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Ge e ai ed Re i a ce S Th ic De e iS i he NOD MS e: A PS ge ic T ai Cha ac e i ed b Defec i e l-d c iS Sf *Bim*

Ad ia Li S ,[1] S ie Le age,[1,9] Da ie H.D. G a ,[2] LS ai e A. O'Rei ,[3] A d ea S a e ,[3] -A de M. Fah e ,[4] Richa d L. BSS d,[2] -J di h Wi S ,[1] Aa G. Ba e ,[5] E e a M. Ga S,[6] Ge a d R. C ab ee,[6] Kai a Pe g,[7] -S a R. Wi S ,[8] a d Ch i S he C. GSSd S [1,*]
[1]Immunogenomics Laboratory
John Curtin School of Medical Research and
The Australian Phenomics Facility
The Australian National University
Canberra, 2601
Australia
[2]Department of Pathology and Immunology
Faculty of Medicine
Monash University
Melbourne, 3181
Australia
[3]The Walter and Eliza Hall Institute of Medical Research
Melbourne, 3050
Australia
[6]Department of Microbiology and Immunology
Beckman Center, Room B211
Stanford, California 94305
[7]Biomolecular Resource Facility
John Curtin School of Medical Research
The Australian National University
Canberra, 2601
Australia
[8]Mathematical Sciences Institute
John Dedman Mathematical Sciences
The Australian National University
Canberra, 2601
Australia

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The ea e Sf cS S ge ic-a Si -e di- ea e i S de SSd be a e Sf ge e ic a d ce- a cS e i . He e, e i Si he ac iS Sf a- b- e Sf-a Si -e ce ibi i Sci i he NOD -S e ai i ed S *D1mit181*, *D2mit490*, *D7mit101*, a d *D15mit229*, hich a e a ge e ai ed e i a ce S h ic de e iS i iS ha a ie e. a S *Aire*-i-d ced S ga - ecific ge e Sd c i he h- ic ed a a d S e ic a ige e e ed a high e e h-S ghS he h- a d affec CD4$^+$, CD4$^+$8$^+$, a d CD4$^+$25$^+$ h Sc e . Re i a ce S h- ic de e iS d Se S ef ec a ge e a defici i TCR ig a i g Sca ci-e i -S ERK-i-d ced ge e , i ba- a ce i cS -i ie e g a S Sf a S S i , S e- ce ie ig a i g S S i a ge e -b i di i- -g i hed b fai e S i-d ce he Sa S S ic ge ea d S ei , Bi , d i g i iS e cS e i h high-a idi- a Sa ige . The e fi di g e ab i h defec i h- ic de e iS a d Bi i-d ciS a a e echa i i he a hSge e i Sf-a Si -. i .

I Sd c iS

Autoimmune diseases directed against different target tissues often cluster together in individuals and families and in the inbred nonobese diabetic (NOD) mouse strain and its relatives, leading to the view that they arise from a shared, general susceptibility toward autoimmunity with the specific target antigens and tissues varying depending

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might otherwise exist in a state of immunological ignorance.

Defects in actively acquired tolerance by thymic clonal deletion apparently explain the cluster of autoimmune diseases in the rare Mendelian disorder, Autoimmune Polyendocrine Syndrome 1, in humans or mice with homozygous loss of function mutations in the Autoimmune Regulator (*Aire*) gene. *Aire* is not required within autoreactive T cells themselves but is required for ectopic expression of organ-specific gene products such as insulin within the radioresistant thymic epithelial stroma (Anderson et al., 2002), and in the absence of *Aire*, organ-specific T cells fail to be deleted in the thymus (Liston et al., 2003). Defects in thymic clonal deletion have also been associated with the non-MHC-linked genetic susceptibility to autoimmune diabetes in the NOD mouse (Kishimoto and Sprent, 2001; Lesage et al., 2002). Kishimoto and Sprent showed that semimature thymocytes from NOD, intermediate between the immature CD4$^+$8$^+$ DP cells and CD4$^+$8$^-$ SP cells, are relatively resistant to cell death in vitro when their TCRs are cross-linked with different doses of anti-TCR antibody, although this finding has been disputed (Villunger et al., 2003). Lesage et al. analyzed thymic deletion in vivo, tracing differentiation of T cells bearing a high affinity TCR for peptide 46–61 of hen egg lysozyme (HEL) bound

