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The Type 1 Diabetes Locus Idd6 Controls TLR1 Expression

David Vallois,† Christina H. Grimm,2* Philip Avner,* Christian Boitard,† and Ute Christine Rogner3*

The Idd6 locus on mouse chromosome 6, which controls the development of type 1 diabetes in the NOD mouse, affects proliferation rates of T cells and the activity of regulatory CD4+CD25+ T cells. Using a transcriptional profiling approach, we show that splenocytes and thymocytes from diabetes-resistant Idd6 NOD.C3H-congenic mouse strains exhibit a constitutive and specific down-regulation of Toll-like receptor 1 (Tlr1) gene expression compared with diabetes prone NOD mice. This phenotype correlates with a diminished proliferation capacity of both CD4+CD25+ effector and CD4+CD25+ regulatory T cells upon in vitro stimulation of the TLR1/TLR2 pathway by the ligand palmitoyl-3-cysteine-serine-lysine 4, and with the constitutive down-regulation of Tnf-α and IL-6 in macrophages of Idd6-congenic mice. These data suggest that TLR1 is involved in the regulation of mechanisms that impinge on diabetes development in the NOD mouse. The Journal of Immunology, 2007, 179: 3896–3903.

Toll-like receptors are type I transmembrane proteins involved in innate immunity by recognizing conserved microbial structures. Signal transduction processes activated by TLRs include the important transcription factor NF-κB and the signals ultimately give rise to increased expression of a multitude of proinflammatory proteins. Recent studies revealed that TLRs also influence the development of adaptive immune responses with several members of the gene family being involved in autoimmune diseases such as lupus (1–4) and type 1 diabetes (T1D)5 (5).

T1D is a multifactorial and polygenic inherited disorder characterized by the autoimmune destruction of the insulin producing β cells of the pancreas. Genetic studies of the NOD mouse (6, 7), probably the best-characterized animal model for T1D, have revealed >30 murine insulin-dependent diabetes (Idd) susceptibility loci, including Idd20, Idd19, and Idd6 on mouse chromosome 6 (8). Recently, we have undertaken a detailed phenotypic analysis of the diabetes-resistant NOD.C3H-congenic strain 6.VIII, carrying C3H alleles at the 5.8 Mb Idd6 interval, and showed that its diabetes resistance is immune dependent. Splenocytes, CD4+ CD62L+ T cells, and regulatory CD4+CD25+ T cells of 6.VIII strain mice all confer enhanced disease protection in diabetes

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*Abbreviations used in this paper: T1D, type 1 diabetes; CO, control; h, human; Parn,C5K4, palmitoyl-3-cysteine-serine-lysine 4; PLN, pancreatic lymph node; Treg, regulatory T cell.

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Materials and Methods

Congenic mouse strains were as described previously (8, 14). Strains were maintained in our animal house by brother-sister mating. The animal studies were approved by the relevant institutional review boards.

