

# Molecular Pathways Altered by Insulin B9-23 Immunization

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**ABSTRACT:** Type 1 diabetes (T1D) in the nonobese diabetic (NOD) mouse can be delayed by administration of insulin or specific insulin peptides. To better understand how insulin treatment delays diabetes development, NOD mice treated with an insulin peptide (B9-23) were compared with age-matched NOD and NOD congenic mice for gene expression changes in spleen using cDNA microarray. Fifty genes were identified that were significantly altered by B9-23 treatment. Thirty-three of these genes are downregulated by the treatment while they are upregulated during the natural disease progression in NOD from immature (3–4 weeks) to mature (10 weeks) stages. Taken together, our data suggest that the B9-23 treatment, like the protective genes in NOD congenic strains, reduces pro-inflammatory activation of lymphocytes that normally occurs in NOD mice. Furthermore, our studies discovered two genes (*Irf4* and *Tra1*) with increased expression in B9-23-treated mice that promote the Th2 response, providing a molecular basis for the B9-23-protective therapy.

**KEYWORDS:** NOD; microarray; B9-23 treatment; insulin; Th1/Th2 balance

## INTRODUCTION

Type 1 diabetes (T1D) arises from complex interactions between susceptibility genes and the environment, resulting in cellular and molecular changes. Extensive studies in the nonobese diabetic (NOD) mouse and human patients have shown that T cells, B cells, macrophages, and dendritic cells are implicated in the disease process.<sup>1–6</sup> Despite increased understanding of the disease, the underlying molecular mechanisms that lead to the development of T1D remain elusive. High throughput microarray technologies allowing simultaneous analysis of the expression of tens of thousands of genes have fundamentally changed how investigators can study the mechanism of disease. Our previous studies have used microarray technology to extensively profile splenic gene expression of NOD mice at different ages. Analysis of longitudinal expression profiles from NOD splenic cells helped establish a key transition (checkpoint) from immature to mature phenotype around 4–5 weeks of age.<sup>7</sup> This checkpoint includes many lymphocyte-specific genes involved in antigen

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presentation, antibody production, and cell proliferation, indicating an increase in immune activation which is consistent with an ongoing autoimmune response. A comparison of age-matched mice from NOD congenic strains protected from the development of diabetes, demonstrated that increased gene expression associated with disease progression in NOD was halted or reduced by protective genes present in the congenic NOD. This is consistent with the reduced autoimmunity in the congenic NOD that have almost no incidence of diabetes.

To further characterize the molecular mechanisms of disease development and protective therapy, we investigated the changes in splenic gene expression after immunization with insulin B chain peptide. Immunization with insulin, inactive insulin, or a peptide from the B chain of insulin (B9-23) have all been able to prevent or delay the onset of type 1 diabetes (T1D) in the NOD mouse model.<sup>8-10</sup> The protective effect is maintained despite the route of administration—whether oral, intranasal, intravenous, or subcutaneous.<sup>11-14</sup> Many possible mechanisms of action have been proposed including induction of tolerance to insulin, restoration of defective T cell function, and alteration of lymphocyte function by signaling through the insulin receptor.<sup>8,10</sup> However, the most popular theory is that insulin treatment induces Th2 cells which correct the imbalance in the immune system that promotes autoimmunity.<sup>9,11,14,15</sup> Our microarray results are consistent with these observations and identified two upregulated genes that may be responsible for the shift from a Th1 to Th2 immune response in B9-23–treated mice.

