Cellular Mechanisms of Restored β-Cell Tolerance Mediated by Protective Alleles of *Idd3* and *Idd5*

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Type 1 diabetes genes within the interleukin (IL)-2, cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), and natural resistance-associated macrophage protein (NRAMP1) pathways influence development of autoimmune diabetes in humans and NOD mice. In NOD mice, when present together, protective alleles encoding IL-2, *Idd3* candidate gene, CTLA-4, NRAMP1, and acetyl-coenzyme A dehydrogenase, long-chain (ACADL) (candidate genes for the *Idd5.1*, *Idd5.2*, and *Idd5.3* subregions) provide nearly complete diabetes protection. To define where the protective alleles of *Idd3* and the *Idd5* subregions must be present to protect from diabetes and tolerize islet-specific CD8$^+$ T cells, SCID mice were reconstituted so that the host and lymphocytes expressed various combinations of protective and susceptibility alleles at *Idd3* and *Idd5*. Although protective *Idd3* alleles in the lymphocytes and protective *Idd5* alleles in the SCID host contributed most significantly to CD8 tolerance, both were required together in both lymphocyte and nonlymphocyte cells to recapitulate the potent diabetes protection observed in intact *Idd3/5* mice. We conclude that genetic regions involved in autoimmune disease are not restricted in their influence to individual cell types. Even a single protective gene product, such as IL-2, must be expressed in both the lymphocytes and dendritic cells to exert its full extent of disease protection. These studies highlight the pleiotropic effects of genes that determine autoimmune disease susceptibility.
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Type 1 diabetes is an autoimmune disease that is caused by T cell–mediated destruction of the pancreatic islet β-cells. Multiple genetic and environmental factors contribute to disease progression in both humans and the NOD mouse model of type 1 diabetes (1). Considerable overlap exists between the mouse and human disease, exemplified by several common genetic risk pathways including genes encoding major histocompatibility complex (MHC)-II, insulin, interleukin (IL)-2/IL-2 receptor α (IL-2RA), cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), and natural resistance-associated macrophage protein (NRAMP1) (2–5). In the mouse, type 1 diabetes susceptibility regions are termed insulin-dependent diabetes (*Idd*) regions and have been identified through the use of congenic strains in which genomic segments from diabetes-protected strains are introgressed onto the NOD background. Here we have examined the mechanisms of protection of four such regions: *Idd3* on chromosome 3 and *Idd5.1*, *Idd5.2*, and *Idd5.3* on chromosome 1.

Idd3 contains the candidate genes *Il2* and *Il21*. The B6-derived *Il2* allele produces twofold more IL-2 protein than the NOD-derived allele (6–8). Although the expression levels of both the *Il2* (6,7,9) and *Il21* (8) genes have been reported to differ between the NOD and B6 alleles, examination of other *Idd3* haplotypes (CZECH, CAST, SWR, and A/J) introgressed onto the NOD background revealed that diabetes protection correlated with high expression levels of *Il2* but no correlation was found with the *Il21* expression level (6). Furthermore, the introduction of a single *Il2* knockout allele onto NOD-*Idd3* congenic mice, which reduces IL-2 production by 50%, abrogates protection despite the presence of two potentially protective *Il21* alleles (6). This experiment proves that reduction of IL-2 causes increased diabetes susceptibility, verifying *Il2* as *Idd3*.

Candidate genes for the *Idd5* region include *Ctla4* (*Idd5.1*) (10,11), *Slc11a1* (formally *Nramp1*, *Idd5.2*) (12,13), and *Acadl* (*Idd5.3*) (13,14). CTLA-4 is a T cell–expressed inhibitory molecule, and enhanced expression of a ligand-independent isoform correlates with the protective allele (11). NRAMP1 is a phagosomal ion transporter expressed in dendritic cells (DCs) (15,16) and macrophages and a loss of function mutation is associated with disease protection (12). ACADL is a ubiquitously expressed enzyme involved in fatty-acid metabolism, and higher expression levels are associated with a protective phenotype (14,16). Despite the identification of candidate genes for these regions, it is not yet clear how they affect the disease process.

We demonstrated previously that protection from disease in mice expressing both *Idd3* and *Idd5* protective alleles (*Idd3/5* mice) is highly correlated with restored CD8$^+$ T-cell tolerance to islet antigens (16–18). *Idd3/5* genes were found to function through both CD4$^+$ T cells and DCs to restore proliferation and accumulation of islet-specific CD8$^+$ T cells to a low level (16). Furthermore, in reconstituted SCID mice, in which the genotype of the donor T cells and host DCs could be manipulated separately, both host cells and donor lymphocytes needed to express protective *Idd3/5* genes to completely protect from the development of insulitis. However, their contribution to diabetes was not explored; neither were the individual contributions of the *Idd3* and *Idd5* regions to donor T cells and host DCs explored. Because both *Idd3* and *Idd5* genes are expressed by both CD4$^+$ T cells and DCs, we aimed to define where the individual *Idd3* and *Idd5* regions are required for the restoration of CD8$^+$ T-cell tolerance, protection from insulitis, and diabetes onset.

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RESEARCH DESIGN AND METHODS

Mice. Experimental procedures were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institutional Animal Care and Use Committee #09-0074). NOD/MrkTac and NOD-SCID mice were purchased from Taconic. *Idd3/5*, *Idd3*, *Idd5*, and *Idd3/5*-SCID congenic strains and NOD-8.3 Thy1.1$^+$ T-cell receptor (TCR) transgenic mice have been described previously (6,16,19–21). The *Idd3* (1098) strain contains a 2.7 Mb genetic interval derived from B6 bounded by the markers D3nds35 and D3nds76. The *Idd3* region has been mapped to a 650 kb region that contains seven genes within line 1098 (6). The *Idd5* (1094) strain contains a 28.3 Mb genetic interval derived from B10 and bounded by the markers D1Mit478 and D1mit134. The *Idd5.1* region has been mapped to a 2.1 Mb region that contains four genes (20); the *Idd5.2* region is 1.52 Mb and contains 45 genes (20) and the *Idd5.3* region has been mapped to a 3.55 Mb region containing 11 genes (13). These strains can be visualized at http://dil.t1dbase.org/page/MouseStrainsHome and enter lines 1094 and 1098. *Idd5*-SCID mice were generated by intercrossing *Idd5* and NOD-SCID mice and selecting mice homozygous for both alleles. Similarly, *Idd3*-SCID mice were made by intercrossing *Idd3* and NOD-SCID mice.

Virus. Recombinant vaccinia virus expressing the H-2K^d restricted epitope VYLKTNVFL, amino acid residues 206–214 of murine endogenous islet antigen, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP; Vac-K^{d}IGRP) was described previously (21). Mice were infected intraperitoneally with 1×10^7 plaque-forming units of virus, and CD8$^+$ T-cell responses were measured in the spleen 7 days later.