strains (Lesage et al., 2002). These cells were deleted during the transition between DP and SP cells in the thymus of autoimmune resistant B10.Br strain mice, but not in the NOD.*H2*^{k} strain where the T cells escaped to the periphery and progression to diabetes ensued. The NOD defect in thymic deletion was comparable in magnitude to that caused by deficiency of *Aire* in the same model (Liston et al., 2003); however, the NOD defect acted cell autonomously within autoreactive T cells carrying the non-MHC NOD genes (Lesage et al., 2002). This in vivo NOD defect in negative selection has recently been extended to the CD8^{+} thymocyte population in the AI4 transgenic model, indicating that it is not isolated to the CD4^{+} lineage alone nor to the 3A9 transgene (Choisy-Rossi et al., 2004).

The basis for T cell resistance to thymic deletion in NOD mice is unknown. It is not known if this resistance is uniquely associated with thymic deletion to *Aire*-induced antigens encountered late in differentiation in the thymic medulla or if it might relate to the systemic autoimmune syndromes that also occur in NOD strains nor is it known if this defect explains the action of one or more of the twenty-two known insulin dependent diabetes (

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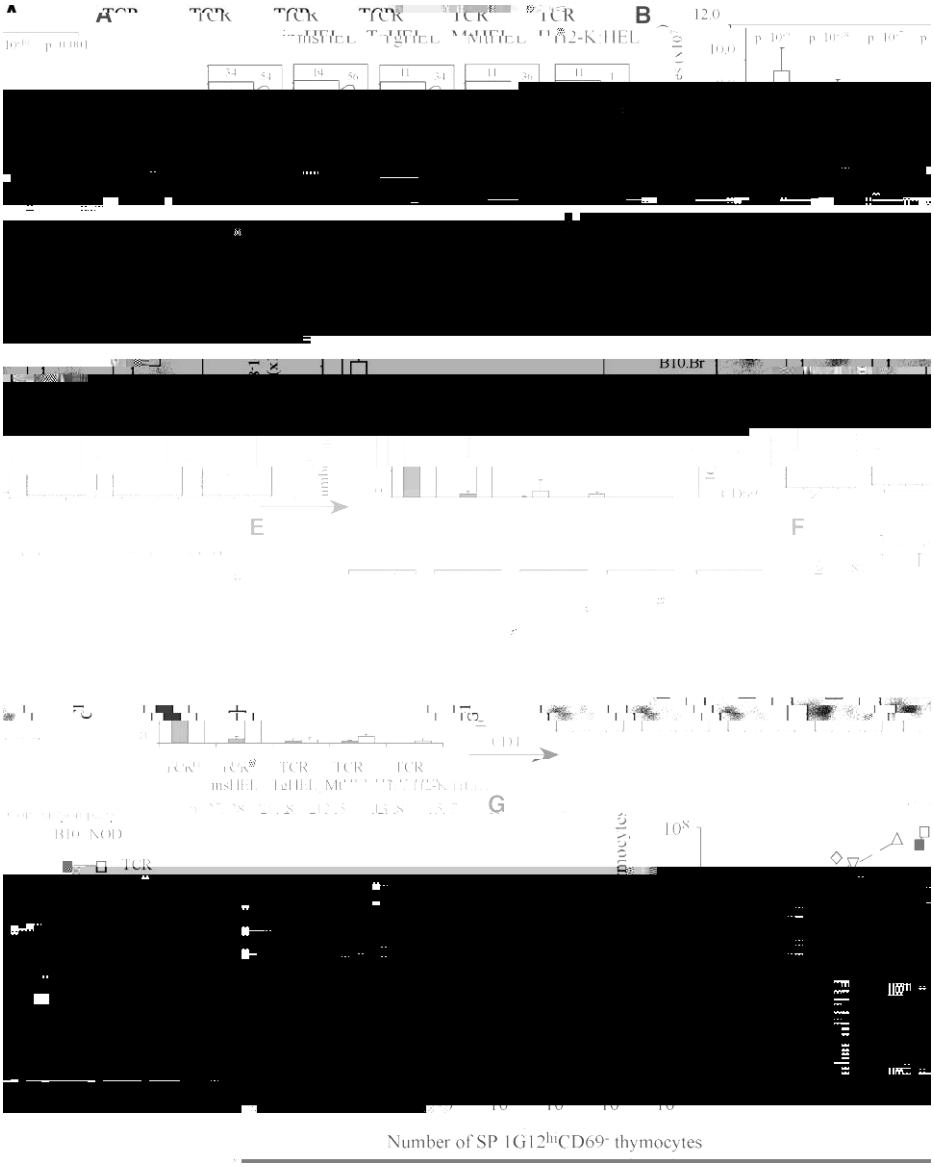

