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Vk polymorphisms in NOD mice are spread throughout the entire immunoglobulin kappa locus and are shared by other autoimmune strains

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Abstract

The diversity of immunoglobulin (Ig) and T cell receptor (TCR) genes available to form the lymphocyte repertoire has the capacity to produce a broad array of both protective and harmful specificities. In type 1 diabetes (T1D), the presence of antibodies to insulin and other islet antigens predicts disease development in both mice and humans, and demonstrate that immune tolerance is lost early in the disease process. Anti-insulin T cells isolated from T1D-prone non-obese diabetic (NOD) mice use polymorphic TCR α chains, suggesting that the available T cell repertoire is altered in these autoimmune mice. To probe whether insulin-binding B cells also possess polymorphic V genes, Ig light chains were isolated and sequenced from NOD mice that harbor an Ig heavy chain transgene. Three insulin-binding V κ genes were identified, all of which were polymorphic to the closest germline sequence matches present in the GenBank database. Additional analysis of over 300 light chain sequences from multiple sources, including germline DNA, shows that polymorphisms are spread throughout the entire NOD Ig κ locus, as these polymorphic sequences represent 43 distinct V κ genes which belong to 14 V κ families. Database searches reveal that a majority of polymorphic V κ genes identified in NOD are identical to V κ genes isolated from SLE-prone NZB $\times$ NZW F1 or MRL strains of mice, suggesting that a shared Ig κ haplotype may be present. Predicted amino acid changes preferentially occur in CDR, and thus could alter antigen recognition by the germline B cell repertoire of autoimmune versus non-autoimmune mouse strains.

Keywords

B cell; Type 1 diabetes; Autoimmunity; Immunoglobulin kappa; Light chain

Introduction

Autoimmunity arises from a breach in tolerance in one or more branches of the immune system, which results in the production and survival of autoreactive cells. Autoimmune damage caused by these cells manifests in a diverse array of autoimmune diseases which afflict 5–8% of the population (The Autoimmune Diseases Coordinating Committee and National Institutes of Health. Progress in Autoimmune Diseases Research 2005). Autoantibodies are present in the prodrome of many autoimmune diseases, including type 1 diabetes (T1D), in which the presence of islet-reactive autoantibodies are predictive of disease development in both humans and mouse models of disease (Yu et al. 2000). These autoantibodies provide direct evidence that B cell tolerance is interrupted in T1D, as effective tolerance should prevent autoreactive B cells from differentiating into the effector plasma B cell stage.

The antigen specificity of a B cell is determined by rearrangement of immunoglobulin gene segments to produce functionally expressed heavy and light chains, which pair to generate a B cell receptor (BCR). Factors which could contribute to the formation of autoreactive B lymphocytes therefore may include differences in the germline repertoire of these gene segments, as well as ineffective removal of autoreactive clones from the mature repertoire. Antigen specificity of T and B cells has been shown to be important for promoting T1D development (Hulbert et al. 2001; Lennon et al. 2009; Silveira et al. 2002). Interestingly, Simone, et al. has found that the T cell receptor α (TCR α) repertoire expressed by anti-insulin T cells is highly restricted and polymorphic in the non-obese diabetic (NOD) mouse (Simone et al. 1997a, b). As TCR α and BCR light chains are structurally similar, we were therefore interested in identifying whether the NOD BCR light chain locus may also be polymorphic. Furthermore, understanding the relationship between germline sequences and V region structures expressed by autoantibodies has important consequences for assessing affinity maturation and T cell dependence.

The studies presented here demonstrate that insulin-binding light chains isolated from NOD mice are polymorphic to germline V κ gene sequences available in the IgBLAST database that is referenced to genome sequencing of C57BL/6 and other common non-autoimmune strains. Polymorphisms do not appear to be restricted to insulin-binding V κ , as sequencing of over 300 V κ clones isolated from various organs as well as germline DNA in NOD mice revealed 43 distinct NOD V κ genes, nearly all of which are polymorphic to reference germline sequences. In striking contrast, the majority of NOD V κ genes were found to be 100% identical to one or more light chains found to be expressed by systemic lupus erythematosus (SLE)-prone MRL/lpr or NZBxNZW F1 mouse strains. The nt differences in the NOD strain encode aa changes that are observed throughout FWR and CDR, but which are preferentially found in the CDR. We therefore propose that portions of the Ig κ haplotype of the NOD mouse may be shared among several autoimmune disease-prone strains of mice, with the potential consequence that alterations in the antigen specificity of the germline repertoire could exist.

Materials and methods

Animals

Conventional (wild-type) NOD mice used in these studies were originally obtained from Taconic Laboratories. The anti-insulin VH125Tg [Cg-Tg(Igh-6/Igh-V125)2Jwt/JwtJ], The Jackson Laboratory) heavy chain transgenic mice used in this study harbor a non-targeted VH transgene on NOD backgrounds as described previously (Hulbert et al. 2001; Rojas et al. 2001). All data are derived from lines that have been backcrossed >20 generations to NOD, and which are hemizygous for all transgenes indicated. All mice were housed under sterile housing conditions, and all studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Hybridoma generation

