-1 expression. *$P < 0.01$ vs. wild-type diabetic mice; #$P < 0.01$ Itgb2 null vs. ItgaL null mice, $n = 6–7$ animals per genotype.

decreases CD11a surface expression. Similarly, Fig. 3*B* shows that genetic deficiency of either molecule also significantly decreases CD18 surface expression. However, robust CD18 surface expression is observed on ItgaL CD3 T-cells compared with absent expression on Itgb2 CD3 cells. Again, these data indicate that other β_2 integrins may be expressed in lieu of ItgaL deficiency. Interestingly, Itgb2 deficiency enhances CD29 surface expression (Fig. 3*D*). CD3 T-cell surface expression of CD62L is similarly decreased in either ItgaL or Itgb2 null mice (Fig. 3*E*). Lastly, surface expression of LPAM-1 was unchanged among wild-type and mutant NOD/LtJ CD3 T-cells (Fig. 3*F*).

Itgb2 integrin deficiency minimally alters NOD/LtJ T-cell development. Previous studies have shown that genetic deficiency of leukocyte integrins can alter T-cell development (14,25). Therefore, flow cytometry was performed on ItgaL$^{-/-}$ NOD/LtJ, Itgb2$^{-/-}$ NOD/LtJ, and diabetic NOD/LtJ wild-type CD3 T-cells at 18 weeks of age to determine the effects of these mutations on different T-cell phenotype populations (Table 1). Wild-type diabetic NOD/LtJ T-cells show 57% of the T-cells expressing CD4, 36.7% expressing CD8, and a small double-negative population (5.3%). Data from ItgaL$^{-/-}$ NOD/LtJ T-cells show essentially the same result. Interestingly, CD3 T-cells from Itgb2$^{-/-}$ NOD/LtJ mice have a statistically greater number of double-negative T-cells (21.3%) compared with wild-type NOD/LtJ mice. Together, these data suggest that genetic deficiency of Itgb2 has a slight impact on NOD/LtJ double-negative T-cell development, which is not observed with ItgaL genetic deficiency.