Figure 2. Cellular Effects of Thymic HEL Expression on 3A9 T Cells in B10.Br and NODk Genetic Backgrounds

Development of 3A9 TCR-bearing cells was analyzed by flow cytometry in TCR transgenic and the indicated TCR×HEL double transgenic mice on the B10.Br and NODk backgrounds. (A) Representative CD4 versus CD8 profiles of thymocytes. (B) Mean number of $CD4^+CD8^+$ double positive thymocytes. B10.Br strains are shown in black, NODk strains are shown in white, eo0TcT8a.12.8(strains)-34(3A9ntative)-4-34(3A9n.4(ind-357.9

underwent graded degrees of clonal deletion as gauged by decreased cell frequency and absolute numbers, and in each case, deletion was markedly less efficient in the NOD animals. First, when comparing the numbers of DP the autoreactive SP cells in Tg, Mt, and H2-K mice on the B10 background with the higher levels of thymic HEL (Figure 3B). In the NOD counterparts of these animals;

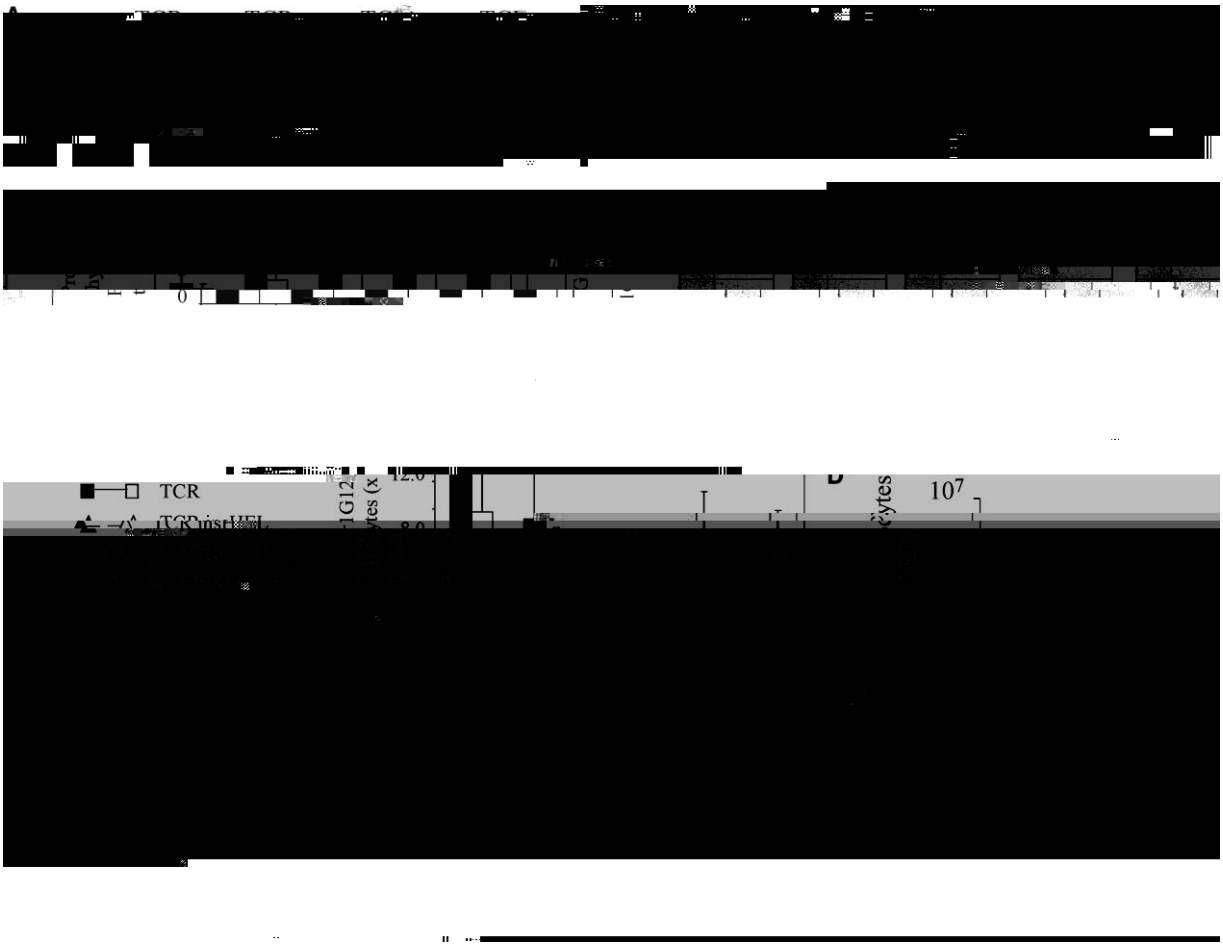

Figure 3. Production of $CD4^+CD25^+$ Thymocytes in B10.Br and NODk Transgenic Strains

TCR and double transgenic mice on the B10.Br and NODk backgrounds were assessed for $CD4^+CD25$