Flow cytometry. CD8$^+$ T cells were stained with H-2K^d-IGRP$_{206–214}$ –PE tetramers (National Institute of Allergy and Infectious Disease [NIAID] MHC Tetramer core facility) for 15 min RT followed by staining with anti-CD8-FITC at 4°C for 15 min. All mAbs were obtained from either eBioscience, BioLegend, or BD Pharmingen (all San Diego, CA). Cells were acquired with either a FACSCalibur or LSRII (Becton Dickinson, Mountain View, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Purification and adoptive transfer of T cells. Naive CD8$^+$Thy1.1$^+$ 8.3 TCR transgenic cells were isolated from lymph nodes and spleen of NOD 8.3 Thy1.1$^+$ TCR transgenic mice. CD8$^+$ T cells were purified using a CD8$^+$ T-cell enrichment kit (BD Biosciences), and carboxyfluorescein succinimidyl ester (CFSE) labeling was performed as described (22). Recipient mice were injected with 4×10^6 purified CD8$^+$ T cells intravenously. Four days after the transfer, pancreatic lymph node (PcLN) cells were prepared for analysis.

Reconstitution of SCID mice. SCID mice were reconstituted with total spleen and lymph node cells prepared from 3- to 5-week-old donor mice. DCs were depleted with Pan-DC microbeads (Miltenyi Biotec) according to the manufacturer's instructions before transfer of 2 to 3×10^7 cells intravenously. Mice were rested for 6–8 weeks before adoptive transfer of CD8$^+$Thy1.1$^+$8.3 TCR cells or 10 weeks before infection with Vac-K^{d}IGRP or harvesting pancreata.

Assessment of diabetes and insulitis. Pancreata were fixed in 10% neutral buffered formalin and paraffin embedded. Hematoxylin and eosin–stained sections were scored for insulitis. At least 20 islets per mouse were scored blind as either 0, no infiltration; 1, peri-insulitis; 2, mild-invasive insulitis; or 3, severe invasive insulitis. Mice having elevated urinary glucose >500 mg/dL (detected using Diastix [Myles, Elkhart, IN]) were classified as diabetic. During the diabetes frequency study, some mice became sick or died without becoming diabetic. This was most likely from the development of thymomas, which commonly occur in SCID mice as they age. Nondiabetic mice were censored from the analysis either four weeks before death or when they became noticeably sick.

Statistical analysis. Kaplan-Meier survival analysis of diabetes was performed for each group of reconstituted mice, and type 1 diabetes frequencies were compared using the log-rank test (GraphPad Prism software). Differences in the proportion or number of tetramer binding cells were compared between groups via the Mann-Whitney *U* test (GraphPad Prism software). Differences in the proportion of divided 8.3 cells were compared via Student *t* test (GraphPad Prism software).

RESULTS

***Idd3* and *Idd5* contribute to restored CD8$^+$ T-cell tolerance to islet antigen IGRP.** Protective alleles at *Idd3* and *Idd5* are associated with reduced CTL responses to IGRP. We chose to examine CD8$^+$ T-cell tolerance to IGRP since these T cells are present at high frequency and their presence is predictive of diabetes progression in individual NOD mice (23). To assess the numbers of IGRP-specific CD8$^+$ T cells, NOD mice congenic for the individual *Idd3* and *Idd5* regions were infected with Vac-K^{d}IGRP and the frequency of IGRP-specific CD8$^+$ T cells that expanded in the spleen were determined by tetramer staining (Fig. 1). Similar to our previous findings measuring CTL killing (18), *Idd3/5* mice had a greatly reduced expansion of autoreactive IGRP-specific CD8$^+$ T cells compared with NOD mice ($P <$ 0.0001). Mice having diabetes-protective alleles at *Idd3* alone (*Idd3* mice) also had a strongly reduced expansion of IGRP-specific CD8$^+$ T cells compared with NOD (P = 0.0037). Mice having diabetes-protective alleles at *Idd5* (*Idd5* mice) showed an intermediate level of CD8$^+$ T-cell expansion compared with NOD (P = 0.0116). Total numbers of IGRP-specific cells were affected similarly (Supplementary Fig. 1). We conclude that diabetes-protective alleles at *Idd3* and *Idd5* both independently contribute to restored CD8$^+$ T-cell tolerance to IGRP and that when diabetes-protective alleles at *Idd3* and *Idd5* are present together, the mechanisms by which tolerance is influenced by each of the regions are additive.

Although *Idd3* (*Il2*) is expressed by DCs (24) in a genotype-dependent manner (16), IL-2 is primarily associated with T-cell functions. Therefore we predicted that *Idd3* expression would be required on T cells. The *Idd5.2* candidate gene product NRAMP1 is only expressed in phagocytic cell types such as DCs and macrophages; therefore, we predicted that *Idd5* rather than *Idd3* would be the primary region required on DCs to facilitate IGRP tolerance. To test these hypotheses, SCID mice expressing NOD, *Idd3*, *Idd5*, or *Idd3/5* alleles were reconstituted with *Idd3/5* or control NOD spleen and lymph node cells and after 10 weeks the mice were infected with Vac-K^{d}IGRP and the expansion of IGRP-specific CD8$^+$ T cells were assessed in the spleen (Fig. 2*A*). NOD->NOD-SCID and *Idd3/5*->NOD-SCID reconstituted mice had very high frequencies of IGRP-specific CD8$^+$ T cells. *Idd3*-SCID mice reconstituted with *Idd3/5* lymphocytes gave intermediate frequencies of

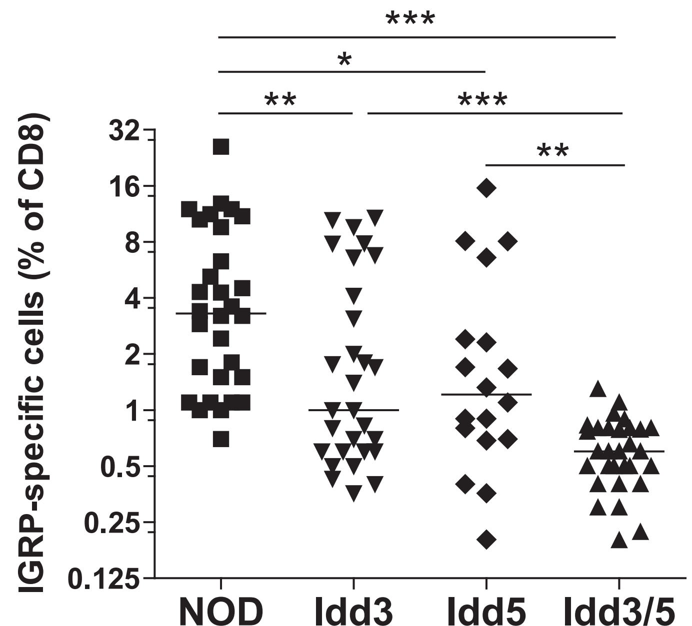

FIG. 1. Restored IGRP-specific CD8$^+$ T-cell tolerance in *Idd3/5* subregion mice. Female, 10- to 14-week-old mice were infected with 1×10^7 pfu Vac-K^{d}IGRP. On day 7 after infection, spleens were analyzed by fluorescence-activated cell sorter for CD8$^+$ IGRP-tetramer$^+$ cells. Results were compiled from five separate experiments with 4–8 mice per strain in each experiment. Horizontal lines depict median value. Pairs of strains were compared with the Mann-Whitney *U* test. *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.0001.