RNA preparation, cDNA synthesis, and microarray analysis

Total RNA from whole tissues, MACS-purified T cells (9), and peritoneal residual macrophages was prepared using RNAble (Eurobio). Random cDNA synthesis was conducted on 10 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s conditions. For microarray experiments, RNA quality was examined using an Agilent 2100 Bioanalyzer (Agilent). DNA microarrays (8k mouse cDNA: Agilent) were hybridized using 10 μg of total RNA transcribed in the presence of Cy3-dCTP or Cy5-dCTP, respectively. Data were analyzed using Feature Extraction and Rosetta resolver software and annotated using SOURCE software (provided by the Genetics Department, Stanford University) and Mouse Genomics Informatics and National Center for Biotechnology Information databases. Quantitative PCR was performed on an Applied Biosystems PRISM 7700 Sequence detector using the SYBR Green PCR Master Mix (PE Biosystems) according to the manufacturer’s conditions. Primers were designed using PrimerExpress software (Applied Biosystems) and used at
optimal concentration. Quantification of the amplification product was done using the \( \Delta \Delta CT \) method and \( Hprt \) or \( TCR \) (for T cells only) as endogenous controls for normalization of the mRNA expression levels. Sequences of the oligonucleotides used were as follows (5’–3’): Tlr1 forward, TCTTCGGCACGTTAGCACTG, Tlr1 reverse, CCAAACCGATCGTAGTGCTGA; Tlr2 forward, TACAGGGATCCGGGTGGTAA, Tlr2 reverse, GCCGAGGCAAGAACAAAGAA; Tlr3 forward, CACGCAGTTCAGCAAGCTATTG, Tlr3 reverse, CGCAAACAGAGTGCATGGTT; Tlr4 forward, GTGATGTGACCATTGATGAGTTCA, Tlr4 reverse, CAGAGACCTTGACGAAACATTCG; Tlr5 forward, CGCTTCGTGTTTTGGACATAAC, Tlr5 reverse, GCCGAACAGGGTGACGTT; Tlr7 forward, ACAGAACATCCCTGAGGGCATT, Tlr7 reverse, TGGTTCAGCCTACGGAAGG; Tlr8 forward, CACGTGTGACATAAGTGATTTTCG; Tlr8 reverse, TTGATCCCCAGGATTGGAA; Tlr9 forward, ACAGGCTGTCAATGGCTCTCA, Tlr9 reverse, CACTGAACGATTTCCAGTGGTACA; Il6 forward, CCCAATTTCCAATGCTCTCC, Il6 reverse, CACTCCTTCTGTGACCTT; Tnf forward, ATGCTGGGACAGTGACCTGG, Tnf reverse, CCTTGATGGTGGTGCATGAG; TCR forward, GTTCTTCACCCTGCCATAGATTTT; TCR reverse, TGTCAACGAGGAAGGATGGAT; and Hprt forward, TTGGTGGAGATGATCTCTCAACTT, Hprt reverse, GGTCCTTTTCACCAGCAAGCTT.

Antibodies

The following mAbs were used purified or conjugated to biotin or FITC: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD25 (7D4), anti-Gr1 (RA3-8C5), anti-B220 (RA3.6B2), anti-Mac-1 (M1/70.15), and anti-Ly76 (TER119). PE-anti-CD4, -anti-CD19 (1D3), -anti-TCRv (KT4), PerCP-anti-CD4 (RM4-5), allophycocyanin-anti-CD4 (RM4-5), and -anti-CD25 (PC61) were purchased from BD Pharmingen Biosciences. Alexa Fluor 647 anti-TLR1 (eBioTR23) was purchased from eBioscience.

Immunofluorescence staining

Cells from thymus, spleen, pancreatic lymph nodes, and peritoneal macrophages were pelleted in 96-well plates and stained for 30 min at 4°C with optimal concentrations of biotin-, PE-, FITC-, PerCP-, or allophycocyanin-labeled reagents in 20 \( \mu l \) of PBS supplemented with 2% FCS and 5 mM sodium azide. Biotin labeling was followed by staining with streptavidin conjugated to the appropriate fluorochrome. Cells were then washed twice and resuspended in PBS containing 1% formaldehyde. Flow cytometric analysis was performed using a FACS Calibur and CellQuest software (BD Biosciences).

Cell proliferation assays

Cell proliferation assays were performed on 96-well plates precoated with 5 \( \mu g/ml \) anti-CD3 Abs using 2.5 \( \times \) \( 10^4 \) CD4^+ CD62L^+ or CD4^+ CD25^+ T cells per well. In some cases, cells were costimulated with human (h) IL-2 (20 ng/ml; R&D Systems) and/or palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4, 2 \( \mu g/ml \); InvivoGen). \([\text{3H}]\)Thymidine incorporation was measured in triplicate 72 h after treatment for each population and condition. CD4^+ cells from spleen and lymph nodes were obtained using the mouse CD4-negative selection kit from Dynal Biotech. CD4^+ CD25^+ and CD4^+ CD25^− T cells were then separated by MACS sorting using biotin-anti-CD25 (7D4) Abs and streptavidin beads. Finally, cells were labeled with streptavidin-PE and anti-Ia-FITC and anti-CD8-allophycyanin Abs before FACS sorting.

