Immunology and Cell Biology (2016) 94, 709–713

www.nature.com/icb

SHORT COMMUNICATION

TCR transgenic mice reveal the impact of type 1 diabetes loci on early and late disease checkpoints

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Linkage analysis studies for autoimmune diabetes have revealed multiple non-major histocompatibility complex (MHC) chromosomal regions linked to disease susceptibility. To date, more than 20 insulin-dependent diabetes (*Idd*) loci linked to diabetes susceptibility have been identified in NOD mice and validated via congenic breeding. Importantly, evidence suggests that *Idd* loci may regulate at least two pathological steps during autoimmune diabetes development, namely the onset of insulitis and the transition from insulitis to overt diabetes. Here we assess the role of various non-MHC *Idd* diabetes-resistance loci, which have been validated in the non-transgenic setting, on autoimmune diabetes progression in the transgenic setting. Specifically, we generated multiple *Idd* congenic strains in the 3A9-TCR:insHEL NOD.*H2*^{k} transgenic model and monitored their diabetes incidence. We show that 3A9-TCR:insHEL NOD.*H2*^{k} mice congenic for *Idd3* or *Idd5* display a reduction in diabetes development, whereas mice congenic for *Idd9* or *Idd13* exhibit an increase, in comparison with 3A9-TCR:insHEL NOD.*H2*^{k} mice. These results suggest that the presence of the 3A9-TCR and hen egg lysosyme transgenes can offset the regulatory function of certain diabetes-resistance genetic variants contained within the *Idd* loci, including *Idd9* and *Idd13*. We propose the antigen-specific 3A9-TCR:insHEL transgenic model as a useful tool for the study of the genetics of autoimmune diabetes development.
Immunology and Cell Biology (2016) **94,** 709–713; doi:10.1038/icb.2016.27; published online 5 April 2016

Autoimmune diabetes results from the immune-mediated destruction of the insulin-producing β cells located within the islets of Langerhans of the pancreas. The investigation of the genetic control of autoimmune diabetes using the NOD mouse, which spontaneously develops the disease, has revealed an interesting parallel to humans.[1] Early studies demonstrated that the major histocompatibility complex (MHC) locus contributes toward defining autoimmune diabetes susceptibility in both mouse and humans.[2,3] However, with the use of congenic mice, the NOD H2^{g7} MHC susceptibility locus was shown to be necessary, but not sufficient to confer diabetes susceptibility,[4] underscoring the importance of non-MHC genes in autoimmune diabetes progression. To date, more than 20 non-MHC insulin-dependent diabetes (*Idd*) loci linked to diabetes susceptibility have been identified using NOD mice.[5] Validation of linkage studies is achieved by substituting different non-MHC chromosomal regions from various diabetes-resistant strains into the NOD strain by congenic breeding.[6–10] Indeed, NOD mice congenic for non-MHC *Idd* loci are sufficient to partially or completely prevent the development of insulitis, characterized by the invasion of islets by immune cell infiltrates, and/or overt diabetes even in the presence of the NOD H2^{g7} MHC alleles.[7–10] This highlights the key contribution of non-MHC loci in modulating autoimmune susceptibility.

To readily track antigen-specific immune responses during autoimmune diabetes progression, various transgenic mouse models have been generated, including the 3A9-TCR:insHEL model. Specifically, the insHEL transgene promotes hen egg lysosyme (HEL) expression under the rat-insulin promoter, thus inducing pancreatic β cell-specific expression, while the 3A9-TCR (Vα3/Vβ8.2) transgene yields T cells recognizing a HEL peptide presented in the context of I-A^k.[11,12] To allow for effective HEL peptide presentation on I-A^k, this pair of insHEL and 3A9-TCR transgenes was introduced onto B10.BR and NOD.*H2*^{k} genetic backgrounds, which are both congenic for the same diabetes-resistant H2^k MHC locus.[4] In the resulting mouse model of spontaneous autoimmune diabetes, almost all 3A9-TCR:insHEL NOD.*H2*^{k} mice progress to diabetes, while very few 3A9-TCR:insHEL B10.BR mice develop the disease,[13] demonstrating that non-MHC loci confer autoimmune diabetes susceptibility. Using this model, we herein study the role of various non-MHC *Idd* diabetes-resistance loci on the antigen-specific immune response during autoimmune diabetes progression.