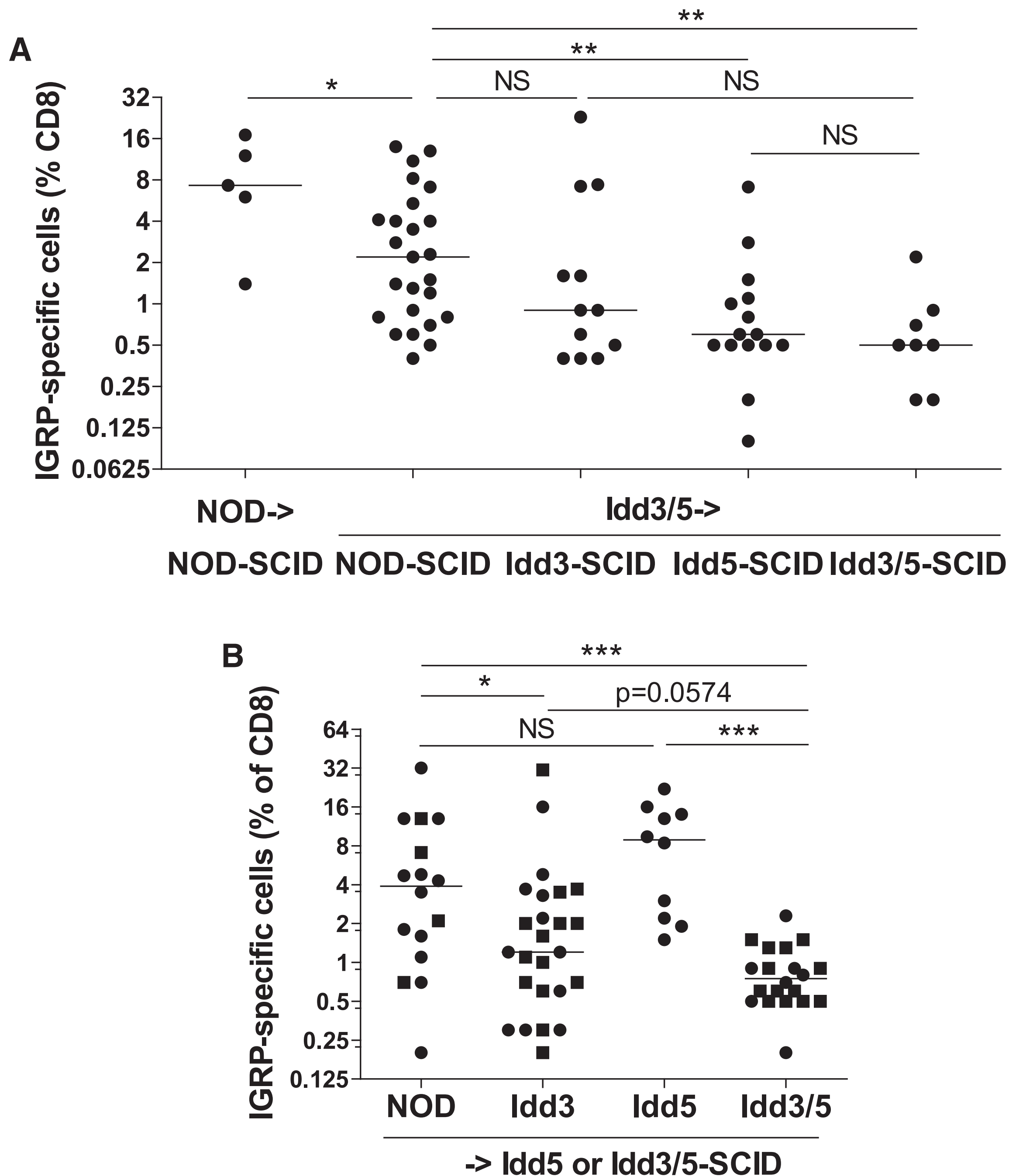

FIG. 2. Expression of *Idd5* genes by nonlymphocytes and *Idd3* genes by lymphocytes is required for restoration of CD8⁺ T-cell tolerance. *A*: NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD or *Idd3/5* donor mice. After 10 weeks, the mice were infected with Vac-K^{d}IGRP, and 7 days later the spleens were analyzed for CD8$^+$ IGRP-tetramer$^+$ cells. Pooled results from four experiments are shown. Horizontal lines depict median value. Pairs of strains were compared with the Mann-Whitney *U* test. *P < 0.05; **P < 0.01. *B*: *Idd5*-SCID (■) and *Idd3/5*-SCID (●) mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD, *Idd3*, *Idd5*, or *Idd3/5* donor mice. After 10 weeks, the mice were infected with Vac-K^{d}IGRP, and 7 days later the spleens were analyzed for CD8$^+$ IGRP-tetramer$^+$ cells. Pooled results from four experiments are shown. Horizontal lines depict median value. Pairs of strains were compared with the Mann-Whitney *U* test. *P < 0.05; ***P < 0.001.

IGRP-specific CD8$^+$ T cells (*Idd3/5*->NOD-SCID vs. *Idd3/5*->*Idd3*-SCID, P = 0.2231; *Idd3/5*->*Idd3*-SCID vs. *Idd3/5*->*Idd3/5*-SCID, P = 0.2143). Expression of *Idd5* genes on the SCID host mice prevented the expansion of IGRP-specific CD8$^+$ T cells (*Idd3/5*->NOD-SCID vs. *Idd3/5*->*Idd5*-SCID, P = 0.0033). There was no significant difference in the level of IGRP-specific CD8$^+$ T cells between *Idd5*-SCID and *Idd3/5*-SCID mice reconstituted with *Idd3/5* lymphocytes (P = 0.4677, not significant [NS]). Thus the presence of diabetes-protective *Idd5* alleles alone in the SCID host in the context of lymphocytes having protective alleles at both *Idd3* and *Idd5* was sufficient to restrain autoreactive, IGRP-specific CD8$^+$ T cells.

To assess the contribution of lymphocyte expression of protective genes, SCID hosts were reconstituted with either NOD, *Idd3*, *Idd5*, or *Idd3/5* donor spleen and lymph node cells depleted of DCs. Both *Idd5*-SCID and *Idd3/5*-SCID hosts were used since they provided equivalent

results (Fig. 2*A*), and no differences were seen between the two hosts (Fig. 2*B*, compare ● and ■). *Idd3*, but not *Idd5*, expressing donor cells were able to reduce the frequency of IGRP-specific $CD8^+$ T cells (Fig. 2*B*, NOD vs. *Idd3*, $P = 0.04$; NOD vs. *Idd5*, $P = 0.22$, NS). However, *Idd3* donor lymphocytes were almost significantly worse than *Idd3/5* donor lymphocytes (*Idd3* vs. *Idd3/5*, $P = 0.0574$, NS), and *Idd3* had a significantly greater variance than *Idd3/5* donor lymphocytes ($P < 0.0001$). This indicates that the presence of diabetes-protective *Idd5* alleles in the donor lymphocytes also had a measurable effect on the frequency of IGRP-specific $CD8^+$ T cells. Taken together these results suggest expression of *Idd5* by DCs and *Idd3* by lymphocytes contributes a highly significant level of tolerance.