## STUDY CONCEPT AND DESIGN

A NOD subtractive splenic cDNA library previously created in our lab was arrayed onto poly-L-lysine–coated glass microscope slides to produce our mouse array 1 (MAR1) slides using methods previously described.<sup>7</sup> The overall goal of this study was to profile splenic gene expression of NOD mice treated with a peptide from the insulin B chain (B9-23) versus age-matched controls. Twenty-three NOD mice at 3.5 weeks of age were immunized with human recombinant insulin B chain, amino acids 9-23 (B9-23) diluted in isophane insulin diluent and mixed with incomplete Freund's adjuvant (IFA). Immunizations were subcutaneously administered in the inguinal, axillary, and dorsal neck regions. A booster set of injections was given 3 weeks later. Four, eight, and eleven mice were sacrificed 4, 11, and 21 days post booster injection, respectively. Splenic RNA was isolated from these mice along with 19 age-matched untreated controls. Total RNA (10 µg) from each sample was indirectly labeled with Cy5. A common reference RNA created from a pool of 10 NOD and 10 C57BL/6 mice was indirectly labeled with Cy3. Hybridizations using the MAR1 slides were performed in which each sample (Cy5) was combined with the common reference (Cy3) for the 16-h hybridization. Slides were then washed, scanned, and analyzed.

## INSULIN B9-23 TREATMENT DOWNREGULATES INFLAMMATORY ACTIVATION

The expression levels of all 23 B9-23–treated NOD mice were compared with four other groups of mice, including 19 age-matched NOD mice with mature pheno-

type (8–10 weeks), 34 immature NOD (3–4 weeks), 23 mature and 34 immature NOD congenic mice (NOD.B10 H<sup>2</sup>b and NOD.Idd3/Idd10, diabetes incidence <1%). The comparison between B9-23-treated mice and age-matched (mature) NOD revealed 50 differentially expressed genes that had a *P* value <0.008 (TABLE 1). Among these 50 genes, 33 were downregulated by B9-23 treatment. These 33 genes were upregulated in mature NOD mice compared to immature NOD mice during a key transition point for disease progression (TABLE 1). Interestingly, most of these genes were not upregulated in the NOD congenic mice from immature to mature ages. These results suggest that both protective genes and insulin immunization can halt the upregulation of gene expression associated with disease progression that normally occurs in the NOD mice. The similarity between expression profiles for mice protected by B9-23 treatment and protective genes in the congenic mice is extraordinary.

The genes downregulated by the B9-23 treatment belong to several interesting functional groups, including antigen processing and presentation, cell proliferation, signal transduction, and transcription/translation. Several genes involved in lymphocyte development and function are also downregulated. For example, IgkV28, Igj, and Igh-6 are decreased in the B9-23-treated NOD, indicating a decrease in B cell activation. Vcam1 known to be involved in autoimmunity through the regulation of lymphocyte migration<sup>16</sup> is also downregulated. The expression of a new member of the CD2 family (Ly108)<sup>17</sup> is decreased by B9-23 treatment. This gene, also known as NTB-A, is believed to be involved in T cell co-stimulation, and its engagement predominantly leads to development of Th1 rather than Th2 cells.<sup>18</sup> Several genes involved in inflammation are downregulated by B9-23 treatment. The first is S100a8, a gene upregulated during the inflammation process.<sup>19</sup> Another gene, Hif1a, plays an important role in macrophages during inflammatory cell recruitment.<sup>20</sup> The reduced expression of multiple genes involved in inflammation and lymphocyte activation indicates B9-23 treatment prevents or reduces pro-inflammatory activation. This complements our previous expression profiling data that suggested an activation in NOD lymphocytes during normal disease progression (from immature to mature phenotype).<sup>7</sup>

### **B9-23 TREATMENT UPREGULATES GENES RESPONSIBLE FOR THE TH2 RESPONSE**

B9-23 treatment also increased the expression of 17 genes (TABLE 1). While some of the genes upregulated by the treatment may be nonspecific and irrelevant to the protection against T1D, the increased expression of two lymphocyte-specific genes, Irf4 and Tra1, may provide novel insight into the molecular mechanism associated with protection conferred by insulin B9-23 treatment. Previous investigations have demonstrated that B9-23 treatment induces a shift from Th1 to Th2 profile for both the lymphocytes isolated from the lesion<sup>21,22</sup> and the spleen.<sup>23,24</sup> However, the molecular mechanism responsible for the cytokine profile shift is unknown. These two upregulated genes in B9-23-treated NOD mice may explain the Th2 profile observed in treated mice.