VH125Tg NOD mice were immunized intraperitoneally with 0.6 mg of porcine insulin covalently coupled to *Brucella abortus* ring test antigen (obtained from the US Department of Agriculture, Ames, IA), 100 μ L injection volume per animal. The coupling procedure was carried out using insulin acylated with m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co.) and *B. abortus* thiolated with methyl-4-mercaptobutyrimidate (Pierce). Splenocytes were harvested after 3 d and fused using polyethyleneglycol to myeloma cells to generate hybridomas (Thomas et al. 1997, 2002). This approach has uniformly identified germline-encoded antibodies. To detect the production of anti-insulin antibody, 96-well microtiter plates (Immulon II) were coated with 1 μ g/mL human insulin in 1 $\times$ borate buffered saline overnight at 37°C. Plates were washed and hybridoma supernatants were applied and incubated 1 h at room temperature. For competitive inhibition of binding, 50 μ g/mL soluble insulin was incubated with hybridoma supernatants. Isotype-specific goat anti-mouse IgM or IgG conjugated to alkaline phosphatase (Southern Biotech) was used for detection. Anti-insulin clone 1F11 (mAb301) was used as a positive control for IgM (Thomas et al. 1997) and mAb125 was used as a control for IgG (Thomas and Hulbert 1996) anti-insulin antibody. All dilutions and washes were conducted at room temperature using 1 $\times$ phosphate buffered saline (PBS) plus 0.2% Tween 20. Results are reported as mean OD₄₀₅ for using paranitrophenyl phosphate (Sigma-Aldrich) as substrate and an automated ELISA reader. Hybridomas producing anti-insulin antibody were cloned by limiting dilution and expanded. ELISA was used to confirm insulin-binding antibody (as above), and RNA was purified from expanded clones and V κ genes were amplified and sequenced as described below.

Cell isolation and flow cytometry

Bone marrow was eluted from the long bones of VH125Tg NOD mice with HBSS (Invitrogen) plus 10% FBS (HyClone). Red blood cells (RBC) were lysed and cells were resuspended at 2×10^6 cells/ml in complete bone marrow culture medium [DMEM plus 10% FBS (HyClone) plus L-glutamine plus HEPES buffer plus MEM sodium pyruvate plus gentamicin plus 2×10^{-5} M 2-ME (Invitrogen) plus 15 ng/ml human rIL-7 (PeproTech)] and cultured for 5 days in a 37°C CO₂ incubator to enrich for B cells. Antibody reagents reactive with B220 (6B2), IgM^a (DS-1), 7-aminoactinomycin D (7AAD), or 4', 6-diamidino-2-phenylindole (DAPI) were used for flow cytometry (BD Pharmingen). Insulin (Sigma) was biotinylated at pH 8.0 in bicine buffer using biotin *N*-hydroxysuccinimide ester (Sigma) and detected with fluorochrome-labeled streptavidin (BD Pharmingen). RNA was subsequently isolated from insulin-binding B cells sorted through flow cytometry with a BD FACS Aria cell sorter. Spleens were harvested and red blood cells were lysed, and splenocytes were resuspended. RNA was directly purified from splenocytes, or following additional purification (flow cytometry sorting of insulin-binding B cells, or separation of B cells bound to plate-bound insulin, as described previously by Woodward and Thomas 2005). Pancreata and pancreatic lymph nodes were harvested as previously described (Kendall et al. 2007) and placed directly into RNeasy lysis buffer (Qiagen). For islet isolation, pancreata were macerated in 1 ml of HBSS. Tissue was agitated with 3 mg/mL collagenase P in HBSS for 12 min at 37°C. Pancreatic material was washed and islets were hand-picked using a dissecting microscope. Islets were placed in culture overnight at 37°C with 5% CO₂, and islets with extruded lymphocytes were selected and placed into RNeasy lysis buffer (Kendall et al. 2007). Germinal center B lymphocytes from pancreatic lymph nodes were sorted with a FACS Aria Cell Sorting System (BD Biosciences), gating on GL7⁺ B220⁺ 7-aminoactinomycin D (7AAD)⁻ lymphocytes. WinMDI 2.8 software (Dr. J. Trotter, Scripps Institute, San Diego, CA) was used for analysis. RNA extraction was performed with the RNeasy spin kit (Qiagen), following the manufacturer's instructions, or using Tri Reagent (Molecular Research Center) for some of the spleen samples.

Determination of V κ usage

First-strand cDNA was generated from total RNA using Superscript II RT (Invitrogen) and 0.67 μ g oligo-dT primer (Amersham Biosciences) in a standard protocol. V κ sequences were amplified from first-strand cDNA using the following primers: murine C κ primer—5'GGA TAC AGT TGG TGC AGC ATC and murine V κ A—5'ATT GTK MTS ACM CAR TCT CCA, V κ B, 5'-GAT RTT KTG RTR ACB CAR RM, or V κ C, 5'-AYA TYN WGM TGA CHC ARW CTM M, where K = G or T, M = A or C, S = C or G, R = A or G. Individual clones are each derived from independent pools of RNA. V κ sequences were amplified, cloned, and sequenced as described previously (Woodward and Thomas 2005). V κ gene segment sequence alignments (excluding sequence provided by the degenerate 5' primer) were assigned using the ImMunoGeneTics (IMGT) database (www.imgt.cines.fr:8104/) and IgBLAST (<http://www.ncbi.nlm.nih.gov/igblast/>). Specific alignments are provided with the genomic C57BL/6 sequence, build 37.1 as reference. Accession numbers for representative sequences from each of the NOD V κ genes are provided in Table 2. Sequence alignments of clones included in this study are provided as online supplemental data.

Germline V κ genes from NOD tail DNA samples were amplified using the following temperatures in PCR: 94°C 1 min, 55, 57, or 60°C 1 min, 72°C 1 min, with the following primers for 40 cycles: V κ 1 GL FWD: TTT GCA TAT TGC TCC CTA GGG A, V κ 1 GL REV: TTG TTA GGG TCT GTATCA CTG TGG GA or V κ 4 GL FWD: TAT TTG CAT ATT TCA TTT TCA GTA ACC ACA, V κ 4 GL REV: TGT TCC AGT CTG TAT CAC TGT GTG; FWD primers recognized the conserved octamer regulatory sequence, REV primers recognized the heptamer and a portion of the nonamer of the RSS. Variation of the annealing temperature aided in amplification of different V κ genes. Germline genes were cloned, sequenced, and identified as above. Accession numbers for germline sequences deposited into GenBank are HM444791-HM444810.