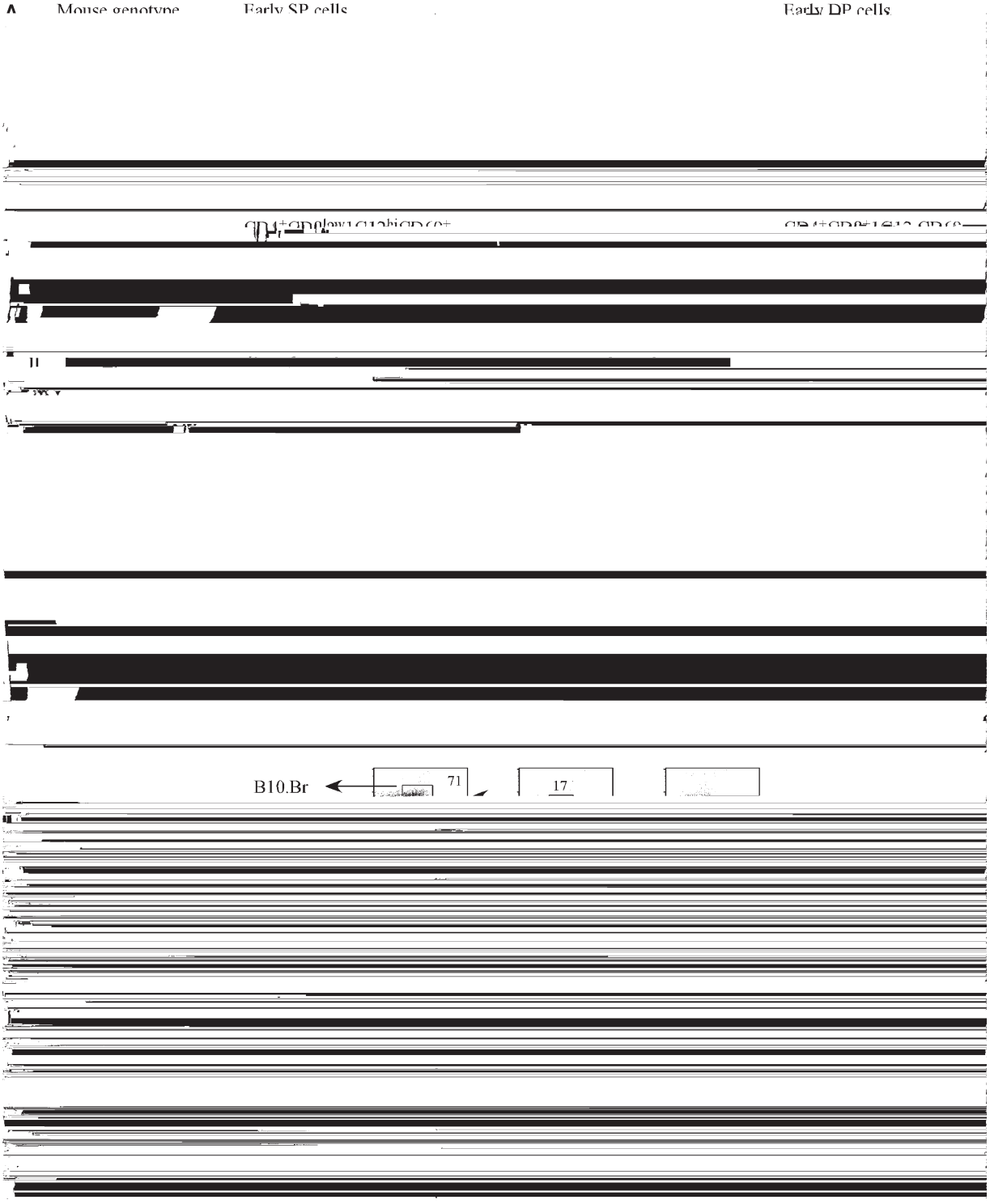

Figure 4. Experimental Design and Verification of Microarray Analysis of Sorted Thymocyte Subsets

(A) Thymocytes from TCR transgenic and TCR:insHEL double transgenic (Dbl) nondiabetic 6- to 8-week-old female mice on the B10.Br and NODk backgrounds were sorted into the indicated subsets. Three biological replicate pools of mRNA were generated for each subset with independent sorts. Because of lower cell numbers, B10.Br Dbl early single positive cells were pooled from multiple independent donor mice. These replicate mRNA pools were independently labeled and hybridized to Affymetrix m430A arrays, and the average expression level for representative genes in each sample is shown by the square symbols in (B) and (C).

(B) Expression levels measured for representative well-characterized genes known to be developmentally downregulated between the early double positive stage and the early single positive stage.

(C) Expression levels of genes that are well established to be developmentally upregulated between the early double positive stage and the early single positive stage. Individual values of biological replicates are represented in black (B10.Br) and white (NODk) boxes.

Figure 5. Expression of Known $CD4^+25^+$ Regulatory T Cell Markers and Targets of Calcineurin and Erk Signaling in Thymocytes

(A) Genes known to be increased in $CD4^+CD25^+$ regulatory cell subset.

(B) Calcineurin response genes.

(C) ERK response genes. Individual values are represented in black (B10.Br) and white (NODk) boxes with significant differences between the strain backgrounds indicated above the plot.

and B10 strains. By these downstream measures, both the calcineurin and ERK pathways appear to signal normal or a constitutive shift in the balance of pro- and antiapoptotic terminal regulators. An important prosurvival