The contribution of *Idd3* and *Idd5* to accumulation of IGRP-specific T cells in the PcLN. The first encounter of naïve T cells in the periphery with islet antigens, such as IGRP, occurs in the PcLN where the cells undergo proliferation. In *Idd3/5* mice this results in deletion of the autoreactive cells, whereas in NOD mice the cells accumulate and can later be found within the islets (16,17). We found previously that the degree of proliferation in the PcLN of transferred islet specific T cells is not influenced by the extent of pre-existing insulitis present in the pancreas (17). Similar to our previous findings (18), 8.3 cells, which are specific for IGRP (25), proliferated significantly more in NOD mice than *Idd3/5* mice ($P < 0.0001$, Fig. 3). When 8.3 cells were transferred into *Idd3* or *Idd5* mice they proliferated at a slightly reduced level compared with 8.3 cells transferred to NOD mice (NOD vs. *Idd3*, $P = 0.0363$; NOD vs. *Idd5*, $P = 0.0302$). However, the presence of both diabetes-protective alleles together reduced the proliferation of 8.3 cells to a very low level (similar to what would be expected in a nondiabetes prone strain [16]). This demonstrates that diabetes-protective alleles together at *Idd3* and *Idd5* have an additive effect on reducing the ability of autoreactive $CD8^+$ T cells to expand in response to islet-derived antigen presented in the regional lymph node, a very early check point in the autoimmune response.

To assess the contribution of protective genes expressed by the donor lymphocytes or host, we repeated the SCID reconstitution experiments described in Fig. 2*A* and after 7 weeks transferred CFSE-labeled NOD 8.3 $CD8^+$ T cells to the reconstituted mice. SCID host mice expressing protective *Idd3* alleles and reconstituted with *Idd3/5* lymphocytes were unable to reduce the proliferation of transferred 8.3 $CD8^+$ T cells in the PcLN (Fig. 4*A*, NOD-SCID vs. *Idd3*-SCID, $P = 0.429$). However, host expression of protective *Idd5* alleles reduced the accumulation of 8.3 $CD8^+$ T cells (Fig. 4*A*, NOD-SCID vs. *Idd5*-SCID, $P = 0.008$). *Idd3/5*->*Idd5*-SCID mice had an equivalent low level of 8.3 T-cell proliferation as *Idd3/5*->*Idd3/5*-SCID mice ($P = 0.241$). We conclude that in the presence of *Idd3/5* lymphocytes, expression of protective *Idd5* alleles in the SCID host cells (including DCs) effectively allows deletion of autoreactive IGRP-specific $CD8^+$ T cells.

To assess the contribution of lymphocyte expression of protective genes to tolerance in the PcLN, *Idd5* or *Idd3/5*-SCID hosts reconstituted as described in Fig. 2*B* were given CFSE-labeled 8.3 cells. Neither *Idd3* nor *Idd5* donor cells were sufficient to fully restore low levels of accumulation of 8.3 cells in the PcLN (Fig. 4*B*). Diabetes-protective *Idd3* alleles on the donor cells gave a partial reduction in 8.3 cell proliferation (NOD vs. *Idd3*, $P = 0.019$; *Idd3* vs. *Idd3/5*, $P = 0.0047$), as did diabetes-protective *Idd5* alleles

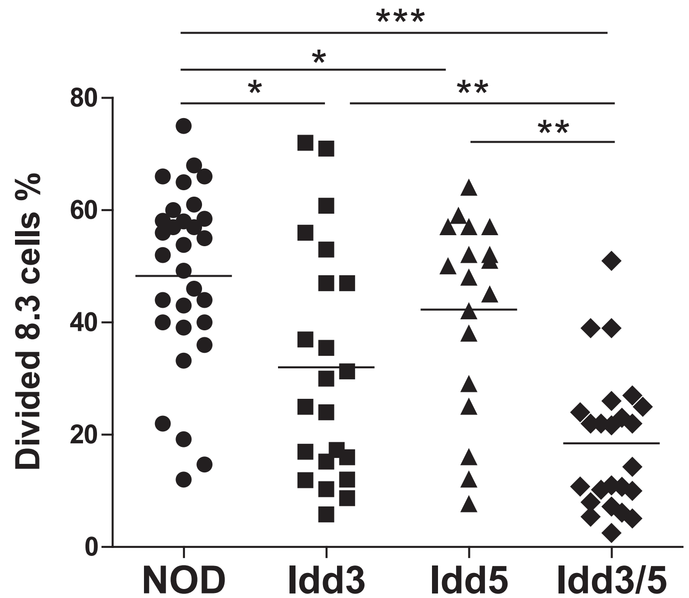

FIG. 3. Reduced proliferation of IGRP-specific 8.3 cells in *Idd3/5* subregion mice. Mice were transferred with CFSE-labeled $Thy1.1^+$ 8.3 CD8 T cells, and four days later the PcLN were analyzed by fluorescence-activated cell sorter. The proportion of $Thy1.1^+$ $CD8^+$ cells with diluted CFSE is plotted. Data are pooled from six independent experiments. Horizontal lines depict mean value. Pairs of strains were compared with the Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

(NOD vs. *Idd5*, $P = 0.0187$; *Idd5* vs. *Idd3/5*, $P = 0.0012$). Only in *Idd3/5* mice was low proliferation and accumulation of IGRP-specific 8.3 cells observed. Thus, tolerance in the PcLNs requires host expression of *Idd5* and lymphocyte expression of both *Idd3* and *Idd5*.