Results

Down-regulation of Tlr1 in the Idd6 NOD.C3H-congenic strain 6.VIII

We recently reported that splenocytes from the diabetes-resistant congenic strain NOD.C3H 6.VIII (6.VIII) confer increased resistance to diabetes when cotransferred to NOD/SCID recipients along with diabetogenic cells. This led to the demonstration that splenocytes, CD4^+ CD25^− and CD4^+ CD25^+ T cells were involved in the diabetes protection mediated by C3H alleles at Idd6 (9).

To evaluate the global transcriptional changes mediated by the Idd6-congenic interval, we performed three independent profiling experiments using pooled RNA from spleens of four 8-wk-old pre-diabetic female mice of each strain on an 8k cDNA microarray set from Agilent (14). Technical replicates were performed for each experiment. 22 down-regulated and 42 up-regulated transcripts (~0.8% of the tested transcripts) were found in the 6.VIII-congenic strain compared with the NOD CO strain in all three experiments (\( p < 0.05 \), t test). Highest fold changes were found for the
TLR1 (Tlr1) (3.15-fold for CO against 6.VIII, \( p = 0.0004 \)) and the heat shock protein 1A (Hsp1A) (3-fold for CO against 6.VIII, \( p = 0.004 \)) genes. All other genes showed average fold changes of <2.5. We did not observe chromosomal or functional clustering of the deregulated genes. This is probably not surprising given that the two mouse strains differ by a 5.8-Mb interval and the complexity of the splenic tissue which contains numerous different cell types (14).

We were able to identify in our data sets several moderately deregulated genes located in the \( \text{Idd6} \) interval (Fig. 1B). Among those were the \( \text{Kras} \) oncogene-associated \( \text{Sarcospan} \) gene (1.6-fold for CO against 6.VIII), the \( \text{Idd6.2} \) candidate gene lymphoid-restricted membrane protein (Lrpm) (1.7-fold for CO against 6.VIII) (15), and the \( \text{Lyr} \) motif containing 5 (\( \text{Lym5} \)) gene (1.4-fold for 6.VIII against CO). Quantitative PCR results have however not confirmed the deregulation of these three genes in the 6.VIII strain (14).

When performing three microarray experiments using pools of CD4\(^+\) T cells, each obtained from the spleens of six- to ten 8-wk-old females, only five genes and two unclassified transcripts were found to be significantly down-regulated in the 6.VIII strain (Table I). Six transcripts, including five known genes, were up-regulated in the 6.VIII strain compared with the NOD CO strain. Our results suggest that the previously described T cell subphenotypes are not related to global transcriptional changes in non-activated CD4\(^+\) T cells.

Only two of these genes (Table I) appear to be associated with a known T cell-specific function: RhoG is a low-molecular-weight GTPase highly expressed in lymphocytes that activates gene transcription and promotes cytoskeletal reorganization in vitro (16). The Neutrophilic granule protein (Ngp) has been described as a Th2 T cell marker and a possible cytokine (17). Ngp and four other genes (Camp, Ltf, Ch1313, and Tlr1) that were down-regulated in the 6.VIII strains are all known to be involved in immune response reactions to pathogens. Interestingly, not only the Tlr1 but also the cation antimicrobial peptide (Camp) and lactotransferrin (Ltf) genes are all involved in TLR-mediated immune responses (18–21).