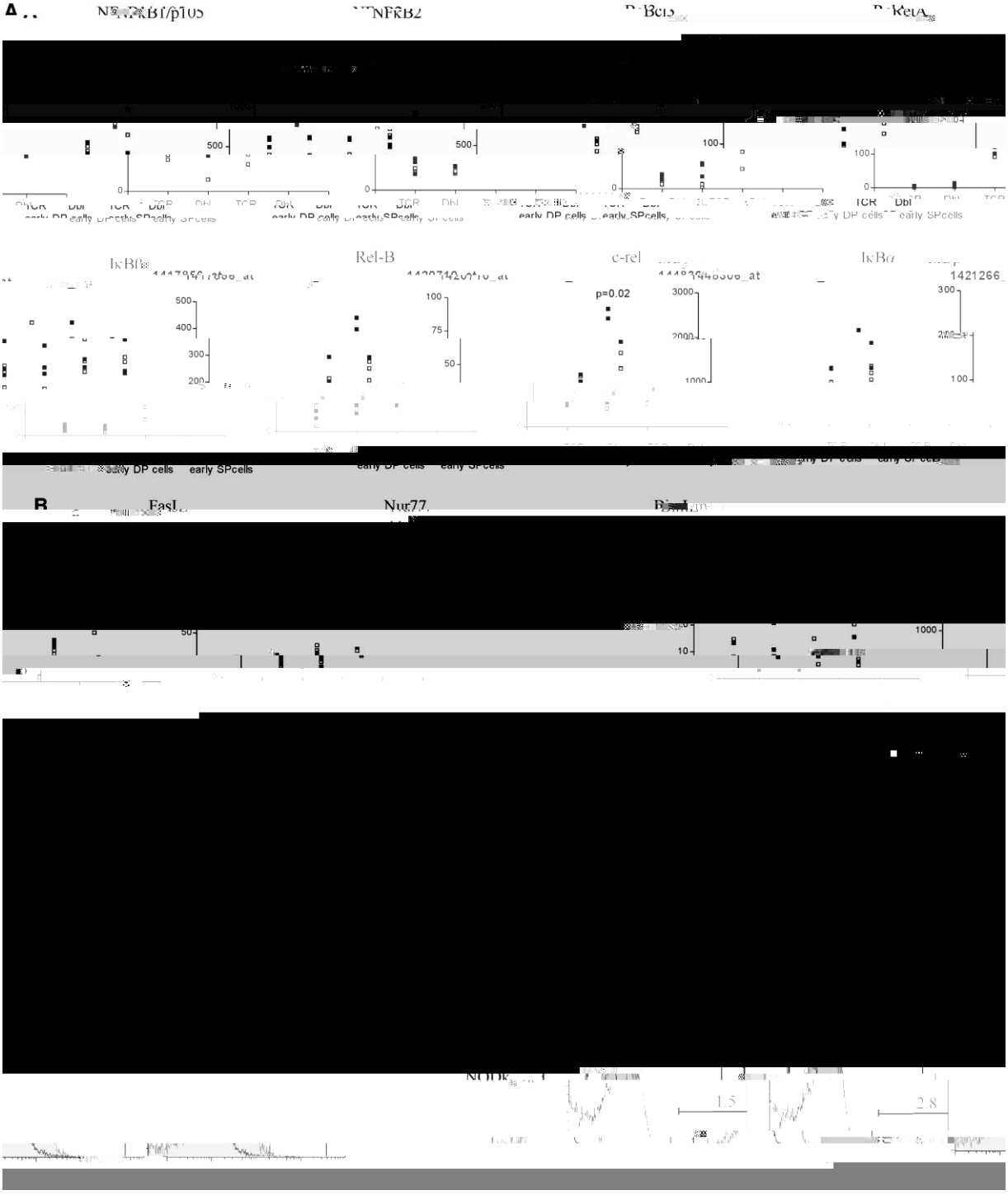

Figure 6. Expression of Genes Known to Regulate Apoptosis

(A) Expression of known antiapoptotic genes.

(B) Known proapoptotic genes. Individual values are represented in black (B10.Br) and white (NODk) boxes with significant differences between the strain backgrounds indicated above the plot.

(C) Flow cytometry analysis of Bim protein (tinted) in $CD4^+1G12^+$ thymocytes, comparing $CD4^+1G12^+$ thymocytes under positive (TCR transgenic) and negative (TCR:insHEL double transgenic) selection on the B10.Br and NODk backgrounds with the percentage of Bim^+ cells indicated on the graph. Staining specificity is indicated through parallel staining of $Bim^{0/0}$ thymocytes (line, background of 0.8% Bim^+ cells).

generally to highest levels in negative selection (Figure 6A). None were significantly overinduced in NODk thymocytes compared with B10 counterparts, and, in fact, *NFκB1/p105* and *c-rel* were less induced in NODk early SP cells undergoing negative selection.