Protective alleles at both *Idd3* and *Idd5* must be present in lymphocytes and nonlymphocytes for protection from diabetes development. We now asked where the individual *Idd3* and *Idd5* regions must be expressed to prevent diabetes. Because *Idd3*-SCID mice had no or minor effects on $CD8^+$ T-cell tolerance to IGRP (Figs. 2*A* and 4*A*), recipient SCID mice expressing protective *Idd3* alleles only were not included in the diabetes study. Instead we compared the effect of *Idd3* in the DCs for its effect on diabetes by comparing the diabetes results obtained with *Idd3/5*-SCID versus *Idd5*-SCID mice. NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with donor spleen and lymph node cells (DC depleted) derived from young NOD, *Idd3*, or *Idd3/5* mice. The frequency of diabetes was then monitored for 25 weeks (Fig. 5). When NOD-SCID mice were reconstituted with NOD lymphocytes, almost all mice became diabetic (Fig. 5*A*). Survival was significantly increased when *Idd3* lymphocytes were used in NOD-SCID hosts ($P < 0.0001$), but *Idd3/5* lymphocytes did not further enhance disease protection (*Idd3*->NOD-SCID vs. *Idd3/5*->NOD-SCID, $P = 0.4467$, NS; NOD->NOD-SCID vs. *Idd3/5*->NOD-SCID, $P = 0.0065$). When NOD, *Idd3*, and *Idd3/5* lymphocytes were used to reconstitute *Idd5*-SCID hosts (Fig. 5*B*), a significant difference between *Idd3* and *Idd3/5* donor cells emerged ($P = 0.0128$), indicating that the presence of diabetes-protective *Idd5* alleles in lymphocytes contributes to protection from type 1 diabetes. *Idd5*-SCID hosts receiving *Idd3* donor lymphocytes were not protected from diabetes as compared with NOD->*Idd5*-SCID reconstituted mice ($P = 0.2059$), although *Idd3*->*Idd5*-SCID mice were significantly better protected than NOD->

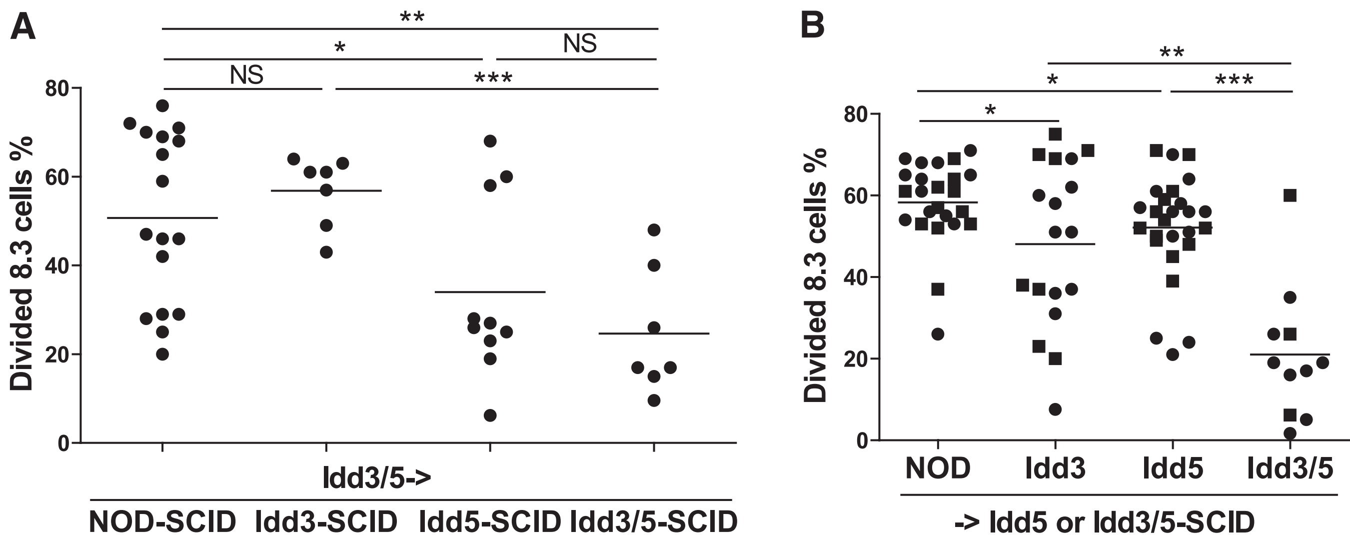

FIG. 4. Expression of *Idd5* genes by nonlymphocytes and both *Idd3* and *Idd5* genes by lymphocytes is required for low proliferation of IGRP-specific T cells. *A*: NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD or *Idd3/5* donor mice. After 7 weeks, CFSE-labeled 8.3 Thy1.1$^+$ CD8 T cells were transferred, and the PcLN was analyzed by fluorescence-activated cell sorter on day 4 for divided 8.3 cells. Pooled results from three experiments are shown. Horizontal lines depict mean value. Pairs of strains were compared by Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *B*: *Idd5*-SCID (■) and *Idd3/5*-SCID (●) mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD, *Idd3*, *Idd5*, or *Idd3/5* donor mice. After 7 weeks, CFSE-labeled 8.3 Thy1.1$^+$ CD8 T cells were transferred, and the PcLN was analyzed by fluorescence-activated cell sorter on day 4 for divided 8.3 cells. Pooled results from four experiments are shown. Horizontal lines depict mean value. Pairs of strains were compared by Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

NOD-SCID mice (P = 0.0017). Finally, *Idd3/5*-SCID mice were reconstituted with NOD, *Idd3*, and *Idd3/5* lymphocytes (Fig. 5*C*), resulting in greatly improved diabetes protection when *Idd3* donor cells were used compared with NOD donor cells (P = 0.0023). *Idd3/5* donor cells were strongly protective when transferred to *Idd3/5*-SCID recipients (NOD->*Idd3/5*-SCID vs. *Idd3/5*->*Idd3/5*-SCID, $P < 0.0001$) and further increased diabetes protection compared with *Idd3* donor cells (P = 0.0610). In conclusion, protective alleles at both *Idd3* and *Idd5* contribute to diabetes protection when expressed by donor lymphocytes. However, the ability of protective alleles at *Idd5* to add to the diabetes protection provided by *Idd3* varies depending on the genotype of the SCID recipient.

We also considered the same data presented in Fig. 5*A-C* to assess the effects of protective alleles at *Idd3* and *Idd5* on the host SCID cells. NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice reconstituted with each lymphocyte type are depicted in Fig. 5*D* (NOD lymphocytes), Fig. 5*E* (*Idd3* lymphocytes), and Fig. 5*F* (*Idd3/5* lymphocytes). Reconstitution of NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice with NOD lymphocytes did not result in significant differences in diabetes incidence (Fig. 5*D*), with relatively rapid diabetes occurring in almost all mice. These results underscore the conclusion that the presence of *Idd3/5* diabetes-protective alleles in the SCID recipients only is not sufficient to provide any level of protection from diabetes. When *Idd3* lymphocytes were used to reconstitute NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice, no increased diabetes protection resulted from expression of *Idd5* on the SCID host (Fig. 5*E*; *Idd3*-> NOD-SCID vs. *Idd3*-> *Idd5*-SCID, P = 0.1042, NS). However, *Idd3*->*Idd3/5*-SCID mice were highly significantly protected from diabetes compared with *Idd3*->*Idd5*-SCID mice (Fig. 5*E*; P = 0.0046). Finally, even though *Idd3/5* donor lymphocytes transferred to NOD-SCID mice provide some diabetes protection as compared with NOD->NOD-SCID recipients (Fig. 5*A*) further decreases in disease were observed, revealing a dramatic effect of host genotype on diabetes incidence (Fig. 5*F*, *Idd3/5*->NOD-SCID vs. *Idd3/5*-> *Idd3/5*-SCID, P = 0.0005). This effect was contributed to by both *Idd5*-SCID hosts (*Idd3/5*->NOD-SCID vs. *Idd3/5*-> *Idd5*-SCID, P = 0.0754) and significantly when *Idd3/5*-SCID hosts were used (*Idd3/5*->*Idd5*-SCID vs. *Idd3/5*->*Idd3/5*-SCID, P = 0.0151). These combined data demonstrate that the presence of protective alleles at both *Idd3* and *Idd5* in the host SCID contributes to protection from diabetes.