The Tlr1 gene was the only gene down-regulated in both the 6.VIII spleen (\( p = 0.0004, \sim 3\)-fold) and enriched 6.VIII CD4\(^+\) T cells (\( p = 0.0005, \sim 2\)-fold) compared with the CO strain. Because the recent literature has strongly implicated the involvement of other TLR members in autoimmune disease, in particular TLR2 which forms a heterodimer with TLR1, we decided to investigate whether the expression control of the Tlr1 gene, mapping to mouse chromosome 5, is associated with the T1D locus \( \text{Idd6} \) on chromosome 6.

Transcriptional down-regulation of Tlr1 is common to Idd6 NOD.C3H-congenic strains

To exclude the possibility that down-regulation of Tlr1 expression in the 6.VIII-congenic strain was due to a mutation in the Tlr1 gene itself, we tested its expression in two other NOD.C3H-congenic strains (6.VII and 6.I; Fig. 1A), both carrying C3H alleles at distal chromosome 6 (8, 22). Our quantitative PCR analysis using spleens and thymi of 8-wk-old females showed that all three \( \text{Idd6} \) congenic strains had the same phenotype when compared with the NOD CO-congenic strain (\( p < 0.0001 \) in the Mann-Whitney U-Test).

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Table I. Summary of the transcripts (\( p < 0.05 \)) identified in three microarray experiments using pooled CD4\(^+\) T cells from CO and 6.VIII mice

| Accession no. | Gene Name or Homology | Symbol | Locus ID | Chromosome | Cytoband or cM | Function | Fold Change\(^b\) |
|---------------|-----------------------|--------|----------|------------|----------------|----------|----------------|---|
| AI323038      | Cathelicidin antimicrobial peptide | Camp | 12796    | 9          | 61.0 cM        | Antimicrobial peptide, defense response to pathogens, up-regulated by TLRs | -3.0 |
| AI158547      | Unclassified TLR1     | Tlr1   | 21897    | 2          | 37.0 cM        | Molecular function unknown, immune response, receptor activity | -1.7 |
| AA019567, AA177549 | Lactotransferrin    | Ltf    | 17002    | 9          | 70.2 cM        | Iron ion transport, antimicrobial activity, activation of TLR4 pathway, Th1/Th2 balance | -3.0 |
| AA673731      | Chitinase 3-like 3, erythroid differentiation regulator 1 | Ch3i3 | 12655    | 3          | 50.5 cM        | Inflammatory response, molecular function unknown | -2.0 |
| AA672784      | Erythroid differentiation regulator 1 | Erdr1  | 170942   | 6          | 75.0 cM        | Cell adhesion, receptor activity | -1.8 |
| AA182334, AA158823 | Ig H chain 6 (H chain of IgM) | IgH-6  | 16019    | 12         | 58.0 cM        | Ig complex | -1.8 |
| AA060282      | Neutrophilic granule protein | Ngp    | 18054    | 9          | 9 F2           | Defense response, Th2 cell marker | -2.9 |
| AI390848      | Ras homolog gene family, member G | RhoG   | 56212    | 7          | 7 F1           | Signal transduction, actin cytoskeleton organization and biogenesis, positive regulation of transcription | +1.8 |
| W15610        | Integrin \( \beta_4 \) | Itgb4  | 192897   | 11         | 76.0 cM        | Cell adhesion, receptor activity | +1.8 |
| W15720        | Synaptogyrin 2        | Syngr2 | 20973    | 11         | 11 E2          | Integral to membrane | +1.3 |
| W18499        | Unclassified          |        |          |        |                |                      | +1.9 |
| W33651        | Platelet-activating factor Acetylhydrolase, isoform 1b, \( \alpha_1 \) subunit | Pfah1b3 | 18476    | 7          | 7 A3           | 1-Alky1-2-acetylglycerophosphocholine esterase activity | +1.3 |
| AA051563      | Core-binding factor, runt domain, \( \alpha_2 \) subunit, translocated to, 3 homolog (human) | Chfa2t3h | 12398    | 8          |                | Hemapoietic development, granulocyte differentiation | +2.1 |

\(^a\)Six to 10 spleens from 8-wk-old females for each experiment is shown. Genes involved in TLR signaling are bold face italic.