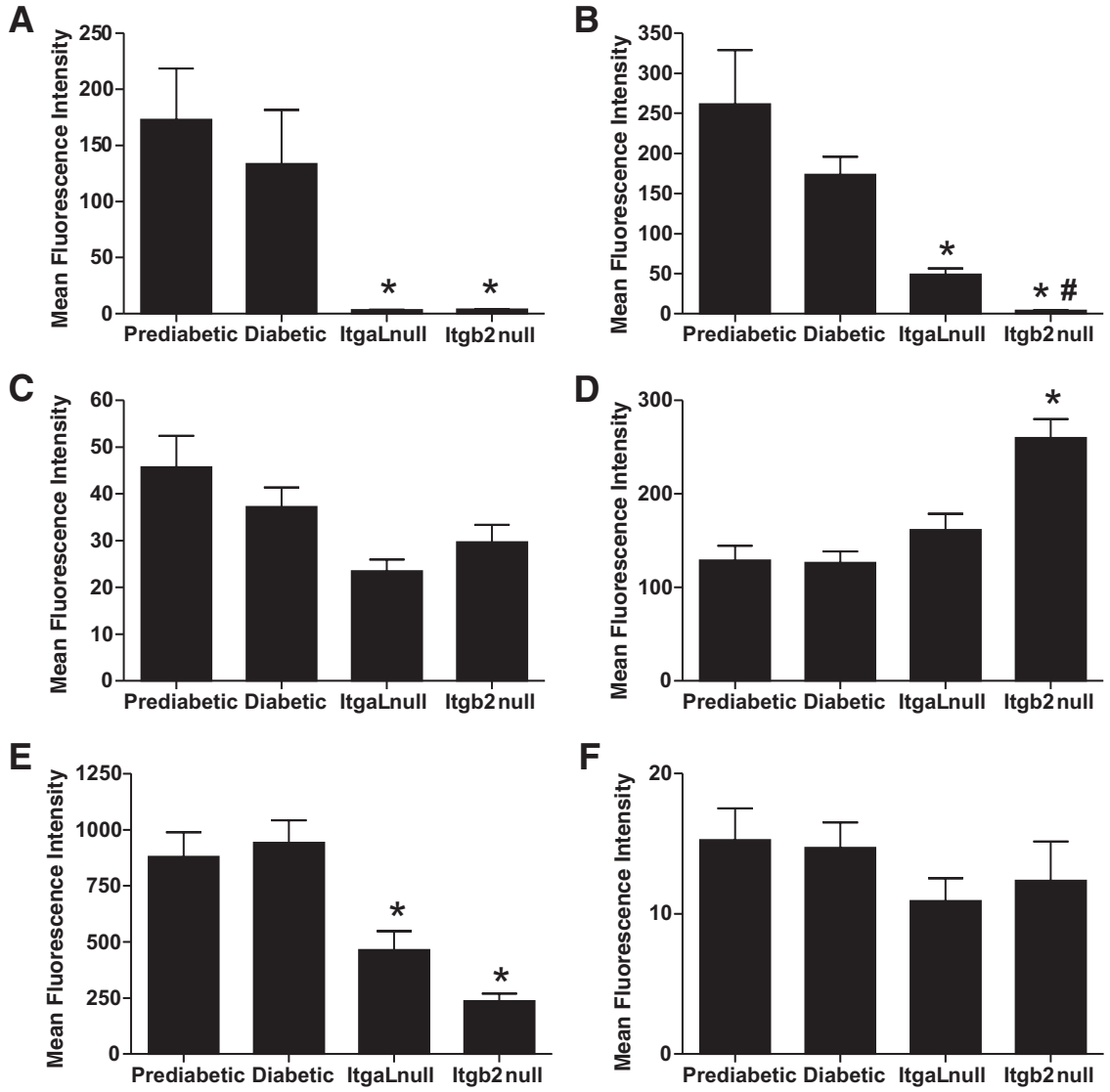

FIG. 3. Loss of Itgb2 or ItgaL expression alters NOD/LtJ T-cell adhesion molecule expression. Whole splenocytes were isolated from wild-type pre-diabetic (12 weeks old), wild-type diabetic (18 weeks old), Itgb2$^{-/-}$ null (18 weeks old), and ItgaL$^{-/-}$ null (18 weeks old) NOD/LtJ mice and stained for CD3 and individual adhesion molecules to determine the amount of surface adhesion molecule expression. *A*: The mean fluorescence intensity for CD11a expression. *B*: The mean fluorescence intensity for CD18 expression. *C*: The mean fluorescence intensity for CD49d expression. *D*: The mean fluorescence intensity for CD29 expression. *E*: The mean fluorescence intensity for CD62L expression. *F*: The mean fluorescence intensity for LPAM-1 expression. *$P < 0.01$ vs. wild-type diabetic mice; #$P < 0.01$ Itgb2 null vs. ItgaL null mice, $n = 6$–7 animals per genotype.

Itgb2 but not ItgaL is necessary for T-cell recruitment. Insulitis data from Itgb2 null and ItgaL null mice suggest that these proteins may diminish islet T-cell infiltration, possibly because of defects in cell capture, rolling, or firm adhesion. To investigate this possibility, T-cells were isolated from NOD/LtJ wild-type, ItgaL$^{-/-}$, and Itgb2$^{-/-}$ mice and adhesion dynamics examined using a parallel plate flow chamber model to emulate physiological leukocyte-endothelial cell interactions. T-cells from the different NOD/LtJ mice were flowed over either control- or TNF-α (10 ng/ml)–stimulated pancreatic islet endothelium. TNF-α stimulation upregulates the expression of P-selectin and E-selectin on islet microvascular endothelial cells, which are involved in the cell rolling process, as we have previously reported (20). TNF-α stimulation of islet endothelial cells resulted in a significant reduction in the average rolling velocity of NOD/LtJ wild-type, ItgaL$^{-/-}$, and Itgb2$^{-/-}$ T-cells compared with unstimulated islet endothelial cells (Fig. 4*A*). Importantly, there was no significant difference between the average rolling velocities between any of the cell groups with or without TNF-α stimulation. This demonstrates that genetic deficiency of