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Received 22 April 2015; revised 3 March 2016; accepted 3 March 2016; published online 5 April 2016

RESULTS AND DISCUSSION

The contribution of specific non-MHC *Idd* loci, known to confer diabetes resistance in non-transgenic NOD mice, could potentially modulate the antigen-specific autoimmune response in the 3A9-TCR:insHEL NOD.$H2^k$ transgenic model.[13] To test this hypothesis, we undertook a diabetes incidence study for 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd3*, *Idd5*, *Idd9* or *Idd3/10/18* loci. Correspondingly, we find that, in comparison with 3A9-TCR:insHEL NOD.$H2^k$ mice, the 3A9-TCR:insHEL NOD.$H2^k$-*Idd3/10/18* mice exhibit a significant reduction in diabetes incidence, whereas we observe a tendency for a reduced incidence in mice congenic for either *Idd3* or *Idd5* alone (Figure 1a). In addition, of the three 3A9-TCR:insHEL NOD.$H2^k$-*Idd3/5* double-congenic mice tested in our study, none developed diabetes by 24 weeks of age (data not shown), suggesting a combined effect of both *Idd3* and *Idd5* loci in providing autoimmune diabetes protection in this transgenic model. Together, these data corroborate the findings from the non-transgenic system,[14–16] and support the importance of both *Idd3* and *Idd5* in conferring autoimmune diabetes resistance. Unexpectedly, however, we observe a significant increase in diabetes incidence for 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd9* in comparison with 3A9-TCR:insHEL NOD.$H2^k$ mice (Figure 1b). These results suggest that the presence of the 3A9-TCR and insHEL transgenes can offset the regulatory function of certain diabetes-resistance genetic variants contained within the *Idd* loci.

To understand why *Idd* loci, which are protective against autoimmune diabetes development in the non-transgenic setting, will either maintain or lose the regulatory function in the 3A9-TCR:insHEL transgenic model, we analyzed various processes involved in disease progression, namely serum autoantibody levels, the proportion of autoreactive $CD4^+$ T cells and the proportion of $CD4^+CD25^+$ regulatory T cells (Tregs). Of note, while serum insulin autoantibody levels are associated with autoimmune diabetes susceptibility,[17] they are not simply bystanders during disease development.[18,19] We observe no differences between the serum anti-HEL IgG levels of 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd* loci that either maintain (*Idd3/10/18*, *Idd3* or *Idd5*) or lose (*Idd9*) their protective function (Figure 1c). In addition, we also find no difference in the proportion of autoreactive T cells, namely 3A9-TCR $CD4^+$ T cells, between the 3A9-TCR:insHEL NOD.$H2^k$ mice congenic, or not, for any of the *Idd* loci in either the spleen (Figure 1d) or the thymus (Figure 1e). Finally, using non-transgenic NOD mice congenic for *Idd* loci, it has been revealed that certain *Idd* loci contribute to immune regulation, at least in part, via $CD4^+CD25^+$ Tregs,[20–22] where Tregs can inhibit autoreactive T-cell function.[23] Thus, we next assessed the proportion of Tregs between the various 3A9-TCR:insHEL congenic mice. Once more, we find no difference in the proportion of Tregs between the 3A9-TCR:insHEL NOD.$H2^k$ mice congenic, or not, for the various *Idd* loci (Figure 1f). Therefore, the reason why these *Idd* loci, which are protective against autoimmune diabetes development in the non-transgenic setting, will either maintain or lose the regulatory function in the 3A9-TCR:insHEL transgenic model, cannot be explained by serum autoantibody levels or the proportion of autoreactive T cells and Tregs.