\(^b\)Average fold change for 6.VIII vs CO.

\( U \)-Test.
Interestingly, the 6.I strain that carries C3H alleles at the Idd19 NOD resistance and at the Idd20 NOD susceptibility loci showed expression levels of Tlr1 comparable to those carrying NOD alleles at Idd19 and Idd20 (6.VIII and 6.VII; p/H11022 0.6). Our results suggest that the Idd19 and Idd20 loci do not influence the control of Tlr1 expression by Idd6. We note however that Idd6 cannot account for the totality of the down-regulation of Tlr1 observed in C3H/HeJ mice (p/H11005 0.01 for 6.VIII against C3H/HeJ, for both spleen and thymus) when compared with NOD CO mice (Fig. 2, A and B).

We further tested Tlr1 expression in six females of each of three recently constructed Idd6-subcongenic strains (6.VIIIa, b, and c; Fig. 1B) (14). None of the disease-protected subcongenic strains showed significant down-regulation of Tlr1 in spleen comparable to the original 6.VIII strain. Highest statistical significance was however obtained for strain 6.VIIIc (p = 0.04). Analysis of gene expression in thymus confirmed the Tlr1 down-regulation in strain 6.VIIIc (p < 0.0001 compared with the NOD CO strain). The intermediate results obtained for the subcongenic strains suggest that several cis-acting genetic elements within the Idd6 interval may be required for the control of Tlr1 expression in the 6.VIII strain (Fig. 2, C and D). However, the results obtained for 6.VIIIc point to a strong contribution of the 700-kb Idd6.3 interval (Fig. 1), recently shown to be associated with diabetes resistance in splenocyte transfer experiments.

Age-dependent expression differences of Tlr1
We next studied the effect of age on Tlr1 expression in NOD control and 6.VIII strain mice. Quantitative PCR on spleen and thymus samples from female animals showed that on average Tlr1 expression was increased in the CO mice compared with 6.VIII animals at 4, 8, and 15 wk of age (p < 0.02 for all ages and tissues; Fig. 3, A and B). The analysis of the thymus suggests that the differences in Tlr1 expression are not restricted to the peripheral immune system, although Tlr1 expression levels appear in general much lower in the thymus than in the spleen. Our result also suggests that Tlr1 expression increases with age (p < 0.002 for comparison of both CO spleen and thymus at 4 and 15 wk). Tlr1 expression also becomes more variable with age and the differences between the two strains tend to become less marked. For example, in thymus the average fold change of 3.1 at 4 wk of age drops to 2.4 at 15 wk of age. Similarly, while at 4 wk of age the average difference in spleen is ~2.2-fold, it is only 1.7-fold at 15 wk of age. This finding may correlate with our previous finding that splenocytes from >15-wk-old 6.VIII mice are as diabetogenic as those from NOD CO mice while significant differences in diabetes protection are found with splenocytes from <8-wk-old 6.VIII mice compared with young NOD CO mice (9).
Table II. Quantitative expression analysis of Tlr genes compared to the Hprt reporter gene in splenocyte and thymocyte samples of 4-wk-old mice†

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Average fold change in spleen CO against 6.VIII (4 wk, n = 6)

Average fold change in thymus CO against 6.VIII (4 wk, n = 6)

Average fold change in CD4\(^+\) T cells CO against 6.VIII (3 experiments)*

Average fold change in CD8\(^-\) T cells CO against 6.VIII (3 experiments)*

Average fold change in peritoneal macrophages CO against 6.VIII (n = 6)

†Fold change values represent average fold changes for the CO strain vs the 6.VIII strain calculated from arbitrary units. Values of p were obtained using the Mann-Whitney U test. The three pooled CD4\(^+\) and CD8\(^-\) T cell preparations tested were from 8-wk-old female mice (n = 6–10/animal; TCR\(\beta\) reporter gene); * p-values for single animals (n = 6) were 0.017 for Tlr1 (CD4\(^+\)) T cells and >0.18 for all other genes. Peritoneal macrophages were from 4-wk-old mice (n = 6; Hprt reporter). Significant differences are boldface.