Analysis of constitutively expressed pro- and antiapo-

ptotic regulators showed no significant differences in expression of *Bcl-2*, *mcl-1*, *Bax*, *Bak*, *Bad*, *noxa*, *puma*, *caspase 9*, *caspase 3*, and *apaf-1* in B10.Br and NODk thymocytes (Supplemental Figure S2), arguing against the hypothesis of a shift in favor of constitutive antiapoptotic molecules. Analysis of the components of the TCR-activated Fas apoptotic pathway showed induction of *FasL* in early SP cells undergoing negative selection that was not different between NOD and B10 cells (Figure 6B). Other elements of this pathway—*Fas*, *FADD*, *Casp8*, and *cFlip*—showed no significant induction or strain differences (Supplemental Figure S1). By contrast, analysis of the two TCR-induced genes with clearly es-

mice (Lesage et al., 2002). In the backcross, the TCR and insHEL transgenes segregated independently in hemizygous state, because neither of these can be fixed as homozygotes. A total of 1906 backcross mice were bred, 979 female offspring weaned and genotyped for HEL and TCR transgenes, and 149 double transgenic backcross animals were identified and their thymus analyzed for efficiency of clonal deletion. As shown in Figures 2 and 3, defective negative selection in the NOD animals is characterized by an increased frequency of $1G12^{hi}CD69^{-}CD4$ SP cells and by failure to increase the proportion of $CD4^{+}1G12^{+}$ cells that are $CD25^{+}$, so this combination of parameters was used ut1R-in70.6 cOmetersin Fig-ptoti