TABLE 1
Genetic deficiency of Itgb2 minimally alters NOD/LtJ T-cell distribution

Genotype	CD4	CD8	Double-negative	Double-positive
Wild type	57.0 ± 1.8	36.7 ± 2.6	5.3 ± 0.9	0.56 ± 0.07
ItgaL null	60.4 ± 3.1	26.4 ± 0.8	12.6 ± 2.6	0.46 ± 0.1
Itgb2 null	50.4 ± 3.8	27.6 ± 3.2	21.3 ± 2.4*	0.31 ± 0.03

Data are reported as percent distributions ±SD. Whole splenocytes were isolated from wild-type diabetic, ItgaL null, or Itgb2 null NOD/LtJ mice and triple-stained for CD3, CD4, and CD8 to evaluate T-cell population distributions. *$P < 0.001$ versus wild-type diabetic NOD/LtJ mice, $n = 5$ per genotype.

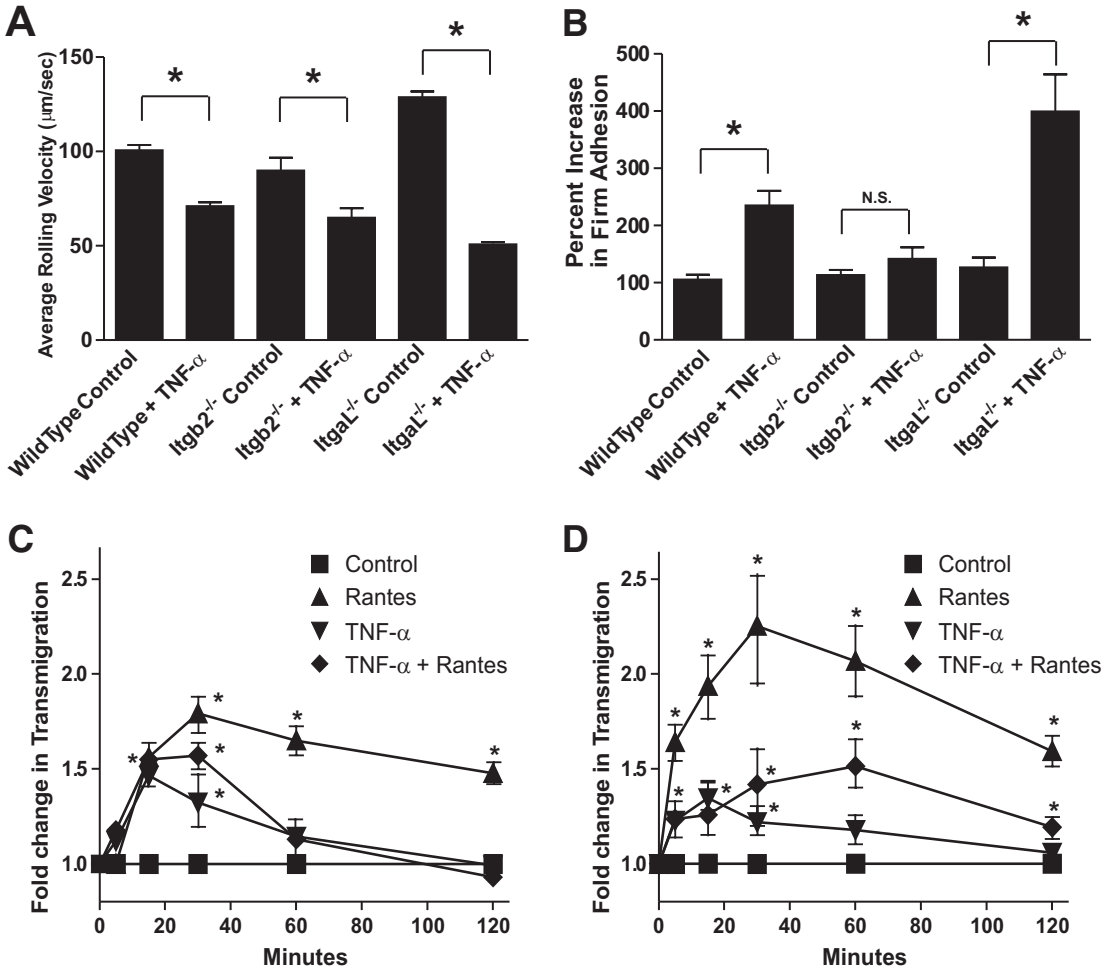

FIG. 4. Genetic deficiency of Itgb2 but not ItgaL blunts NOD/LtJ T-cell firm adhesion under hydrodynamic flow conditions. CD3 T-cells were isolated from either diabetic wild-type, Itgb2$^{-/-}$ null, or ItgaL$^{-/-}$ null NOD/LtJ mice and perfused over either unstimulated or TNF-α–stimulated (10 ng/ml) islet microvascular endothelial cells at physiological shear stress. *A*: The average rolling velocity of the different T-cells on unstimulated or TNF-α–stimulated islet endothelial cells. *B*: The percent increase in T-cell firm adhesion to either unstimulated or TNF-α–stimulated islet endothelial cells. CD3 T-cells from diabetic wild-type and ItgaL null mice were evaluated for their ability to transmigrate across either unstimulated or TNF-α–stimulated islet endothelial cells toward a chemotactic gradient of RANTES (100 ng/ml). *C*: Wild-type diabetic NOD/LtJ T-cell transmigration. *D*: ItgaL$^{-/-}$ null NOD/LtJ T-cell transmigration. *$P < 0.01$, $n = 8$.

ItgaL or Itgb2 does not alter the ability of the T-cells to tether and roll on islet endothelium.

We have previously reported that treatment of islet microvascular endothelium with TNF-α also increases the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on the islet endothelial surface, which is crucial for firm adhesion (20). Figure 4*B* shows that NOD/LtJ CD3 T-cell adhesion is significantly increased as