Expression analysis of other members of the Tlr family

We tested whether down-regulation of Tlr1 in the congenic strain 6.VIII was specific to this member of the Tlr gene family by performing quantitative PCR on eight other family members using spleen and thymus of six 4-wk-old female mice (Table II). We detected only small expression differences (less than average fold change 1.7) for most Tlr genes. In no case was the deregulation as marked as that for Tlr1 (2.2-fold in spleen and 3.1-fold in thymus).

When three independent pools of RNA of enriched CD4\(^+\) T and CD8\(^-\) T cell subsets were tested (each pool was obtained from five to ten 8-wk-old female mice and tested in two replicates), we detected an average difference for Tlr1 that was >4-fold for CD4\(^+\) T cells. No other Tlr family members were strongly down-regulated in 6.VIII CD4\(^+\) T cells, except for Tlr9 (2.2-fold). Statistical analysis on CD4\(^+\) T and CD8\(^-\) T cell subsets from individual female mice confirmed significant down-regulation solely for Tlr1 (p = 0.017 for Tlr1 and p > 0.18 for all other genes in both CD4\(^+\) T and CD8\(^-\) T cell populations; Mann-Whitney U test).

Tlr1 was strongly down-regulated in 6.VIII macrophages (n = 6, 3.2-fold; Table II). The down-regulation of Tlr5, Tlr6, and Tlr9 was also found to be significant albeit at lower levels (≤ 2.2-fold). When pooled RNA from four different mice was examined, the down-regulation of Tlr1 (5.1-fold; Fig. 4) and Tlr9 (1.7-fold) in CO macrophages could be confirmed, whereas that of Tlr5 (1.0-fold) and Tlr6 (1.2-fold) could not.

Our results indicate that Idd6 probably does not influence an overall mechanism that is common to the regulation of the entire Tlr family, but rather acts specifically on Tlr1 regulation with variations according to cell type and tissue.

Tissue-dependent expression of TLR1 protein in macrophages

The TLR1 has been shown to be expressed mainly in myeloid cells (23). In agreement with these data, both strong general Tlr1 other genes in both CD4\(^+\) T and CD8\(^-\) T cell populations (Mann-Whitney U test).

FIGURE 4. A. FACS analysis of TLR1 protein expression in CD11b\(^+\) macrophages isolated from spleen (>3,000 cells), PLNs, and peritoneum (>15,000 cells) of 8-wk-old female mice (n = 5). Anti-HCD8 was used for isotypic control. Values correspond to percentages ± SD; p values were obtained using the Mann-Whitney U test. Graphs show data for one representative individual of the five in each group. B. Quantitative PCR analysis of Tlr1, Il6, and Tnfa expression in peritoneal macrophages from four 4-wk-old 6.VIII and CO mice ± SD (Hprt reporter gene). AU, Arbitrary units; FC, fold change. * Differences for Tlr1 (p < 0.032) and Il6 (p < 0.0001) were significant in a second experiment using five individual animals (Mann-Whitney U test).
expression and expression differences for \( \text{Tlr1} \) were observed between NOD CO and 6.VIII macrophages. FACS analysis confirmed the results obtained by quantitative RT-PCR and showed that TLR1 protein expression was much lower in 6.VIII macrophage cell populations than in NOD CO macrophages (Fig. 4). Interestingly, this difference was stronger in peritoneal macrophages and macrophages isolated from pancreatic lymph nodes (PLNs) than in those isolated from spleen \((p < 0.01\) in the Mann-Whitney \(U\) test). We also noticed that there were more macrophages expressing high TLR1 levels in PLNs and the peritoneum than in the spleen. This result may indicate that TLR1 expression levels in macrophages depend on their tissue of origin.