Results

Insulin-binding NOD V κ genes are polymorphic to reference germline V κ genes

VH125Tg NOD mice, which harbor an anti-insulin heavy chain transgene, show an increased percentage of insulin-binding spleen B cells when compared to VH125Tg C57BL/6 mice (Hulbert et al. 2001; Woodward and Thomas 2005). Differences in the endogenous light chain repertoire between the two strains, as is found among insulin-binding, structurally homologous TCR α chains (Simone et al. 1997a, b), could help explain this difference. VH125Tg NOD mice were therefore immunized with insulin conjugated to *B. abortus* ring test antigen and hybridomas were generated from splenocytes harvested on d3. Hybridomas were cloned by limiting dilution and insulin-binding specificity was confirmed by screening the supernatants of the clones by ELISA (Fig. 1a). Three different insulin-binding V κ light chain sequences were identified from multiple hybridomas by RT-PCR. These sequences showed conserved nt changes when compared to germline sequences present in the IgBLAST and IMGT databases. For consistency, sequences were compared to the C57BL/6 reference strain, for which complete genome sequencing is available. Each of these light chains is observed to differ at multiple nucleotides within both framework (FWR) and complementarity determining regions (CDR), boundaries for which are designated by the Kabat system (Fig. 1b – d), when compared to counter-part C57BL/6 V κ genes 3–4, 4–57-1, and 4–74. Insulin-*B. abortus* activates B cells independent of T cell help; thus these finding suggest that either anti-insulin NOD V κ genes are polymorphic to C57BL/6 V κ genes, or that the immunization procedure captured mutated V κ genes. The term “polymorphism” is defined as follows: “genetic polymorphism is

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variability at a gene locus in which the variants occur at a frequency of greater than 1%” (Janeway et al. 2001), and refers to the observed nt changes present in the NOD strain compared with the C57BL/6 reference strain, many of which encode aa changes.

Polymorphisms are observed in NOD V κ genes throughout the Ig κ locus

To determine the origin of nt differences observed in the anti-insulin NOD V κ genes isolated from insulin-specific hybridomas, RNA was isolated from B cells harvested from multiple organs of non-immunized NOD mice, including the bone marrow, spleen, lymph nodes, and pancreas and V κ genes were amplified and sequenced. V κ sequences included in this analysis are those for which three or more independent sequences show 100% identity, either with other sequences determined in these studies or with sequences published in the 2009 GenBank database. Using these criteria, 43 unique V κ genes were identified which belong to 14 different V κ families based on the IMGT classification system (Table 1). Over 300 light chain sequences were isolated from various organs of non-immunized mice, as well as germline DNA; these studies thus provide the widest coverage of NOD V κ gene sequencing published to date. For ease, the closest reference C57BL/6 V κ gene match from the IMGT database for each NOD V κ gene, as well as the Zachau system designation are listed in Table 1.

The nt changes observed among the three insulin-binding hybridoma V κ genes depicted in Fig. 1 are shared among corresponding V κ clones derived from multiple organs isolated directly from non-immunized mice (SD Figs. 14, 19 and 24). Furthermore, nt changes are present in 93% of the NOD V κ genes identified (Table 1). The NOD V κ genes presented in Table 1 range from 91.2% to rarely 100% nt identity with the closest C57BL/6 germline matches (SD Figs. 1–43). The number of nt differences present range from 0 to 24, with an average of 8 nt differences per V κ gene (Fig. 2 and Table 2). The majority of the observed nt polymorphisms predict amino acid replacement, however silent changes are also predicted (Fig. 2). There were four pairs of NOD V κ genes which were distinct from each other, but which showed the highest sequence identity with the same reference germline V κ genes (V κ 1–133, V κ 1–135, V κ 3–4, and V κ 4–57-1), thus complicating assignment of independent V κ gene identities. The closest C57BL/6 match is thus insufficient to formally assign NOD V κ gene identities; rather, this sequence information will need to be combined with Ig κ locus position information derived from sequencing the entire NOD Ig κ locus to make individual gene assignments.

Despite originating from multiple different organs, it is possible that the nt changes observed in the NOD V κ gene sequences arise from somatic hypermutation (SHM), perhaps due to specific autoantigen-driven selection. However, nearly 70% of the identified NOD V κ genes show 100% sequence identity with other published V κ gene sequences identified from other autoimmune mouse strains, particularly NZBxNZW F1 and MRL/lpr mouse strains. As SLE results from targeting of a different cohort of autoantigens, it is improbable that all of the nt changes observed are due to SHM. Furthermore, the nt changes observed are also found in a panel of NOD V κ gene sequences produced by another laboratory, confirming that they are not specific to our colony of NOD mice (Table 1, Carrillo et al. 2008). To confirm that the nt changes observed were germline-encoded, and not due to SHM, light chains were amplified and cloned from NOD genomic tail DNA. The same nt changes that were observed in sequences isolated from B cells in various organs of the NOD mouse were present in 15/15 corresponding unique germline sequences retrieved from tail DNA (Table 2 and SD Figs. 2–11, 18–19, 21, 23, 25–26, and 33–34), confirming that the observed nt changes are germline-encoded polymorphisms. These data suggest that the majority of NOD V κ genes are polymorphic to the reference database of V κ genes compiled from C57BL/6 and other non-autoimmune strains of mice, many of which are identical to V κ sequences isolated from other autoimmune disease-prone NZBxNZW F1 and MRL strains of mice.