Non-transgenic NOD mice bearing the *Idd3* and *Idd5* loci are protected from diabetes as a result of a decrease in insulitis,[16] whereas NOD.*Idd9* mice show extensive insulitis.[15] Indeed, in non-transgenic NOD mice, *Idd9* specifically controls events in the disease process associated with the transition from insulitis to overt diabetes rather than the initial islet infiltration.[15] Accordingly, our group has recently shown that *Idd2*, which influences the degree of insulitis,[9,24] also protects NOD.$H2^k$ mice from diabetes development in the 3A9-TCR:insHEL transgenic model.[25] Together, these results suggest that *Idd* loci containing genes that regulate islet infiltration by autoreactive T cells prevent diabetes development, whereas *Idd* loci regulating the transition to overt diabetes without influencing the severity of insulitis, such as *Idd9*, may not be able to protect from diabetes development, in the 3A9-TCR:insHEL transgenic model. Interestingly, the *Idd13* locus, similar to *Idd9*, is known to provide protection from autoimmune diabetes, but not from insulitis, in the non-transgenic setting.[7,8] We thus monitored the impact of the *Idd13* locus on diabetes incidence in the 3A9-TCR:insHEL transgenic model. Similar to our findings for 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd9*, we observe an increase in the diabetes incidence of 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd13* in comparison with 3A9-TCR:insHEL NOD.$H2^k$ mice (Figure 2a). Importantly, as observed in the non-transgenic setting,[7,8] we find that *Idd13* does not provide protection from insulitis in the 3A9-TCR:insHEL transgenic model (Figure 2b). Together, these results further suggest that *Idd* loci that regulate the transition to overt diabetes without decreasing the severity of insulitis in non-transgenic NOD mice may not be able to protect from diabetes development in 3A9-TCR:insHEL NOD.$H2^k$ mice.

In an attempt to explain the observed increase in diabetes incidence in 3A9-TCR:insHEL NOD.$H2^k$-*Idd13* congenic mice relative to 3A9-TCR:insHEL NOD.$H2^k$ mice, we again investigated the various mechanisms involved in disease progression. In comparison with 3A9-TCR:insHEL NOD.$H2^k$ mice, there are no significant differences in anti-HEL IgG autoantibody serum levels (Figure 2c). We again find no difference in the proportion of either autoreactive 3A9 TCR^+ $CD4^+$ T cells or of Tregs in both the spleen and the thymus of 3A9-TCR:insHEL NOD.$H2^k$-*Idd13* mice relative to 3A9-TCR:insHEL NOD.$H2^k$ mice (Figures 3a–d). Finally, we find that 3A9-TCR:insHEL NOD.$H2^k$ mice bearing the *Idd13* locus present with a decrease in the proportion of activated autoreactive 3A9 TCR^+ $CD4^+$ T cells in both the thymus and the spleen relative to 3A9-TCR:insHEL NOD.$H2^k$ mice (Figures 3e and f). Similar results were observed in the lymph nodes (not shown). Thus, the reason why *Idd13*, which is protective against autoimmune diabetes development in the non-transgenic setting, loses its protective function in the 3A9-TCR:insHEL transgenic model cannot be explained by autoantibody levels, the proportion of Tregs or the proportion and activation autoreactive T cells. However, these results do not preclude potential differences in peripheral tolerance mechanisms that may be observed directly within the pancreas. Additional experiments, such as investigating the effectiveness of Tregs and the quality of the islet infiltrates, are needed to address the impact of both *Idd9* and *Idd13* loci in conferring diabetes susceptibility in the 3A9-TCR:insHEL NOD.$H2^k$ setting. Nevertheless, the combination of our results suggests that the 3A9-TCR:insHEL NOD.$H2^k$ transgenic model is a useful model to determine whether a chromosomal region is essential for the regulation of the early (islet infiltration) or later (transition from insulitis to overt diabetes) stages of diabetes development, where genetic loci affecting the early, but not the later, stages of disease development can modulate disease susceptibility in the 3A9-TCR:insHEL NOD.$H2^k$ model. Notably, a potential explanation as to why the *Idd9* and *Idd13* loci differentially impact on diabetes incidence in the NOD and 3A9-TCR:insHEL NOD.$H2^k$ strains relates not only to the transgenes but also to the MHC locus. Indeed, the *Idd9* and *Idd13* loci may confer diabetes protection only in the context of the $H2^{g7}$ MHC locus. Experiments addressing this issue are difficult to perform as NOD mice congenic for other MHC loci are protected from disease.