Recently, macrophages from \( \text{Tlr1} \)-deficient mice stimulated with mycobacteria or with a mycobacterial 19-kDa lipoprotein were shown to have impaired production of TNF-\( \alpha \) and IL-6 (24). We therefore tested whether the constitutive down-regulation of \( \text{Tlr1} \) that we observed in the \( \text{Idd6} \)-congenic strain 6.VIII was associated with down-regulation of \( \text{Tnf-} \alpha \) and \( \text{Il6} \) expression using RNA pooled from four mice (Fig. 4). In this experiment, both genes were found to be down-regulated in peritoneal macrophages, and the \( \text{Il6} \) down-regulation was particularly striking. The data were confirmed using RNA from five individual mice with a \( p \) value of 0.095 for \( \text{Tnf-} \alpha \) and \( p < 0.0001 \) for \( \text{Il6} \). Because high levels of IL-6 can block immunosuppression mediated by CD4\( ^{+} \)CD25\(^{+} \) T cells (25, 26), the \( \text{Tlr1} \) and \( \text{Il6} \) down-regulation could be of some advantage to the activity of 6.VIII-derived CD4\(^{+} \)CD25\(^{+} \) T cells (9).

\( \text{Idd6} \) controls T cell proliferation and \( \text{Tlr1} \) down-regulation affects T cell proliferation

Previous in vivo results have shown that the \( \text{Idd6} \) locus affects the proliferation rates of T cells in the thymus (13). Our own unpublished results point to a more widespread control of T cell proliferation occurring in other tissues such as PLNs. This can be shown by in vivo proliferation assays using Ag-specific CD4\(^{+} \)CD25\(^{+} \) T cells from PLNs of NOD.BDC 2.5 mice, which show significantly higher proliferation rates in the PLNs of injected NOD CO mice than in PLNs of injected 6.VIII mice. Control of T cell proliferation could be mediated by cell-intrinsic factors as well as by extrinsic factors.

\( \text{Idd6} \) also affects the activity of regulatory CD4\(^{+} \)CD25\(^{+} \) T cells in the peripheral immune system (9) and extrinsically controlled down-regulation of T cell proliferation could, at least in part, be due to the higher activity of Tregs in 6.VIII mice. The TLR2 pathway has recently been implicated in the regulation of proliferation rates and activity of CD4\(^{+} \)CD25\(^{+} \) Tregs (27). Because TLR1 dimerizes with TLR2, we studied whether \( \text{Tlr1} \) down-regulation in 6.VIII mice affects T cell proliferation (Fig. 5). Both FACS-sorted CD4\(^{+} \)CD25\(^{+} \) and CD4\(^{+} \)CD25\(^{+} \) T cells were stimulated in vitro by anti-CD3 Abs in the presence or absence of hIL-2 and/or the TLR2 ligand Pam3CSK4. No general deficiency in T cell activation was observed and neither the TCR pathway nor the IL-2 pathway was significantly affected. The TLR1/TLR2 pathway was however strongly affected and proliferation was down-regulated in both 6.VIII CD4\(^{+} \) T cell subsets. This effect could not be overcome by additional stimulation of the IL-2-mediated pathway.

Our data suggest that the down-regulation of \( \text{Tlr1} \) is associated with lowered proliferation of stimulated CD4\(^{+} \) T cells. In the case of pathogenic CD4\(^{+} \)CD25\(^{+} \) T cells, this may contribute to a increased basal immune activation threshold in 6.VIII mice. Because rapid cellular expansion diminishes their immunoregulatory function, the lower proliferation rates of CD4\(^{+} \)CD25\(^{+} \) T cells might allow these cells to maintain their suppressor activity for longer (27).