The nucleotide polymorphisms in NOD V κ genes are found in FWR, but occur most frequently in CDR

CDR are responsible for antigen-binding specificity of the antibody molecule, thus we were interested in identifying whether the NOD V κ nt differences occurred in these antigen-contacting regions. The location of nt changes were categorized as occurring in either FWR1, CDR1, FWR2, CDR2, FWR3, or CDR3, based on the Kabat system-defined boundaries for each germline V κ gene (Table 2). To analyze whether the nt differences were preferentially located in the FWR or CDR portions of the antibody molecule, the per site rate of nt changes was calculated. For example, the total number of nt changes in FWR1 was divided by the total number of nt in FWR1, and the average value observed for all 43 V κ genes was calculated to produce the average frequency of nt changes in FWR1. This was repeated for FWR2, FWR3, CDR1, CDR2, and CDR3. The average frequency of changes in FWR1 was divided by the sum of the average frequency of changes in all of the FWR and CDR to calculate the relative proportion of nt changes that were occurring in FWR1, which was repeated for the other regions. As shown in Fig. 3, the relative proportion of nt polymorphisms found in the CDR (69%) was greater than that found in the FWR (31%), and was distributed among each of the CDR1–3 without substantial bias. These data therefore show that nt differences are present in both the FWR and CDR, but are preferentially targeted to the CDR.

Amino acid replacements are found throughout the FWR and CDR, but occur most frequently in CDR

To identify whether the nt polymorphisms observed in the NOD V κ genes resulted in changes in the protein sequence, aa alignments of NOD and reference V κ genes were compared. The aa sequences of NOD V κ genes presented are predicted translations of experimentally determined nt sequences. In this analysis, replacement (R) or silent (S) changes were enumerated for each gene segment (Table 3) within Kabat-defined FWR and CDR boundaries. The number of amino acid changes per NOD V κ gene is shown in Fig. 4. The sum of R and S aa changes encoded by nt was calculated for all 43 V κ genes analyzed and these totals were divided to yield an average R/S for each FWR and CDR. The average R/S was as follows: FWR1: 0.8, FWR2: 1.9, FWR3: 1.0, CDR1: 3.9, CDR2: 3.2, CDR3: 12.8; the average R/S for FWR=1.3, CDR=6.6; Table 4). Further analysis of conservative (e.g., T>S) and non-conservative (e.g., E>K) aa changes was performed as follows: the sum of the total number of conservative and non-conservative aa changes for each NOD V κ gene in each FWR or CDR was calculated, as well as the percentage of non-conservative aa changes (number of non-conservative aa changes divided by the total # of aa changes) for each region, and these data are shown in Table 4. These data show that many of the aa replacements are non-conservative, that they occur more frequently in CDR, and that they are particularly concentrated in CDR3.

Discussion

The findings presented here suggest that many, and likely the large majority of NOD V κ genes are polymorphic to V κ genes in the reference database of common mouse strains and the sequenced genome of C57BL/6 mice. The sequences published here are believed to be germline, and not the result of SHM, as they are identical among clones isolated from independent animals and B cells derived from multiple NOD organs. Among the 43 NOD V κ genes analyzed, 15/15 unique corresponding sequences amplified from germline DNA confirm 100% of the polymorphisms observed in the other sequences (Table 2 and SD Figs. 2–7, 11, 18–19, 21, 23, 25–26, and 33–34). Furthermore they share identity with sequences found in other autoimmune disease-prone strains of mice such as MRL and NZBxNZW F1, which target a different cadre of autoantigens. Polymorphisms are observed in all of the different V κ families represented in this study, and are spread throughout the Ig κ locus (Table 1, Figs. 2 and 5). The NOD mouse therefore possesses a unique Ig κ locus that is polymorphic

to Igk reference sequences derived primarily from non-autoimmune strains, such as C57BL/6; however, portions are shared with SLE-prone mouse strains. It is possible that the altered available germline V κ genes may shape the repertoire of antigen specificities generated in the developing B cell repertoire, as the per site rate of nt changes and the ratio of R/S is increased for the CDR (Fig. 3 and Table 4). These polymorphisms may thus contribute to B cell autoimmunity in the NOD mouse; however, whether or not these changes enhance repertoire autoreactivity requires further investigation.

The presence of polymorphisms in the Igk locus have been previously reported in a widespread analysis of multiple strains (Kofler et al. 1989), however this is the first study to demonstrate the broad array of polymorphisms present in the NOD mouse. Many previously published V κ gene sequences derived from autoimmune strains were thought to be derived from SHM. In light of our findings that many of these “mutated” sequences show 100% identity with other autoimmune V κ sequences, we propose that in many cases, these differences arise from germline polymorphisms, rather than from SHM. It is unlikely that B cells isolated from different autoimmune mice share the same autoantigen specificity, as T1D and SLE arise from pathogenic targeting of a different cohort of autoantigens. Given the likely diversity in the antigens responsible for driving selection, it is improbable that SHM would produce identical nt changes in both T1D and SLE-prone strains of mice.

The IMGT and Kabat gene assignments given to particular NOD V κ genes are provided throughout figures and tables to give a helpful frame of reference. These assignments cannot yet be confirmed however, as NOD Igk locus has not yet been sequenced, and thus the position and therefore identity of individual NOD V κ genes needs to be verified. Many germline V κ genes do not greatly differ in nt sequence from one another, particularly among the V κ 4 family. Eight NOD V κ genes identified in this study (NOD V κ gene 6, 7, 8, 9, 13, 14, 19, 20, Table 1) showed the closest sequence identity to the same 4 reference C57BL/6 germline genes, despite clearly being distinct sequences from one another. For these reasons, the classification and positional assignment we have given to NOD V κ genes along the Igk locus in this report is tentative, as the high degree of polymorphism in many sequences prevents fully confident assignment. We compile here the results of multiple different sequencing efforts in our lab centered around the NOD mouse, therefore the relative abundance of given light chains is likely due to sampling bias for light chains of particular binding specificities not discussed here, rather than to bias of amplification of particular V κ genes.