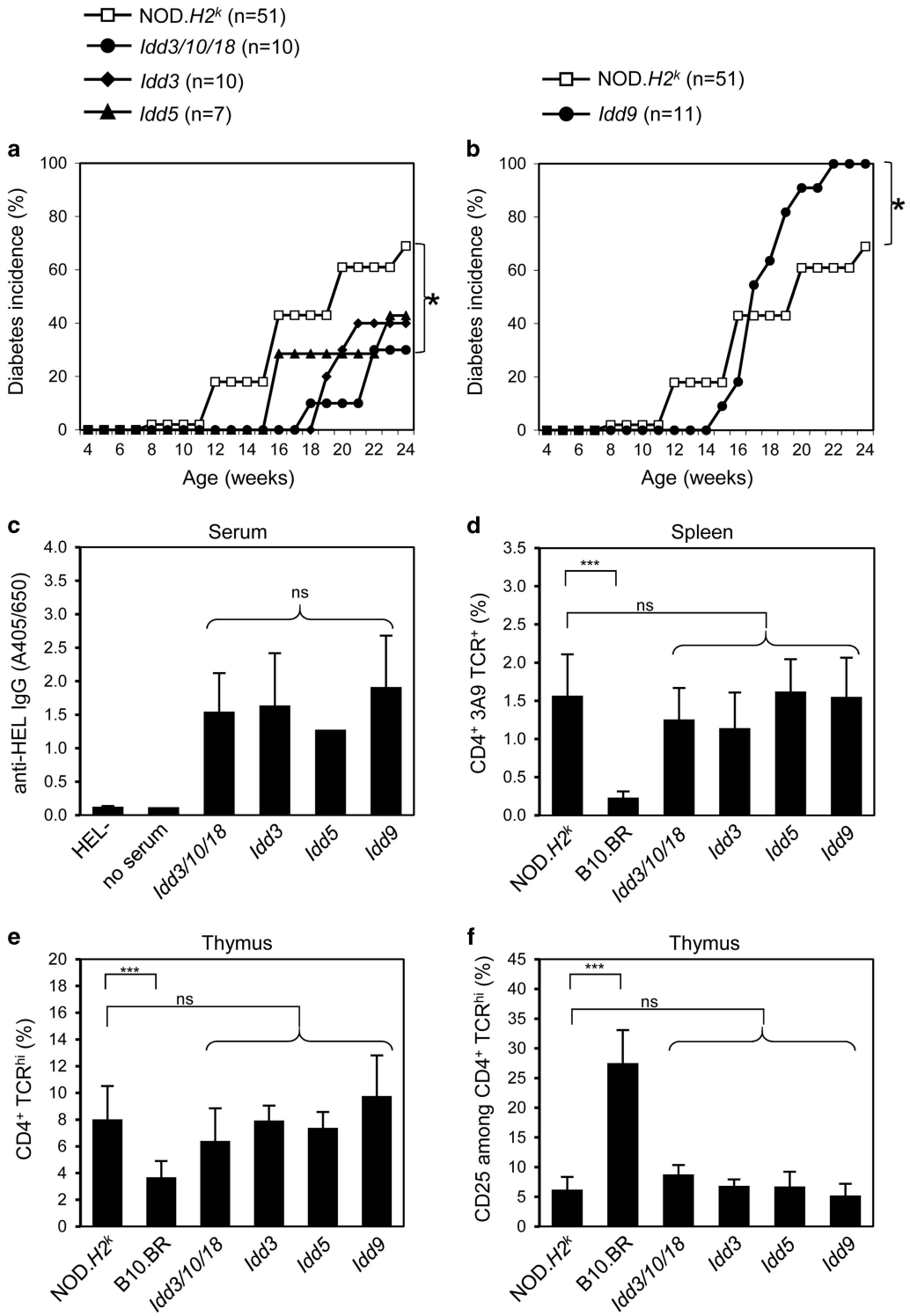

Figure 1 *Idd* loci confer either resistance or susceptibility to diabetes in 3A9-TCR:insHEL NOD.*H2*^{k} mice. (**a**) Diabetes incidence is shown for female 3A9-TCR:insHEL NOD.*H2*^{k} mice congenic for *Idd3* ($n=10$, closed diamond), *Idd5* ($n=7$, closed triangle) or *Idd3/10/18* ($n=10$, closed circle) in comparison with female 3A9-TCR:insHEL NOD.*H2*^{k} mice ($n=51$, open square). *$P\leqslant 0.05$, according to log rank (Mantel–Cox) test. (**b**) Diabetes incidence is shown for female 3A9-TCR:insHEL NOD.*H2*^{k} mice congenic for *Idd9* ($n=11$, closed circle) in comparison with female 3A9-TCR:insHEL NOD.*H2*^{k} mice ($n=51$, open square). *$P\leqslant 0.05$, according to log rank (Mantel–Cox) test. (**c**) Relative serum anti-IgG HEL levels are shown for all congenic strains. HEL− and no serum, respectively, indicate that no HEL protein ($n=5$) or no serum ($n=2$) was added. *Idd3* ($n=7$), *Idd5* ($n=1$), *Idd3/10/18* ($n=16$) and *Idd9* ($n=9$). (**d**) The proportion of CD4$^+$ 3A9 TCR$^+$ T cells in the spleen is shown. (**e**) The proportion of CD4$^+$ 3A9 TCR^{hi} mature