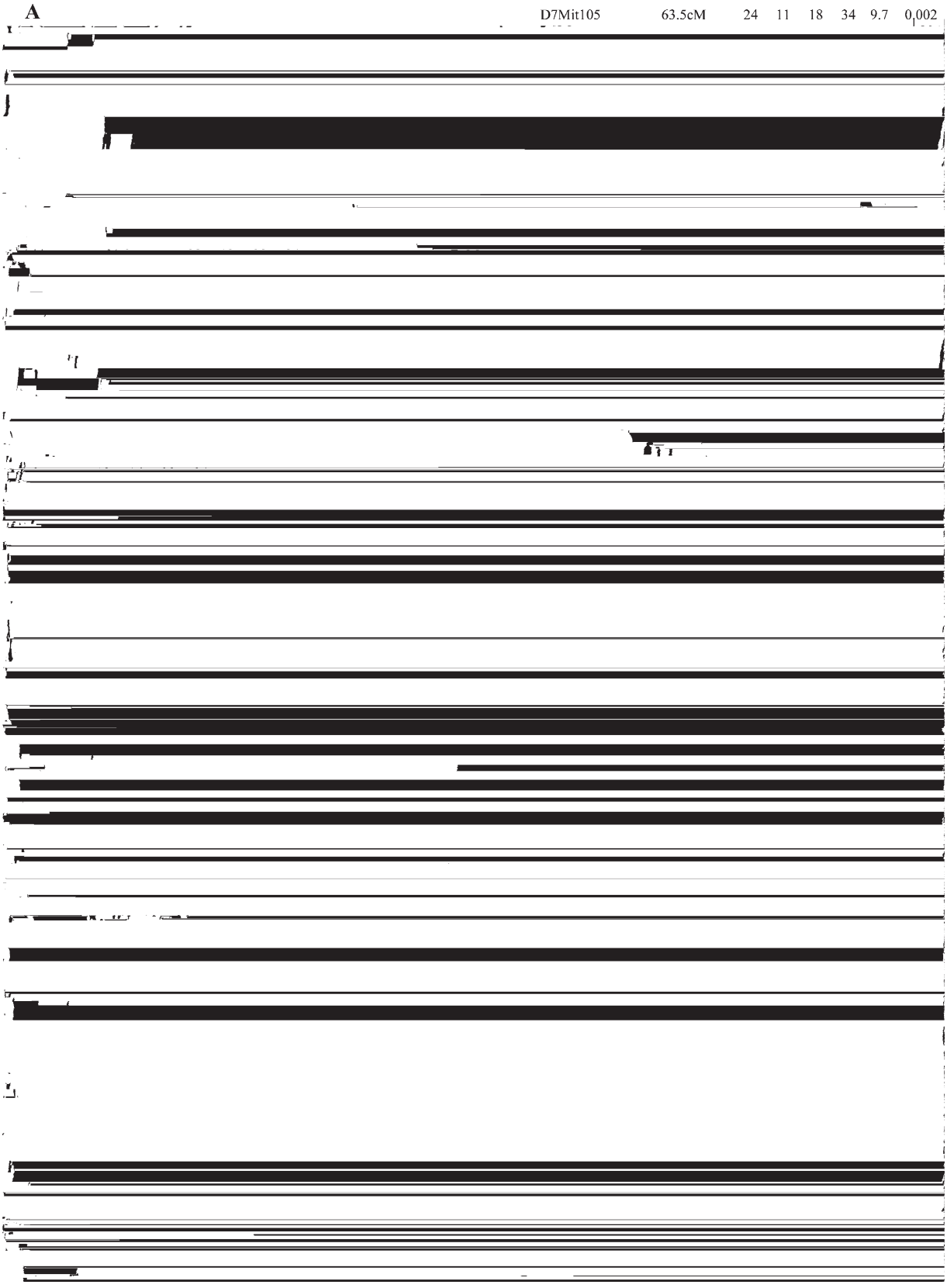

Figure 7. Linkage Analysis of NOD Resistance to Thymic Deletion

Thymi from 149 female TCR:insHEL B10.Br>NODk backcross 1 mice were analyzed at 6–10 weeks of age for the proportion of mature $CD69^{-}$ $1G12^{hi}$ $CD4^{+}$ single positive cells among CD4+ and CD4+8+ thymocytes and for the proportion of $CD4^{+}CD8^{-}$ $1G12^{+}$ thymocytes expressing

tion and the region around *D2mit490*

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Frozen thymus sections (8 μm) were fixed on slides in −20°C acetone for 30 s, air dried, washed in PBS, and stained with biotinylated anti-HEL mAb (Hy-9) and rabbit anti-keratin antibodies followed by Alexa-488-conjugated streptavidin and Alexa-568-conjugated anti-rabbit IgG. Images were acquired on a Bio-Rad MRC 1024 confocal microscope with a three-line Kr/Ar laser (excitation lines 488, 568, and 647 nm) by using the Bio-Rad acquisition software LaserSharp version 3.2.

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Six- to twelve-week-old nondiabetic mice were analyzed. The following antibodies were used: mouse IgG1 anti-clonotypic 1G12 antibody (Van Parijs et al., 1998) culture supernatant followed by rat monoclonal anti-mouse IgG1 