As shown in Table 1, NOD V κ genes which match with published NZBxNZW F1 sequences typically do not also match with MRL sequences, and vice versa. Furthermore, if the proposed IMGT-based gene identity is considered and NOD V κ genes are arranged based on their proposed location in the Igk locus, NZBxNZW F1 sequences which match NOD sequences localize to the 5' portion of the locus, whereas MRL sequence matches localize to the 3' portion of the locus, proximal to the J κ segments (Fig. 5). Different strains of mice have been previously reported to possess different Igk haplotypes (Kofler et al. 1989). It is tempting to speculate that the acquisition of these polymorphic regions may have occurred differently among the three strains. Sequencing of the Igk locus has not been completed for NOD, NZBxNZW F1 or MRL strains of mice, thus the apparent positional bias could also be due to a bias in the sequences that have been deposited into GenBank. It is also possible that polymorphisms are not restricted to V genes in the NOD Igk locus, but may also extend to regulatory elements. If so, this might influence the efficiency of processes such as receptor editing which are critical for tolerance maintenance. Polymorphisms in the recombination signal sequence have previously been recognized to affect recombination efficiency of particular V κ genes, which ultimately influence host immunity (Atkinson et al. 1996; Feeney et al. 1996). Furthermore, MRL/lpr mice, which appear to share at least a portion of polymorphic V κ genes present in the NOD locus, show reduced receptor editing (Lamoureux et al. 2007). Complete sequencing of the

NOD Igk locus will therefore be useful in identifying whether such additional differences are present.

We find polymorphisms in 43 independent V κ genes spread throughout the Igk locus, many of which are shared among SLE-prone strains of mice. The disease relatedness of these findings is questioned by the observation that neither the Igk or TCR loci are present among the identified T1D disease susceptibility loci. However both B cell and T cell antigen specificity have been shown to play a key role in the development of T1D (Hulbert et al. 2001; Lennon et al. 2009; Silveira et al. 2002). It has previously been suggested that TCR α polymorphisms in the NOD mouse promote the formation of anti-insulin T cells to drive T1D (Simone et al. 1997b), and it is possible that the V κ polymorphisms identified may similarly skew the B cell repertoire in the NOD strain. Multiple tolerance defects in the NOD mouse may fail to cull B cell autoimmunity, which combine with multigenetic influences throughout other cellular compartments to promote T1D. We speculate that the presence of an autoimmune Igk haplotype might serve as a compounding risk factor for T1D development, and may thus potentially serve as a useful biomarker in identifying individuals who are at elevated risk for developing disease. However, further work is required to prove this possibility, as it is equally possible that the observed polymorphic V κ genes may not directly contribute to enhanced disease development in NOD mice, but may have simply been inherited alongside IDD loci genes involved in disease progression.

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Fig. 1.

Insulin-binding Vκ genes are polymorphic to C57BL/6 germline sequences. Hybridomas generated from VH125Tg NOD splenic B cells were cloned at limiting dilution. **a** Hybridoma clone supernatants were tested by ELISA to identify insulin-binding antibody production. Tenfold excess insulin competitor was incubated in solution with the supernatants to detect an antigen-specific interaction. The percent inhibitable binding is shown. **b–d** Sequence alignment of insulin-binding hybridoma sequences with the closest C57BL/6 germline sequence matches. Kabat-defined CDR are boxed, putative polymorphisms are indicated by asterisks

Fig. 2.

Polymorphisms are present in the majority of identified NOD Vκ genes and are spread throughout the Igκ locus. NOD Vκ genes for which three or more sequences that differed from each other by ≤ 2 nt changes are included in the analysis, the total number of sequences analyzed are indicated in Table 1. The closest C57BL/6 germline match was identified by IgBLAST and *Mus musculus* build 37.1. Chromosomal locations, and thus Vκ gene identity of NOD Vκ sequences are unconfirmed. Polymorphisms are defined as those nt which are conserved among NOD sequences and differ from the sequence of the closest C57BL/6 germline match. The total number of nt polymorphisms for each NOD Vκ gene is shown, aa replacement (*black*) and silent nt changes (*white*) are shown as portions of the total bar

Fig. 3.

The frequency of NOD V κ nucleotide changes is increased in CDR regions. The per site rate of nt changes in each FWR and CDR was calculated, e.g., the total number of nt changes in FWR1 was divided by the total number of nt in FWR1 for each of the NOD V κ genes, and the average value observed for all 43 V κ genes was calculated to produce the average frequency of nt changes in FWR1 (shown in parentheses). This was repeated for FWR2, FWR3, CDR1, CDR2, and CDR3. The average frequency of changes in FWR1 was divided by the sum of the average frequency of changes in all of the FWR and CDR to calculate the relative proportion of nt changes that were occurring in FWR1, which was repeated for the other regions, shown as percentage of the total

Fig. 4.