thymocytes and (**f**) the proportion of CD25 among these mature thymocytes are shown. (**d**–**f**) No significant differences were observed among the congenic mice relative to the 3A9-TCR:insHEL NOD.*H2*^{k} parental strain. B10.BR ($n=16$), NOD.*H2*^{k} ($n=18$), *Idd3* ($n=10$), *Idd5* ($n=11$), *Idd3/10/18* ($n=11$) and *Idd9* ($n=9$). A one-way analysis of variance applying a Bonferroni *post hoc* adjustment was used to determine *P*-values. NS (non-significant), *P*-value >0.05; ***P-value <0.001.

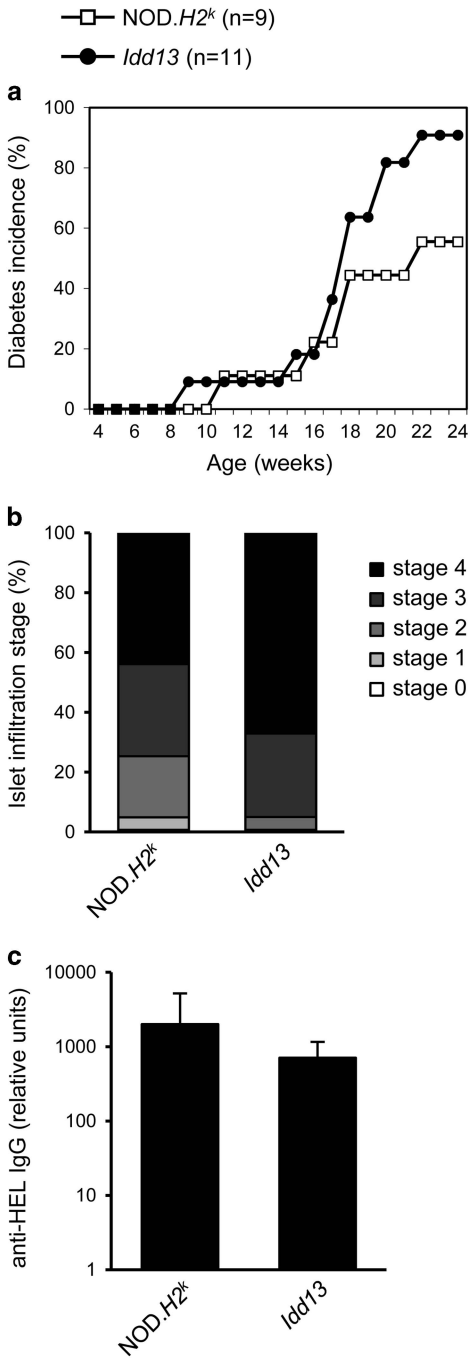

Figure 2 3A9-TCR:insHEL NOD.*H2*^{k}-*Idd13* mice display an increase in diabetes development. (**a**) Diabetes incidence is shown for female 3A9-TCR:insHEL NOD.*H2*^{k} mice congenic for *Idd13* ($n=11$, closed circle) in comparison with female 3A9-TCR:insHEL NOD.*H2*^{k} mice ($n=9$, open square). (**b**) The degree of islet infiltration between the two strains is depicted ($n=6$ for both strains). $P>0.05$, non-significant. (**c**) Relative serum anti-IgG HEL levels are shown. NOD.*H2*^{k} ($n=7$) and *Idd13* ($n=11$). $P>0.05$, non-significant. (**b**, **c**) Student's *t*-test was applied.