expected. In contrast, Itgb2$^{-/-}$ T-cells showed no statistically significant increase in the percentage of firmly adherent cells on stimulated versus unstimulated endothelium. These data suggest that the protection offered by genetic deficiency of Itgb2 is a result of the inability of the T-cells to firmly adhere to the endothelium. Surprisingly, T-cells from ItgaL$^{-/-}$ NOD/LtJ mice still showed an increase in T-cell firm adhesion on TNF-α–stimulated endothelium along with a slight but insignificant increase in firm adhesion over that of wild-type NOD/LtJ T-cells. These results demonstrate that ItgaL expression is not essential for T-cell firm adhesion in the NOD/LtJ mouse, suggesting that this molecule alters the progression of diabetes through other mechanisms.

Having observed that ItgaL was not necessary for NOD/LtJ T-cell adhesion, we next evaluated whether ItgaL null T-cells do not infiltrate the pancreatic islets because of a defect in their ability to transmigrate (26). NOD/LtJ wild-type CD3 T-cells transmigrated across islet microvascular endothelium toward the chemoattractant RANTES (100 ng/ml) at a much higher rate than toward HBSS control treatment (Fig. 4*C*). When islet endothelial cells were activated with TNF-α, with or without RANTES chemoattractant, T-cell transmigration was also increased, but to a lesser degree. The same effect was seen when CD3 T-cells from ItgaL$^{-/-}$ NOD/LtJ mice were used, with a greater increase in transmigration of T-cells in response to RANTES (Fig. 4*D*). These data demonstrate that genetic deficiency of ItgaL does not inhibit the ability of NOD/LtJ T-cells to transmigrate across pancreatic islet endothelial cell monolayers.

ItgaL deficiency increases NOD/LtJ T-cell CD11b expression and function. Flow cytometry analysis of ItgaL null T-cells revealed the unexpected finding of residual CD18 (Itgb2) expression on the surface coupled with the observation that ItgaL null T-cells are still capable of firm adhesion and transmigration. Therefore, experi-