Nucleotide polymorphisms result in amino acid changes in NOD Vκ genes. NOD Vκ aa sequences are based on predicted translations from NOD nt sequences. The number of aa changes in each NOD Vκ gene is shown, along with the putative IMGT Vκ gene identity along the top of the graph. These data are compiled from results presented in Table 3

	Vk NOD Gene ID	IMGT Vk Gene	NOD Strains	GenBank Accession Numbers	
				MRL Strains	NZBxNZW F1 Strains
5' region of Igκ locus (distal to Jκ)	11	2-137	EU568253		AF072780
	8	1-135			Z22077, U55679
	9	1-135			Z22029, Z22092
	7	1-133	EU568274		AF321950
	6	1-133			
	5	1-132			U55685
	40	9-124	EU568242		AF321948
	34	1-122			Z22083, z22081
	4	9-120			
	33	1-117	EU568261		Z22035, Z22135
	3	14-126			
	2	1-110			M28132
	10	2-109	EU568267		
	42	16-104	EU568255	X14621, L09043, L09038	U87809
	41	15-103	EU568282		Z22052, U55584
	39	14-100			
	1	1-99	EU568245, EU568250		
	35	10-94	EU568273, EU568238	AF346760, AF441459, AY081858	
	43	19-93	AK157689	U27012	
	27	4-91			
	26	4-81		U28349, M18239, M18237	
	25	4-78	EU568264, EU568263	M20834, U21065	
	24	4-74		AF045525, AF045526, AF045523	
	23	4-70			
	22	4-69			
	21	4-59			
	19	4-57-1			
	20	4-57-1			
	18	4-56	EU568256	M31273	
	17	4-53	EU568252		
	16	4-50			
	38	12-46			
	29	5-45		AF139842	
	37	12-41	EU568265	U27003, U27010, AY081856	
	36	12-44	EU568244		
3' region of Igκ locus (proximal to Jκ)	28	5-37			
	30	6-32	EU568243, EU568270, EU568271	L16981, X14622, M24101	
	32	8-28	EU568281	L17135, L17137, L09029	
	31	8-24	EU568247		
	15	3-10		M31266, AY081862, X63809	
	13	3-4		L09042, L09040	
	14	3-4		X63812	
	12	3-1		U28352, L18942	

Fig. 5.

NOD Vκ sequence matches with NZB x NZW F1 Vκ cluster to the 5' region, whereas matches with MRL Vκ cluster to the 3' region of the Igκ locus. The closest C57BL/6 germline match was identified by IgBLAST, and NOD Vκ genes are positioned from the Jκ distal to Jκ proximal region of the locus as indicated, based on the positions of the C57BL/6 sequence matches in NCBI Map Viewer, *M. musculus* build 37.1. Chromosomal locations of NOD Vκ sequences are unconfirmed

Table 1

Light chain polymorphisms are present in the majority of NOD V κ genes, which are shared among other autoimmune strains of mice

NOD V κ Gene	Reference germline V κ match		Total number of sequence matches ^a	C57BL/6 % identity ^b	GenBank nucleotide accession numbers			Other strains
	IMGT	Zachau			NOD strains	MRL strains	NZBXNZW F1 strains	
1	1-99	cv1	5	98.2	EU568245, EU568250			
2	1-110	bb1	25	97.5			M28132	
3	1-117	cr1	16	99.6	EU568261		Z22035, Z22135	
4	1-122	bl1	6	98.6			Z22083, Z22081	
5	1-132	bi2	7	97.8				
6	1-133	bj2	9	99.3				
7	1-133	bj2	7	95.3	EU568274		AF321950	
8	1-135	bd2	4	96.4			Z22077, U55679	
9	1-135	bd2	5	95.3			Z22029, Z22092	
10	2-109	he24	3	96.5	EU568267			M80410 (M. m. castaneus)
11	2-137	hf24	5	93.7				
12	3-1	21-1	4	98.5		U28352, L18942		
13	3-4	21-4	4	96.7		L09040, L09042		
14	3-4	21-4	6	91.2		X63812		
15	3-10	21-10	6	99.3		M31266, AY081862, X63809		
16	4-50	4-50	3	97.3				
17	4-53	kh4	9	98.9	EU568252			
18	4-56	ao4	11	96.1	EU568256	M31273		
19	4-57-1	4-57	38	95.1				M15593-6 consensus (RF)
20	4-57-1	4-57	3	92.8				
21	4-59 ^c	kk4 ^c	3	97.9				
22	4-69 ^c	km4 ^c	5	96.6				
23	4-70 ^c	kn4 ^c	4	96.1				
24	4-74 ^c	ai4 ^c	39	94.7		AF045523, AF045525-6		

NOD V _k Gene	Reference germline V _k		Total number of sequence matches ^a	C57BL/6 % identity ^b	GenBank nucleotide accession numbers			Other strains
	IMGT	Zachau			NOD strains	MRL strains	NZBXNZW F1 strains	
25	4-78	ad4	17	97.7	EU568263, EU568264	M20834, U21065		
26	4-81	ag4	9	96.3		U28349, M18237, M18239		
27	4-91	kf4	5	95.1				
28	5-37	23-37	3	96.5				
29	5-45	23-45	8	99.6		AF139842		
30	6-32	19-32	8	95.8	EU568243, EU568270-1	L16981, X14622, M24101		M14361 (C.c58)
31	8-24	8-24	8	98.2	EU568247			
32	8-28	8-28	5	97.1	EU568281	L17135, L17137, L09029		
33	9-120	bv9	36	96.6				
34	9-124	cw9	38	98.5	EU568242		AF321948	
35	10-94	cp9	8	97.7	EU568238, EU568273	AF346760, AF441459, AY081858		
36	12-41	12-41	4	97.3	EU568265	U27003, U27010		AY437016 (NZM2410)
37	12-44	12-44	7	100.0	EU568244			
38	12-46	12-46	4	96.9				
39	14-100	cf9	4	99.6				
40	14-126	br9	9	100.0				AY436984 (NZM2410)
41	15-103	gr32	5	97.7	EU568282		Z22052, U55584	
42	16-104	RF	21	100.0	EU568255	X14621, L09038, L09043	U87809	AY437018, AY437031 (NZM2410)
43	19-93	gj38c	12	98.1	AK157689	U27012		