Discussion

TID results from the autoimmune destruction of insulin-producing pancreatic \( \beta \) cells. A widely accepted scheme for defective aut immunity postulates that environmental factors somehow foul up the immune system by triggering the activation and expansion of autoreactive lymphocytes. Although the role of environmental factors in modulating diabetes development has been clearly established for many experimental models of TID, the role of a unique triggering event, however, is still disputable. TID is a multifactorial disease in which environmental factors concur with a highly multigenic susceptibility background to allow the escape from immune tolerance of islet \( \beta \) cells. A possible hypothesis is that numerous genes assemble to contribute to the stochastic activation of the autoimmune reaction to \( \beta \) cells. Immune activation through TLRs might be expected to contribute to the overall immune activation of this complex genetic set up, irrespective of whether the activation is provoked by internal or external ligands. Our data open up the possibility that the TLR1 is such one contributing factor.

Members of the TLR family are involved in early responses to pathogens that allow the activation of the immune system via a NF-\( \kappa B \)-dependent pathway. Some of these receptors are known to be involved in autoimmune diseases and especially in their initial triggering, either through direct responses to pathogens such as viruses within a target tissue (5) or through activation by endogenous ligands such as necrotic cells, DNA, or RNA in systemic autoimmune diseases (5).
TLR1 is involved in the innate immune responses to the outer surface lipoprotein A of the Lyme disease pathogen *Borrelia burgdorferi* (28). Activation and regulation of TLR1 has also been confirmed to have a role in human leprosy. TLR1 can form heterodimers with TLR2 that are responsible for the cellular activation mediated by *Mycobacterium leprae* and triacylated lipoproteins (29). Macrophages from *Tlr1*-deficient mice stimulated with mycobacteria or with a mycobacterial 19-kDa lipoprotein show impaired production of TNF-α and IL-6 (24) due to deficiencies in the NF-κB activation pathway. The importance of the TNF-α and IL-6 molecules in the regulation of the immune system may be supportive of a role of TLR1 in autoimmune disease.

The T1D-associated *Iddd* locus overlaps with two other autoimmune loci, including the experimental autoimmune myocariditis locus *Eamdc2* (30) and the lupus susceptibility locus *Lmw4* (31, 32). We previously showed that *Iddd* is capable of modulating the activity of regulatory CD4⁺CD25⁺ T cells (Tregs). The regulatory function of this cell population is known to be mediated by cell-intrinsic factors, such as the transcription factor Foxp3 (33), and to be dependent on interaction with APCs and soluble molecules. In particular, IL-6 has been shown to override the suppressive effects of CD4⁺CD25⁺ Tregs (34). The constitutive low Il6 expression observed for 6.VIII strain macrophages correlates with this finding and the observation of enhanced activity of this Treg population in 6.VIII mice. Recently, it has been shown that the expansion and suppressive activity of CD4⁺CD25⁺ Tregs is directly controlled by TLR2. TLR2-deficient mice contain significantly fewer Tregs, and administering TLR2 ligands to wild-type mice results in increased Treg numbers. In the presence of TLR2 ligand, the suppressive phenotype of Tregs is temporarily abrogated, thereby enabling the enhancement of the immune response in vitro and in vivo. Following removal of the TLR2 trigger, in vitro-expanded Tregs then fully regain their phenotype and suppressive capabilities (35). These data establish a direct link between TLRs and the control of immune responses through Tregs. Such findings and our present results lead us to suggest that the TLR1/TLR2 pathway may be involved in the *Iddd* Treg phenotype. This hypothesis is also supported by the finding that the *Iddd* 3. subinterval contributes strongly to the *Tlr1* expression phenotype. *Iddd* 3 has recently been shown to control diabetes resistance in splenocyte transfer experiments, since *Iddd* maps to the Idd6.3 subinterval contrib-

**Disclosures**

The authors have no financial conflict of interest.

**References**