Cellular requirements for protective *Idd3* and *Idd5* alleles to reduce insulitis development. Disease progression is characterized by increasing insulitis. To assess the contribution of *Idd3* and *Idd5* on the lymphocyte and nonlymphocyte cell types to insulitis, NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with NOD, *Idd3*, or *Idd3/5* donor spleen and lymph node cells, and after 10 weeks, pancreata were harvested and assessed for insulitis development (Fig. 6). When the three SCID hosts were reconstituted with NOD lymphocytes and compared, little difference in insulitis was found (Fig. 6), a similar result to diabetes (Fig. 5*D*). Reconstitution of *Idd5*-SCID mice with *Idd3* lymphocytes significantly decreased insulitis compared with NOD->*Idd5*-SCID mice (P = 0.0143), confirming a role for *Idd3* on lymphocytes. However, *Idd3*->*Idd5*-SCID mice had significantly more insulitis than *Idd3/5*->*Idd5*-SCID mice (P = 0.0323), demonstrating a role for *Idd5* on lymphocytes also. When *Idd3/5* lymphocytes were used to reconstitute NOD-SCID and *Idd5*-SCID mice, no difference in the amount of insulitis was observed. However, when *Idd3/5*->*Idd5*-SCID and *Idd3/5*-> *Idd3/5*-SCID mice were compared, although no significant difference in the median value was found using the Mann-Whitney U test, significantly more *Idd3/5*->*Idd3/5*-SCID mice had no insulitis (score 0, P = 0.0198, Fisher's exact test). These data support our finding that the expression of protective *Idd3* alleles by the host SCID mouse contributes to protection against type 1 diabetes.

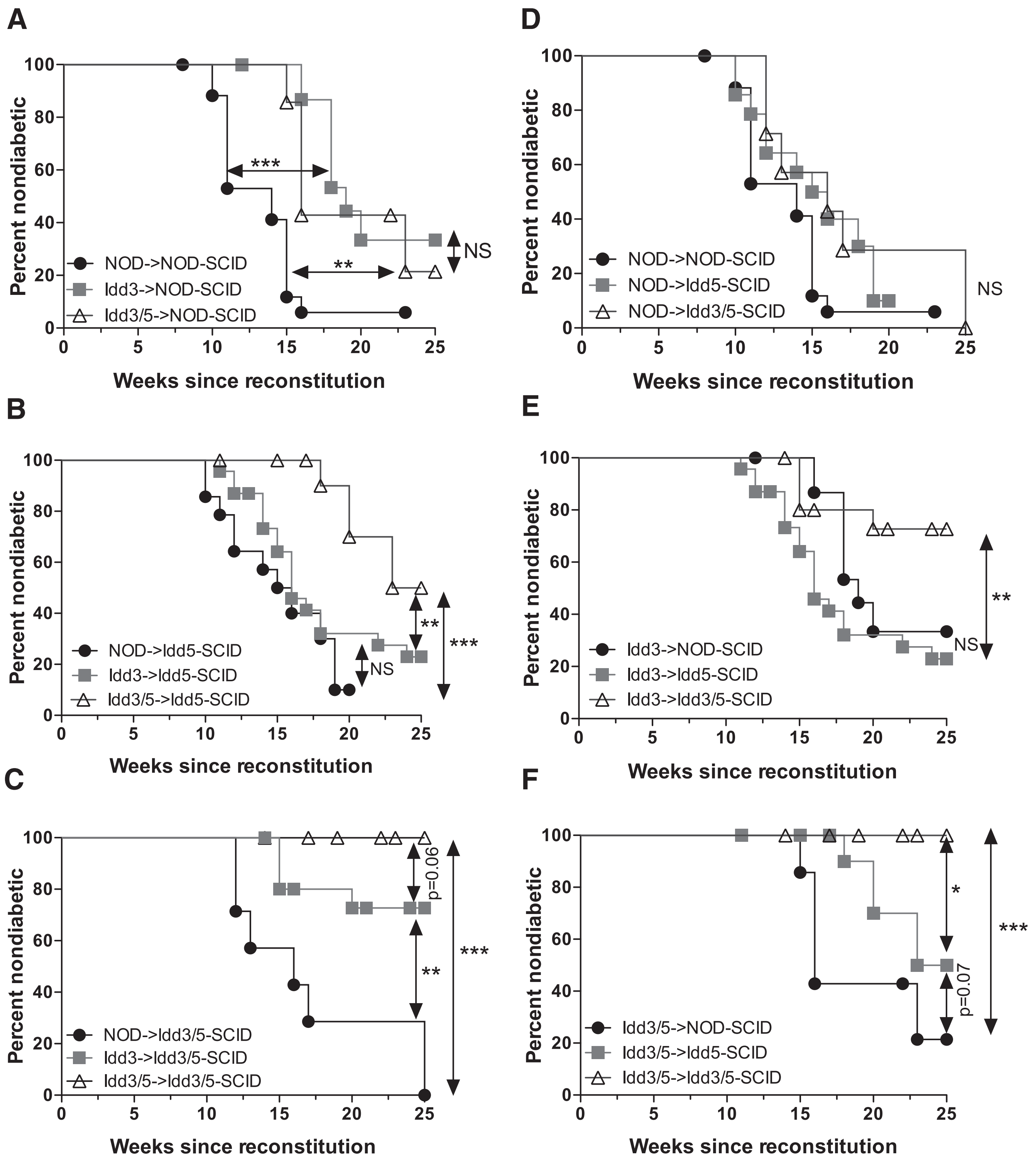

FIG. 5. Diabetes is dependent on both *Idd3* and *Idd5* region genes, expressed on both lymphocytes and nonlymphocytes. NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD, *Idd3*, or *Idd3/5* donor mice. NOD->NOD-SCID (*n* = 18), *Idd3*->NOD-SCID (*n* = 16), *Idd3/5*->NOD-SCID (*n* = 7), NOD->*Idd5*-SCID (*n* = 14), *Idd3*->*Idd5*-SCID (*n* = 23), *Idd3/5*->*Idd5*-SCID (*n* = 15), NOD->*Idd3/5*-SCID (*n* = 7), *Idd3*->*Idd3/5*-SCID (*n* = 16), and *Idd3/5*->*Idd3/5*-SCID (*n* = 13) mice were monitored for 25 weeks for elevated urine glucose. *A*: NOD-SCID hosts. *B*: *Idd5*-SCID hosts. *C*: *Idd3/5*-SCID hosts. *D*: NOD donor lymphocytes. *E*: *Idd3* donor lymphocytes. *F*: *Idd3/5* donor lymphocytes. Data are pooled from three experiments. Kaplan-Meier survival curves were compared with the log-rank test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