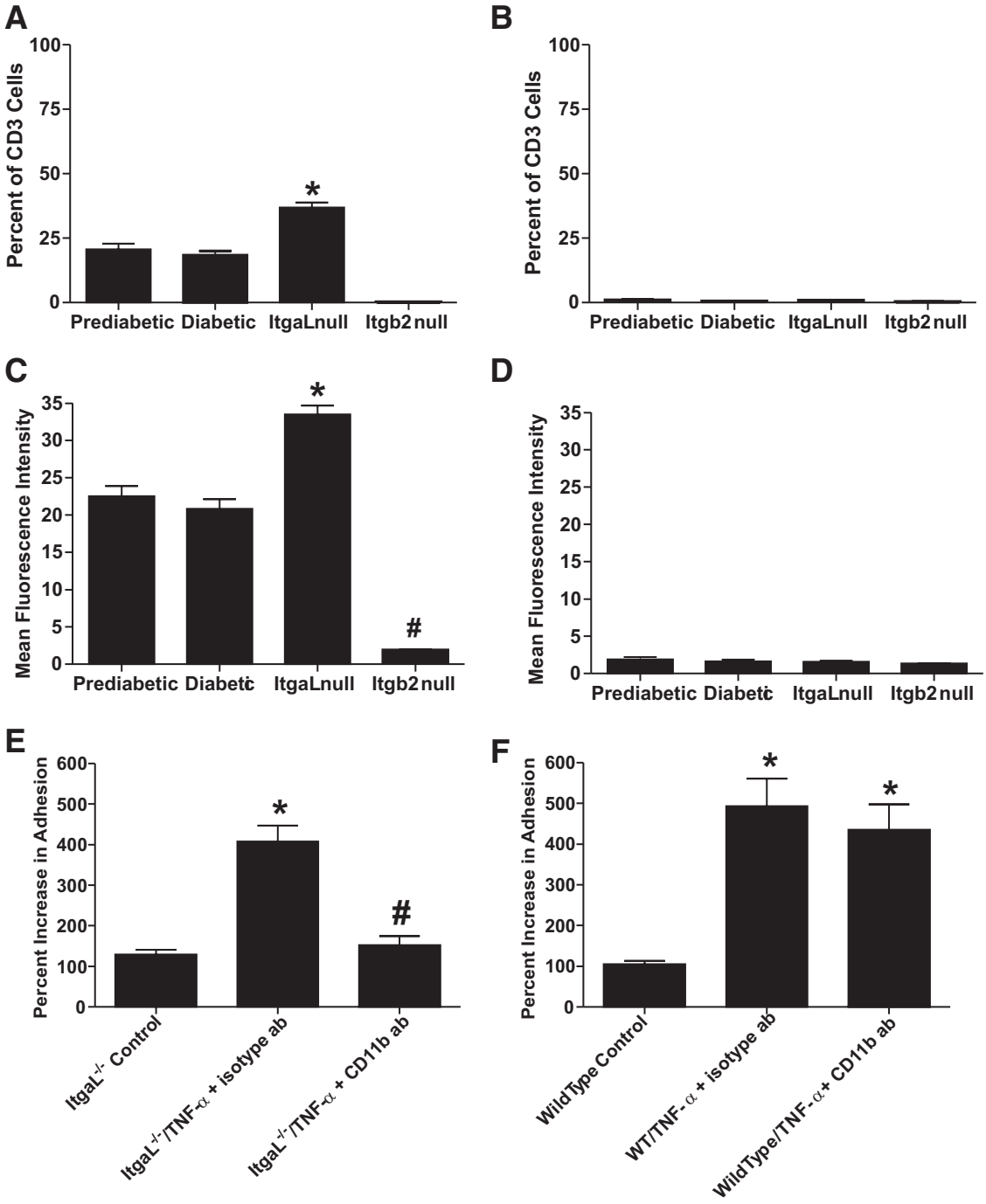

FIG. 5. Genetic deficiency of ItgaL enhances CD11b expression and regulates T-cell adhesion. Whole splenocytes were isolated from wild-type pre-diabetic (12 weeks old), wild-type diabetic (18 weeks old), Itgb2$^{-/-}$ null (18 weeks old), and ItgaL$^{-/-}$ null (18 weeks old) NOD/LtJ mice and stained for CD3 and either CD11b or CD11c to determine what other β_2 integrins could be expressed in ItgaL null cells. *A* and *B*: The percent of CD3 T-cells that were positive for CD11b and CD11c, respectively. *C* and *D*: The mean fluorescence intensity for CD11b and CD11c expression, respectively. *P < 0.01 vs. wild-type diabetic CD3 T-cells, #P < 0.01 vs. wild-type diabetic CD3 T-cells. *E*: The effect of anti-CD11b blockade on ItgaL null NOD/LtJ T-cell adhesion to TNF-α–stimulated islet endothelial monolayers under hydrodynamic flow conditions. *F*: The effect of anti-CD11b blockade on wild-type diabetic NOD/LtJ T-cell adhesion to TNF-α–stimulated islet endothelial monolayers under hydrodynamic flow conditions. *P < 0.01 vs. control; #P < 0.01 vs. isotype antibody control, n = 6 per treatment group. ab, antibody.