In summary, several genetic intervals that confer autoimmune diabetes susceptibility have been identified using the non-transgenic NOD mouse model. However, the mechanisms by which they protect against development of diabetes have not been fully elucidated. Evidence suggests that genetic variants encoded within *Idd* loci may

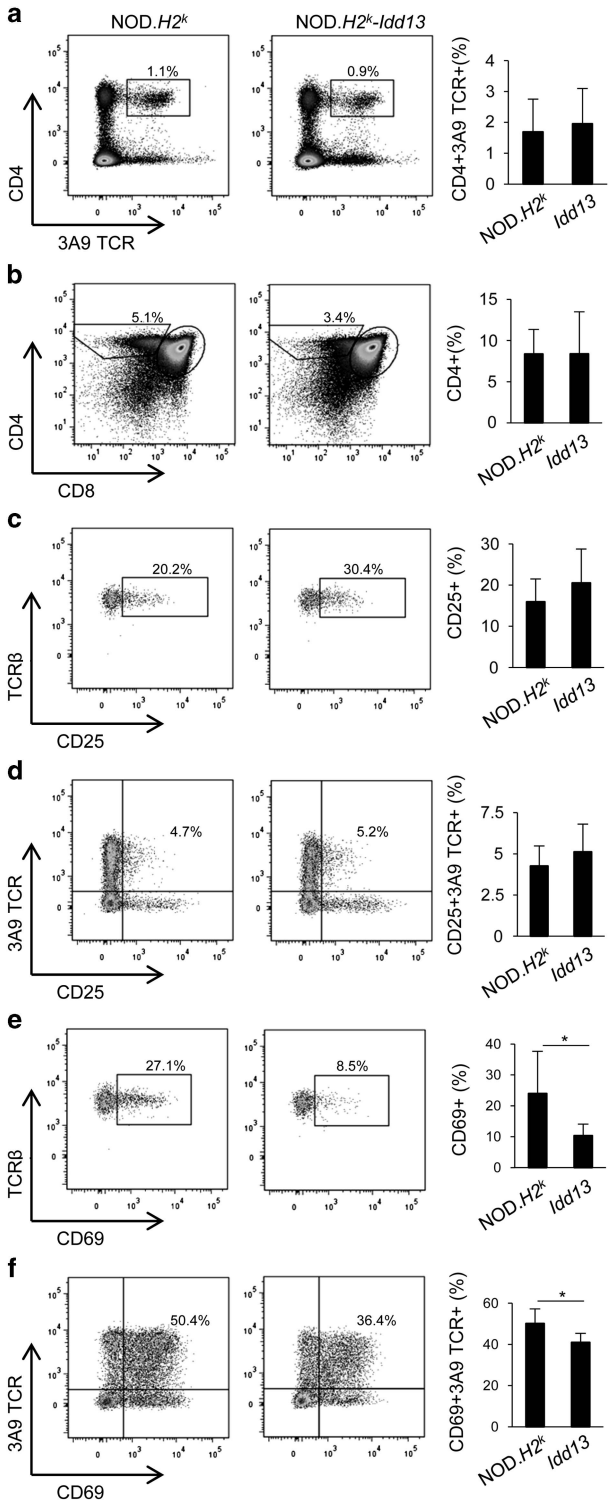

Figure 3 3A9-TCR:insHEL NOD.*H2*^{k}-*Idd13* mice do not exhibit an increase in autoreactive T cells or a decrease in Tregs. (**a**) The proportion of CD4$^+$ 3A9 TCR$^+$ T cells in the spleen, (**b**) CD4$^+$ thymocytes, (**c**) CD25 and (**e**) CD69 among CD4$^+$ 3A9 TCR$^+$ T cells in the spleen, and (**d**) 3A9 TCR$^+$ CD25$^+$ and (**f**) 3A9 TCR$^+$ CD69$^+$ among CD4$^+$ thymocytes are shown for 3A9-TCR:insHEL NOD.*H2*^{k}-*Idd13* mice relative to 3A9-TCR:insHEL NOD.*H2*^{k} mice. A compilation of data from at least three independent experiments is shown on the right panels. (**a**–**d**) NOD.*H2*^{k} ($n=4$) and *Idd13* ($n=9$); (**e**, **f**) NOD.*H2*^{k} ($n=3$) and *Idd13* ($n=7$). Student's *t*-test was applied. **P*-value <0.05.

regulate at least two pathological steps leading to overt diabetes, namely the accumulation and infiltration of autoreactive lymphocytes into the pancreas and the transition from insulitis to overt diabetes. We believe that the antigen-specific 3A9-TCR:insHEL transgenic model is a useful model to determine whether a chromosomal region, and ultimately a given gene, is essential for the regulation of the early (insulitis) or later stages (progression to overt diabetes) of disease development, where a decrease in diabetes incidence would suggest that the particular chromosomal region regulates the early stages of autoimmune diabetes. To that effect, the 3A9-TCR:insHEL transgenic model represents a useful tool for the study of the genetic facets of autoimmune diabetes and the given stage of disease development.

METHODS

Mice and diabetes incidence