DISCUSSION

Expression of protective alleles of *Idd3* and *Idd5* almost entirely abrogates diabetes development (19,26). In this study, we sought to define the cell type where each individual protective region acts alone as well as in combination. Because there is very strong evidence that the causative gene in the *Idd3* locus is *Il2* (6), which is most commonly associated with T-cell function, we hypothesized that *Idd3*

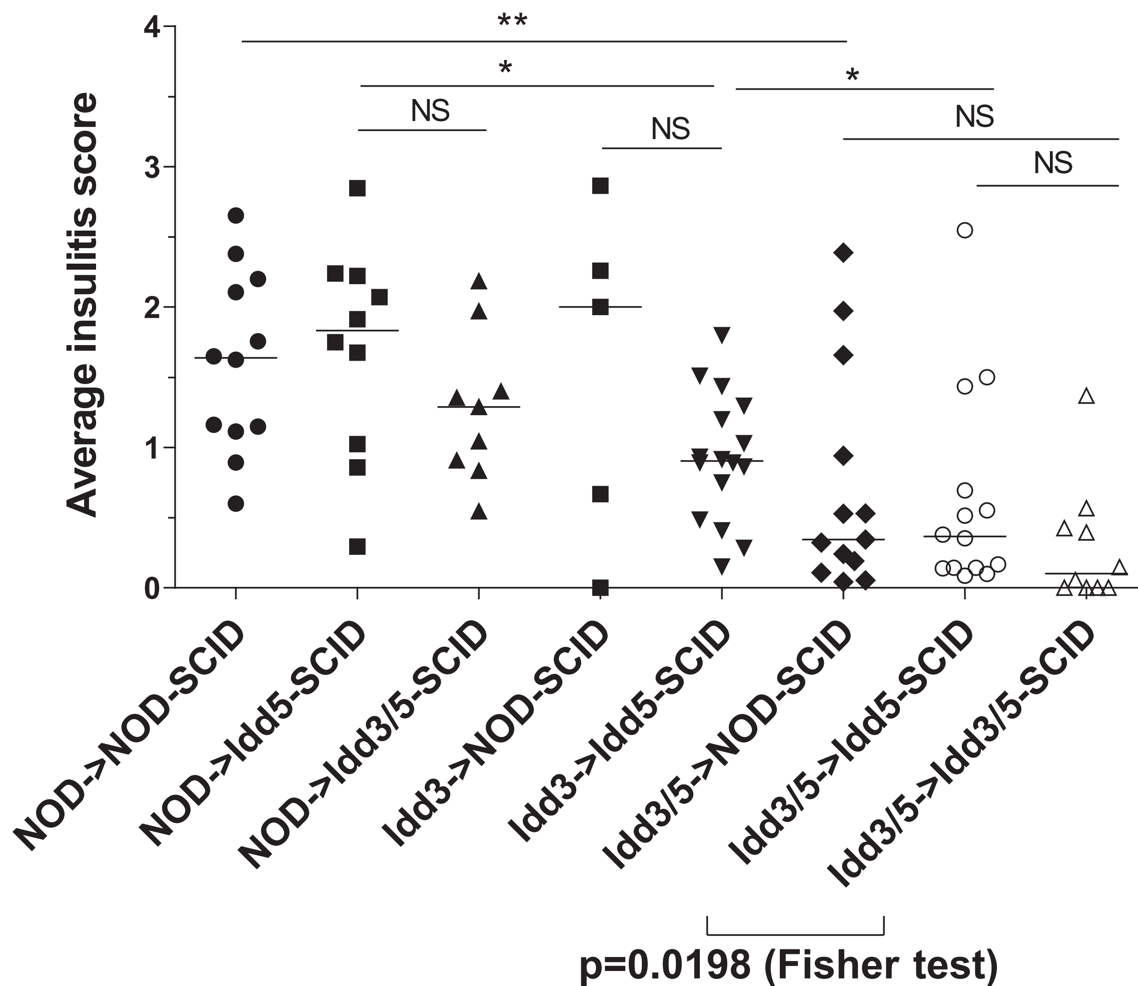

FIG. 6. Requirements for expression of protective *Idd3* and *Idd5* subregions for protection from insulitis development. NOD-SCID, *Idd5*-SCID, and *Idd3/5*-SCID mice were reconstituted with spleen and lymph node cells depleted of DCs isolated from 3- to 4-week-old NOD, *Idd3*, or *Idd3/5* donor mice. After 10 weeks, pancreata were harvested and hematoxylin-eosin–stained sections were assessed for insulitis. Data are pooled from four experiments. Horizontal lines depict median value. Pairs of strains were compared with the Mann-Whitney *U* test. *$P < 0.05$; **$P < 0.01$.

would function in the lymphocyte compartment. Because the *Idd5* subregion with the strongest individual diabetes protective effect is *Idd5.2* (13), and substantial evidence points to the DC and macrophage-expressed protein NRAMP1 (12), we hypothesized that the DC/host component of diabetes protection would be mediated by *Idd5*. The protective form of *Slc11a1* (encoding NRAMP1) contains a loss of function mutation of this phagosomal ion transporter, which has been postulated to alter phagosome acidification and may influence antigen processing (15). Alternatively, NRAMP1 expression alters cytokine production in macrophages and DCs, which could alter differentiation or effector responses of phagocytic cells (27). We did find that *Idd3*->*Idd5*-SCID mice had significantly delayed diabetes as compared with NOD->NOD-SCID mice; however, it was not to the degree that we had expected since 80% of *Idd3*->*Idd5*-SCID mice still became diabetic. Therefore, significant *Idd3/5* protective effects were missing in this combination.

In addition to the *Idd3* candidate *Il2*, the *Idd5.1* and *Idd5.3* candidate genes *Ctla4* and *Acadl* are also expressed by lymphocytes. The protective allele of *Ctla4* produces increased amounts of a ligand-independent splice form (liCTLA-4). Expression of liCTLA4 inhibits TCR signaling by dephosphorylating the TCR-ζ chain, although it is not clear how this modulates disease (28); but one possibility is that liCTLA-4 and IL-2 both act to enhance Treg function. ACADL is a ubiquitously expressed enzyme involved in fatty acid metabolism. T cells have been shown to switch between primarily utilizing fatty acid metabolism to glycolytic metabolism upon proliferation, and regulation of this switch may be involved in the transition from effector to memory T cells (29). Therefore the study of ACADL in T-cell function is an area of interest, and we are currently testing protective alleles at individual *Idd5* subregions together with *Idd3* protective alleles for their effects on the diabetes protection mediated by donor cells. An additional complexity and caveat of these studies is that different subregion genes may act in different cell types. For example *Idd5.1*, *Idd5.2*, and *Idd5.3* may act separately in lymphocytes and host cells and, furthermore, additional unidentified weak subloci may also contribute.