^aConsists of independent NOD sequences obtained from various organs, plus the number of published GenBank Sequences indicated, that differ from the representative NOD V_k sequences by 0-2 nt

^bThe percent identity of the NOD sequence with the C57BL/6 germline reference correlate was calculated by subtracting the total number of nt changes from the total nt length of the V_k gene, and dividing by the total length of the gene, excluding the sequence encoded by the degenerate 5' primer portion of FWR1 and J_k

^cTwo NOD V_k genes showed the same % sequence identity with an additional germline reference V_k gene, as follows: 21: 4-70/kn4, 22: 4-62/ab4, 23, 4-59/kk4, 24: 4-57-1/4-57

Table 2

Location of NOD polymorphisms and GenBank accession numbers

NOD V κ gene	Reference germline V κ match	Isolated from	Number of nucleotide polymorphisms ^d						Total # of nt Changes	C57BL/6 % Identity	GenBank accession number ^c
			FWR1	CDR1	FWR2	CDR2	FWR3	CDR3			
1	1-99	cv1	P	0	2	0	0	3	0	5	GU563166
2	1-110	bb1	BM, S, PLN, P, I, T	1	2	0	2	0	2	7	AY675527
3	1-117	cr1	S, P, PLN, T	0	0	1	0	0	0	1	GU563167
4	1-122	bl1	P, T	0	0	0	0	4	0	4	GU563168
5	1-132	bi2	S, P, T	0	1	3	0	2	0	6	GU563169
6	1-133	bj2	H, I, T	1	1	0	0	0	0	2	AY089720
7	1-133	bj2	S, P, PLN, I, T	5	1	2	1	3	1	13	GU563170
8	1-135	bd2	S	0	3	2	1	2	2	10	GU563171
9	1-135	bd2	S, H	2	1	1	1	5	3	13	GU563172
10	2-109	he24	S	1	1	2	1	1	4	10	GU563173
11	2-137	hi24	T	0	9	1	3	4	2	19	HM444797
12	3-1	2l-1	P, PLN	2	1	0	1	0	0	4	GU563174
13	3-4	2l-4	P, PLN	0	4	1	0	2	2	9	GU563175
14	3-4	2l-4	S, H, P	1	8	3	2	6	4	24	GU563176
15	3-10	2l-10	S, PLN	1	0	0	1	0	0	2	GU563177
16	4-50	4-50	S, P	1	1	1	1	3	0	7	GU563178
17	4-53	kh4	S, PLN, I	0	1	1	1	0	0	3	GU563179
18	4-56	ao4	S, PLN, P, I, T	0	1	1	3	5	0	10	GU563180
19	4-57-1	4-57	BM, S, H, PLN, P, I, T	2	2	1	2	1	5	13	GU179059
20	4-57-1	4-57	PLN, P, I	3	2	2	2	4	6	19	GU563181
21	4-59 ^b	kk4 ^b	T	0	1	0	1	1	3	6	HM444800
22	4-69 ^b	km4 ^b	PLN, P, I	0	1	3	0	5	0	9	GU563182
23	4-70 ^b	kn4 ^b	T	0	2	3	2	2	2	11	HM444801
24	4-74 ^b	ai4 ^b	BM, S, H	4	2	1	1	3	3	14	GU179060
25	4-78	ad4	S, PLN, P, I, T	3	1	1	0	1	0	6	GU563183

NOD V κ gene	Reference germline V κ match	Isolated from	Number of nucleotide polymorphisms ^a										Total # of nt Changes	C57BL/6 % Identity	GenBank accession number ^c
			IMGT	Zachau	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3					
26	4-81	ag4	S, I, T		0	2	3	1	4	0			10	96.3	GU563184
27	4-91	kf4	S, P		3	1	3	0	3	3			13	95.1	GU563185
28	5-37	23-37	S, PLN, P		0	2	3	2	0	2			9	96.5	GU563186
29	5-45	23-45	S, PLN, I		0	1	0	0	0	0			1	99.6	GU563187
30	6-32	19-32	H		2	3	1	0	4	1			11	95.8	AY089727
31	8-24	8-24	S, H, P, I		0	1	0	0	4	0			5	98.2	GU563188
32	8-28	8-28	S		1	2	2	0	2	1			8	97.1	*EU568281
33	9-120	bv9	BM, S, P, PLN, I, T		2	3	3	1	0	0			9	96.6	AY675540
34	9-124	cw9	BM, S, P, PLN, I, T		0	0	0	3	0	1			4	98.5	AY731704
35	10-94	cp9	S		0	3	1	1	1	0			6	97.7	*EU568238
36	12-41	12-41	-		0	0	0	2	1	4			7	97.3	*EU568265
37	12-44	12-44	S, PLN		0	0	0	0	0	0			0	100.0	GU563189
38	12-46	12-46	S, PLN, P		1	0	0	1	4	2			8	96.9	GU563190
39	14-100	cf9	S, PLN, P		0	0	0	1	0	0			1	99.6	GU563191
40	14-126	br9	S, PLN, P		0	0	0	0	0	0			0	100.0	GU563192
41	15-103	gr32	PLN		1	2	0	0	2	1			6	97.7	*EU568282
42	16-104	RF	I		0	0	0	0	0	0			0	100.0	GU563193
43	19-93	gj38c	S, PLN, P, I		0	1	3	0	1	0			5	98.1	GU563194

Organ designations refer only to sequences determined by our lab

BM bone marrow, *S* spleen, *LN* lymph node, *PLN* pancreatic lymph node, *P* pancreas, *I* islet, *T* tail DNA, *H* hybridoma