3A9-TCR transgenic and insHEL (ILK-3) transgenic mice on B10.BR and NOD.$H2^k$ backgrounds have been previously described.[13] The generation of 3A9-TCR:insHEL NOD.$H2^k$ mice congenic for *Idd3*, *Idd5*, *Idd3/5*, *Idd9* and *Idd13* has been previously described.[26] NOD.*Idd3/10/18* congenic mice were bred to 3A9-TCR:insHEL NOD.$H2^k$ mice. Experiments involving 3A9-TCR:insHEL NOD.$H2^k$-*Idd3*, -*Idd5*, -*Idd3/5*, -*Idd9* and -*Idd3/10/18* congenic mice and their appropriate controls were performed at the Mouse Genome Center, Canberra, ACT, Australia, while 3A9-TCR:insHEL NOD.$H2^k$-*Idd13* congenic mice and their appropriate controls were performed at the Maisonneuve-Rosemont Hospital, Montreal, QC, Canada. Diabetes incidence was monitored for female 3A9-TCR:insHEL mice and the congenic derivatives as previously described.[13] All animal procedures performed at the Mouse Genome Center were approved by the Animal Ethics and Experimentation Committees of the Australian National University, whereas those performed at the Maisonneuve-Rosemont Hospital Research Center were approved by the Animal Ethics Committee overseen by the Canadian Council for Animal Protection.

Enzyme-linked immunosorbent assay and insulitis

Quantification of serum anti-HEL IgG levels and the degree of insulitis were quantified as previously described.[13]

Flow cytometry

Flow cytometry was performed for all congenic mice as previously described,[13] except for the *Idd13* congenic strain, for which the CD25 antibody was purchased from Caltag (Carlsbad, CA, USA) and other commercial antibodies were purchased from Biolegend (San Diego, CA, USA). The data for experiments with *Idd13* congenic mice were acquired on a FACSCanto I or LSR II (BD Biosciences, Mississauga, ON, Canada) and analyzed on FlowJo X software (TreeStar, Ashland, OR, USA).

Statistical analysis

Data were tested for significance using a one-way analysis of variance applying a Bonferroni *post hoc* adjustment or using a two-tailed unpaired Student's *t*-test, with a minimal threshold of 0.05, as specified in the figure legends. Diabetes incidence were subjected to a log rank (Mantel–Cox) test. All statistical analyses were performed using the SPSS 19.0 software (IBM, Montreal, QC, Canada).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Fany de Wilde and Judi Wilson for expert technical assistance in animal care. This work was supported by a grant from the Canadian Diabetes Association (OG-3-13-4018) to SL.

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