ments were performed to identify the nature and function of these residual integrins. Figure 5*A* shows that a significantly greater percent of ItgaL null CD3 T-cells are positive for CD11b expression, whereas there was no difference in CD11c positivity among the different strains of mice (Fig. 5*B*). Consistent with this finding, Fig. 5*C* illustrates that the mean fluorescence intensity of CD11b expression was significantly enhanced on ItgaL null T-cells compared with CD11c (Fig. 5*D*). Interestingly, blockade of CD11b function with a neutralizing antibody completely prevented ItgaL null T-cell adhesion to TNF-α–activated islet microvascular endothelial cell monolayers under flow conditions (Fig. 5*E*). Conversely, anti-CD11b antibody treatment did not alter diabetic wild-type T-cell adhesion to TNF-α–activated islet endothelium (Fig. 5*F*). These data indicate that genetic

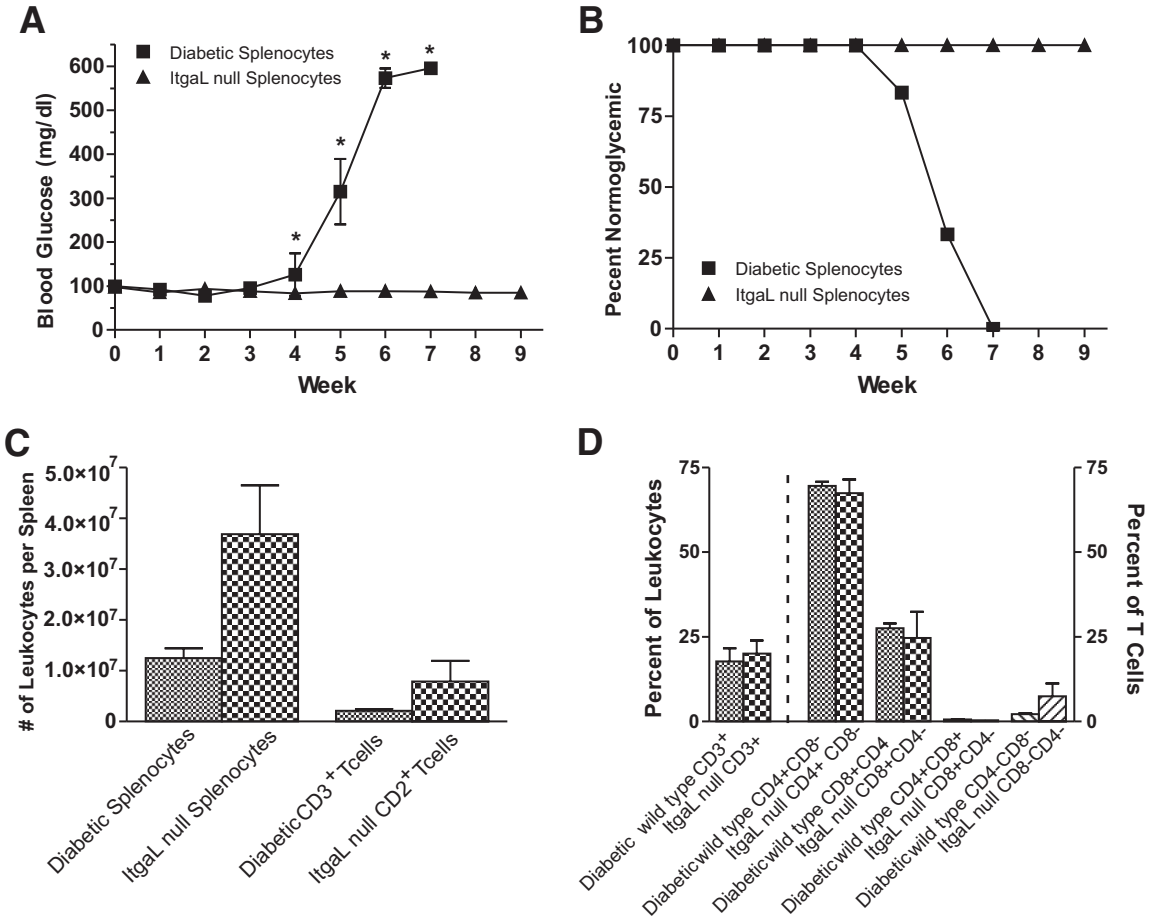

FIG. 6. Adoptive transfer of ItgaL splenocytes does not elicit autoimmune diabetes. Splenocytes from wild-type diabetic or ItgaL$^{-/-}$ null NOD/LtJ mice were adoptively transferred into Rag-1 null NOD/LtJ mice. *A*: Changes in blood glucose on adoptive transfer between diabetic splenocytes and ItgaL$^{-/-}$ null splenocytes. *$P < 0.01$, $n = 8$. *B*: The percent normoglycemia during adoptive transfer between wild-type diabetic or ItgaL$^{-/-}$ null splenocytes ($n = 8$). *C*: The number of leukocytes and CD3 T-cells found within the spleens of adoptively transferred mice between diabetic and ItgaL$^{-/-}$ null splenocytes ($n = 4$). *D*: T-cell phenotypic distribution between Rag-1 null NOD/LtJ mice reconstituted with either diabetic or ItgaL$^{-/-}$ null splenocytes ($n = 4$).

deficiency of ItgaL leads to a preferential increase of functional CD11b on NOD/LtJ T-cells.

Adoptive transfer of ItgaL splenocytes does not elicit autoimmune diabetes. Data from ItgaL$^{-/-}$ NOD/LtJ mice suggest that the primary reason for protection against the development of autoimmune diabetes is likely attributable to defects in immune cell activation rather than deficiencies in T-cell recruitment across islet microvascular endothelial cells. Moreover, ItgaL is also expressed on antigen-presenting cells