^aFramework (FWR) and complementarity-determining regions (CDR) are defined by the Kabat system

^bTwo NOD V κ genes showed the same % sequence identity with an additional germline reference V κ gene, as follows: 21, 22: 4-62/ab4, 23, 24: 4-57-1/4-57

^cSequences published by other labs are indicated by asterisks

Table 3

Location of NOD V κ nt polymorphisms which result in aa replacements (R), or are silent (S) in FWR and CDR

NOD V κ Gene	Reference germline V κ match		Number of replacement (R) or silent (S) nt polymorphisms ^a						C57BL/6% aa identity
	IMGT	Zachau	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3	
1	1-99	cv1	0	SR	0	0	SSS	0	98.9
2	1-110	bb1	S	SR	0	R ²	0	RR	95.7
3	1-117	cr1	0	0	S	0	0	0	100.0
4	1-122	bl1	0	0	0	0	SSSS	0	100.0
5	1-132	bi2	0	R	SRR	0	SS	0	96.7
6	1-133	bj2	S	S	0	0	0	0	100.0
7	1-133	bj2	SSSSS	R	RR	R	SRS	S	94.6
8	1-135	bd2	0	SRR	SR	R	RS	RR	92.4
9	1-135	bd2	R ²	R	RR	R	SRRRS	RR ²	90.2
10	2-109	he24	S	R	RS	R	RS	R ² R ²	93.6
11	2-137	hf24	0	RSRR ² R ²	S	R ³	RR ² S	R ²	91.0
12	3-1	21-1	RR	S	0	R	0	0	96.7
13	3-4	21-4	0	R ² RS	S	0	SS	RR	95.6
14	3-4	21-4	S	S R ² RR ² RR	RRS	RS	SR ² SRS	SRR ²	86.8
15	3-10	21-10	R	0	0	R	0	0	97.8
16	4-50	4-50	R	RR	R	R	RRR	0	90.7
17	4-53	kh4	0	S	R	S	0	0	98.9
18	4-56	ao4	0	R	R	R ² R	RR R ² S	0	91.9
19	4-57-1	4-57	SSSSS	RR	S	RS	S	S R ² RR	93.2
20	4-57-1	4-57	RRS	RR	SS	RS	RSRS	R ³ R ² R	88.6
21	4-59	kk4	S	0	0	R	S	R ² R	96.8
22	4-69	km4	0	R	SRR	0	RSRSR	0	93.2
23	4-70	kn4	S	R	RR ²	RR	SR	RR	91.5
24	4-74	ai4	RSR ²	R ²	S	S	R ² S	RRR	92.0
25	4-78	ad4	S R ²	R	S	0	R	0	96.6
26	4-81	ag4	0	RR	RRR	S	RSRR	0	91.0
27	4-91	kf4	SRS	S	R ² S	0	RRS	RRR	92.1

NOD Vκ Gene	Reference germline Vκ match		Number of replacement (R) or silent (S) nt polymorphisms ^a					C57BL/6% aa identity	
	IMGT	Zachau	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3	
28	5-37	23-37	0	RR	RRR	SR	0	R ² S	91.9
29	5-45	23-45	0	S	0	0	0	0	100.0
30	6-32	19-32	RR	RRS	S	0	RSRR	R	90.8
31	8-24	8-24	0	R	0	0	R ² SR	0	96.8
32	8-28	8-28	R	RS	RS	0	SS	R	95.7
33	9-120	bv9	RS	R ² R	RRR	R	0	0	92.0
34	9-124	cw9	0	0	0	R ² R	0	R	96.6
35	10-94	cp9	0	RSR	S	R	R	0	95.4
36	12-41	12-41	0	0	0	SR	S	RRR ²	95.4
37	12-44	12-44	0	0	0	0	0	0	100.0
38	12-46	12-46	R	0	0	S	RSSR	R ²	95.4
39	14-100	cf9	0	0	0	R	0	0	98.9
40	14-126	br9	0	0	0	0	0	0	100.0
41	15-103	gr32	S	RR	0	0	RS	R	95.4
42	16-104	RF	0	0	0	0	0	0	100.0
43	19-93	gj38c	0	R	R ³	0	S	0	97.7

^aEach S or R denotes a single nt polymorphism, R² denotes 2 nt changes which resulted in the same aa change, R³ denotes nt changes throughout the entire codon. FWR and CDR boundaries are defined by Kabat. Changes in aa sequence are based on predicted translations of nt sequences

Total number of replaced (R) and silent (S), as well as conservative and non-conservative amino acid changes present in FWR and CDR in NOD Vκ genes, based on predicted amino acid sequences

Table 4

	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3
Total R nt Polymorphisms ^a	19	54	33	29	43	51
Total S nt Polymorphisms ^a	23	14	17	9	41	4
Average R/S ^b	0.8	3.9	1.9	3.2	1.0	12.8
Total conservative aa changes ^c and (% of total changes)	13 (81%)	13 (29%)	10 (36%)	10 (43%)	19 (50%)	9 (24%)
Total non-conservative aa changes ^d and (% of total changes)	3 (19%)	32 (71%)	18 (64%)	13 (57%)	19 (50%)	29 (76%)

^aTotal number of R or S nt changes present in NOD Vκ genes 1–43 for each FWR and CDR

^bTotal R was divided by Total S for each FWR and CDR to calculate Average R/S

^cConservative aa changes are those for which a polar aa is changed to another polar aa, and so forth. Polar, non-polar, acidic, and basic were the categories which aa were subdivided into

^dNon-conservative aa changes are those for which a polar aa is changed to another category of aa, such as acidic aa, and so forth