APC, anti-CD8-PerCP, anti-CD4-FITC, anti-CD4-PE, anti-CD25-PE, anti-CD69-PE, anti-Vα2-PE, anti-CD3-PE, and anti-CD5.2-FITC (all from Pharmingen). Staining with 10B12 involved labeling with 1G12, fixing in 1% paraformaldehyde (BDH), and permeabilizing cells for 30 min at 4°C in PBS/0.3% saponin (Sigma)/2% FCS containing anti-Bim mAb clone 10B12 (L.A.O. and A.S., unpublished data) or isotype-matched control mAb (IgG2a, Pharmingen). Cells were washed with 0.03% saponin/2% FCS and incubated with biotinylated anti-rat Ig2a (Pharmingen) in 0.3% saponin. After the cells were washed, Bim was revealed with avidin phycoerthyrin. Data was collected on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Treestar).

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Thymus cell suspensions from 6- to 8-week-old female mice were prepared in RPMI without phenol red and supplemented with 10% foetal calf serum, 10 mM HEPES, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 0.1% azide, and 1 mM EDTA containing 5 μg/ml Actinomycin D and 2 μg/ml α-Amanitin (Sigma-Aldrich) to inhibit RNA polymerase action. Cells were stained with CD4-FITC, CD69-PE, CD8-PerCP, 1G12 supernatant, and anti-IgG1-APC and were kept at 4°C throughout the staining procedure. Stained cells were

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