and is involved in immune synapse formation (27), leaving the possibility that protection in the ItgaL mice could involve other immune cell responses independent of T-cell recruitment. Therefore, we performed adoptive transfer experiments using ItgaL$^{-/-}$ splenocytes into NOD/LtJ Rag-1$^{-/-}$ mice to directly address whether T-cell behavior is altered. NOD/LtJ Rag-1$^{-/-}$ mice injected with 2×10^7 splenocytes from diabetic NOD/LtJ mice developed hyperglycemia in 5–7 weeks, whereas those receiving cells from ItgaL-deficient NOD/LtJ mice were normoglycemic and did not develop diabetes, as shown in Fig. 6*A* and *B*. Insulitis scores confirm that splenocytes from diabetic NOD/LtJ mice transferred into NOD Rag-1$^{-/-}$ mice promoted insulitis quickly, resulting in islets that were completely obliterated (insulitis score of 4) in <2 weeks after onset of hyperglycemia. However, no insulitis was observed in any ItgaL$^{-/-}$ transfer experiments (data not shown). A greater number of leukocytes and CD3$^+$ T-cells were found in NOD Rag-1$^{-/-}$ mice spleens injected with cells from ItgaL$^{-/-}$ NOD/LtJ mice than from diabetic wild-type NOD/LtJ mice (Fig. 6*C*), indicating that T-cells from ItgaL$^{-/-}$ mice are retained in the spleen. Lastly, Fig. 6*D* shows that ItgaL deficiency does not alter the percent distribution of leukocytes that were CD3$^+$ or the ratio of CD4$^+$ to CD8$^+$ T-cells, confirming that loss of ItgaL does not differentially alter different lymphocyte populations.

DISCUSSION

Regulation of leukocyte recruitment into specific tissue niches is a critical innate immune response event. However, it has become increasing more apparent that immune cell recruitment is important for acquired immune responses involved in autoimmunity (12,13,28–33). Several different adhesion molecules facilitate the process of leukocyte recruitment, and many of these have been implicated in the development of autoimmune diabetes (9,34,35). However, identification of key proteins and their mechanisms of action still remain elusive. In this study, we determined the importance of leukocyte integrins Itgb2 and ItgaL for the development of autoimmune diabetes using gene-targeted null mutations of either gene.