Our most surprising finding was that protective alleles at *Idd3* as well as *Idd5* contributed to diabetes protection when expressed by SCID host cells. Other than T cells, IL-2 can be produced by NK and NKT cells (30,31,32) as well as DCs after stimulation with microbial products (16,24), and DC-produced IL-2 has been shown to have a role in T-cell priming (24). Very recently, it has been shown that DCs provide IL-2 to T cells very early after activation, before

the T cells produce their own IL-2, enhancing T-cell activation (33). However, an in vivo requirement for DC-produced IL-2 has not been shown previously in an animal model, and this is the first demonstration in which it may have a role in autoimmune disease. It is possible that DCs provide IL-2 to Tregs, enhancing their homeostatic proliferation or suppressor function.

The protective effect provided by the host cells was completely masked when NOD lymphocytes were present. It is noteworthy, however, that even when protective *Idd3* alleles were expressed by T cells, a high frequency of diabetes occurred unless the host expressed *Idd3* and *Idd5* protective alleles. Thus the effects of both the lymphocytes and host are critical, and both must express protective alleles to dramatically reduce the incidence of diabetes.

To gain more information about what stage of the disease process the *Idd3* and *Idd5* genes function, we also examined the frequency of IGRP-specific $CD8^+$ T cells in the reconstituted SCID mice as well as the proliferation of IGRP-specific 8.3 T cells in the PcLN. We found that host-expressed *Idd5* was sufficient to tolerize IGRP-specific $CD8^+$ T cells. This was in contrast with diabetes where host-expressed *Idd5* was not able to provide as protective an environment as *Idd3/5*. This suggests that the effective $CD8^+$ T-cell tolerance seen 7 and 10 weeks after reconstitution may not be maintained since the mice age to 17–25 weeks when they became diabetic.

On the lymphocytes, *Idd3* alone had a strong effect on $CD8^+$ T-cell tolerance, whereas *Idd5* alone had no effect or only a slight effect (Fig. 2). Because of these minimal effects of diabetes-protective alleles at *Idd5*, we did not test whether protective alleles at *Idd5* alone in the lymphocyte donor protect from diabetes. Similarly, because *Idd5* hosts strongly promoted $CD8^+$ T-cell tolerance, we did not include *Idd3*-SCID mice in the diabetes studies but compared *Idd3* host effects in combination with *Idd5*.

These studies demonstrate that the protective alleles of type 1 diabetes susceptibility genes act in multiple cell types and stages of disease to prevent diabetes. Our findings for each phenotype we have analyzed are summarized diagrammatically in Fig. 7. The important implication of these studies is that although *Idd5* was sufficient on the host cells in the prediabetic period represented by early proliferation and tolerance of islet-specific $CD8^+$ T cells, *Idd3* on the host was critical to prevent conversion into clinical disease. This may have therapeutic consequences since, for example, treatments that enhance IL-2 production by DCs may be valuable at the time of disease onset.

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E.E.H.-W. conceived and designed the study, performed experiments, analyzed data, and wrote the manuscript. J.C. performed experiments. E.E.H.-W., L.S.W., and L.A.S. are the guarantors for this article. D.B.R. and K.M.H. developed congenic mouse strains. L.S.W. developed

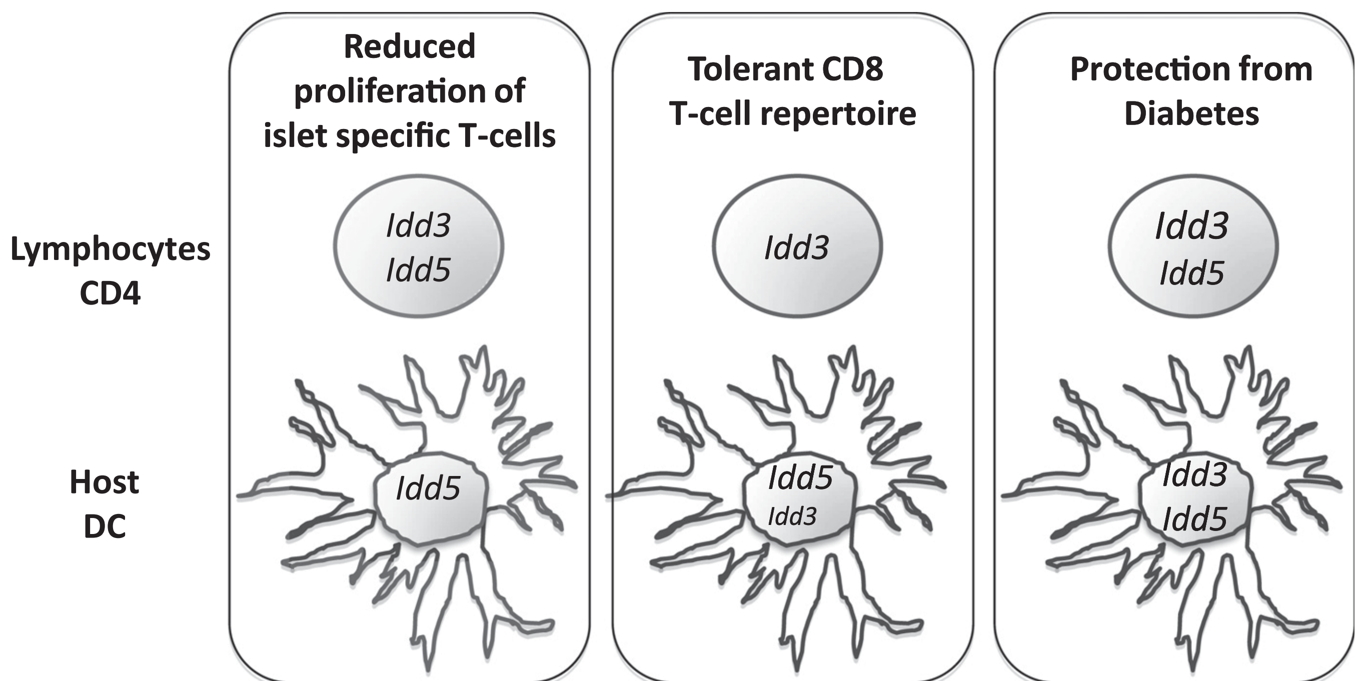

FIG. 7. Diagram of the phenotypic effects of the individual *Idd3* and *Idd5* regions. The individual protective alleles of the *Idd3* and *Idd5* regions have been identified to act in either the lymphocytes (including $CD4^+$ T cells) or SCID host (including DCs) to restore a protective phenotype. Two subclinical phenotypes were examined: proliferation of transferred IGRP-specific $CD8^+$ T cells and tolerance of the $CD8^+$ T-cell repertoire to IGRP as well as the clinical phenotype of diabetes incidence.

congenic mouse strains, conceived the study, and edited and revised the manuscript. L.A.S. conceived the study and edited and revised the manuscript.

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