Mice genetically deficient in either of the two integrin subunits were protected from diabetes, as evidenced by both blood glucose measurements and histopathology. These findings were striking for two reasons. First, members of the β_1 integrin family have long been suspected to play an important role in modulating autoimmune cell infiltration into tissues (36–39). That genetic deficiencies of either Itgb2 or ItgaL in NOD/LtJ mice prevented disease suggests that Itgb2 play a dominant role in autoimmune diabetes pathogenesis because expression of Itgb1 was still observed and actually enhanced in Itgb2 null NOD/LtJ mice, yet these mice were protected against autoimmune diabetes. Second, these results suggest that Itgb2 heterodimeric association with ItgaL is likely the pathophysiological heterodimer of the Itgb2 family necessary for autoimmune diabetes. This is due to the fact that genetic deficiency of Itgb2 results in loss of all four Itgb2 integrin proteins (ItgaL, ItgaM, ItgaX, and ItgaD) from the cell surface because intracellular chain pairing is necessary for surface localization and that other Itgb2 heterodimers (ItgaM/Itgb2) are enhanced in the ItgaL null mutation (13). Together, these data demonstrate that functional disruption of these molecules is a highly effective means by which to halt the development of autoimmune diabetes.

Another important result of this study was the manner in which gene-deficient mutations of either Itgb2 or ItgaL conferred protection against the development of diabetes. Genetic deficiency of Itgb2 inhibited autoimmune diabetes because of defective T-cell recruitment and adhesion to islet microvascular endothelial cells. However, deficiency of ItgaL likely limits T-cell activation with lesser effects on T-cell adhesion or transmigration apparently because of increased surface localization of CD11b. This finding could be caused by increased CD11b gene expression; however, additional studies are needed to better understand this surprising observation. Moreover, these results are an excellent example of differential integrin functions during disease. Previous studies investigating the role of adhesion molecules (e.g., lymphocyte function–associated antigen-1/intercellular adhesion molecule-1) have all used immunoblockade approaches with combined antibody therapies (40–43). Although this approach may be useful, it has not provided a clear understanding of how and to what extent each integrin chain contributes to disease pathogenesis. Moreover, immunoneutralization approaches against adhesion molecules have been reported to involve off-target effects and responses (44,45). Thus, our work using gene-targeted deficiency of specific leukocyte integrin chains provides a precise understanding of the importance of these molecules during disease and the molecular mechanisms by which they act.

Adoptive transfer experiments using ItgaL null splenocytes into Rag-1–deficient NOD/LtJ mice suggest that this integrin may also be involved in immune cell homing to lymphoid tissue necessary for antigen-dependent activation. It has been reported that ItgaL-deficient T-cells exhibit defective homing to peripheral and mucosal tissue lymph nodes, whereas homing to the spleen is less altered (46). Our data are suggestive of a defective homing response with ItgaL null splenocytes, as seen by a threefold increase in total leukocytes and a twofold increase in CD3 T-cells in spleens of adoptive transfer Rag-1 null NOD/LtJ mice. Importantly, deficiency of ItgaL does not alter the distribution of different T-cell populations (CD4 vs. CD8) during adoptive transfer, reinforcing the notion that loss of this molecule does not adversely affect cell proliferation and survival in this model. This finding coupled with the fact that ItgaL deficiency still results in some (albeit minimal) insulitis along with intact adhesion responses strongly suggests that diminished T-cell activation plays a large role in the protection against autoimmune diabetes. However, future experiments are necessary to precisely determine how loss of ItgaL specifically alters NOD/LtJ T-cell activation and homing.

In summary, our work demonstrates that Itgb2 or ItgaL serve as dominant adhesion molecule regulators of autoimmunity, highlighting the importance of innate immune responses, which clearly influence disease progression. Moreover, these results suggest that therapeutic intervention aimed at these molecules could be clinically useful for autoimmune diabetes. Indeed, eflaizumab (anti-ItgaL antibody) is a well-established and -tolerated therapy for autoimmune psoriasis that is currently in clinical trials for islet transplantation in type 1 diabetic patients; however, eflaizumab therapy could also be useful in patients at high risk of type 1 diabetes or those recently diagnosed with type 1 diabetes.

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