Interferon regulatory factor 4 (Irf4) was decreased threefold during the natural progression of autoimmunity in NOD mice as shown by our microarray analysis and

**TABLE 1. Gene expression changes in NOD, congenic NOD, and B9-23-treated NOD mice**

Name	B9-23/mNOD		B9-23/imNOD		mCON/imNOD		imCON/imNOD		mNOD/imNOD	
	ratio	p-value	ratio	p-value	ratio	p-value	ratio	p-value	ratio	p-value
Down-regulated by B9-23 treatment										
Antigen processing and presentation										
Hspa8	0.4	0.002	0.9	NS	1.0	NS	1.0	NS	2.4	0.0001
Cst3	0.6	0.003	1.5	0.0002	2.5	<1E-07	1.2	0.03	2.8	2.11E-07
H2-Aa	0.7	0.0009	0.9	NS	1.1	NS	1.1	NS	1.3	0.0007
histocompatibility 2, class II antigen A, alpha										
Lymphocyte development and function										
IgkV28	0.6	0.007	2.7	<E-07	3.7	<1E-07	1.4	0.01	4.6	<1E-07
immunoglobulin kappa chain variable 28 (V28)										
Igj	0.6	0.006	2.7	<1E-07	3.5	<1E-07	1.6	0.003	4.4	<1E-07
Igh-6	0.6	0.006	2.0	1.6E-07	2.5	<1E-07	1.4	0.002	3.2	<1E-07
Vcam1	0.6	0.0002	1.1	NS	1.4	0.002	1.3	NS	1.8	0.00002
Ly108	0.7	0.004	1.2	NS	2.1	0.006	1.9	0.0	1.8	0.04
Inflammation										
Hif1a	0.4	0.002	0.9	NS	0.9	NS	1.8	0.0002	2.3	0.0008
hypoxia inducible factor 1, alpha subunit										
S100a8	0.5	0.001	0.6	0.03	1.1	NS	0.9	NS	1.1	NS
Tmsb4x	0.6	0.002	2.1	<1E-07	2.8	<1E-07	1.2	NS	3.9	<1E-07
Cell proliferation										
Mki67	0.6	0.008	1.2	NS	1.1	NS	1.1	NS	2.0	8.28E-06
antigen identified by monoclonal antibody Ki 67										
Ppm1g	0.6	0.002	1.0	NS	1.2	NS	1.2	NS	1.6	0.01
protein phosphatase 1, G gamma isoform										

TABLE 1. (*continued*) Gene expression changes in NOD, congenic NOD, and B9-23--treated NOD mice

Name	B9-23/mNOD		B9-23/imNOD		mCON/imNOD		imCON/imNOD		mNOD/imNOD	
	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value
Signal transduction										
Grb2 growth factor receptor bound protein 2	0.5	0.006	1.8	0.0005	2.6	6.2E-07	1.1	NS	3.9	2.11E-06
Transcription/ translation										
Naca nascent polypeptide-associated complex alpha polypep.	0.5	0.0003	1.5	0.00004	1.7	3.1E-06	1.0	NS	2.8	<1E-07
Ddx5 DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	0.5	0.0001	1.5	0.0004	2.2	<1E-07	1.1	NS	3.2	<1E-07
Hnrpa2b1 heterogeneous nuclear ribonucleo-protein A2/B1	0.5	0.0003	1.1	NS	1.0	NS	0.9	NS	2.1	0.00001
Mbn1 muscblind-like 1 (Drosophila)	0.6	0.006	1.5	0.001	1.6	0.0008	1.2	NS	2.5	3.42E-07
Tex189 testis expressed gene 189	0.6	0.005	1.6	0.001	2.1	2.7E-06	1.0	NS	2.7	2.66E-06
Rab1 RAB1, member RAS oncogene family	0.6	0.004	1.3	NS	1.4	NS	1.4	NS	2.3	0.0008
RpL21 ribosomal protein L21	0.6	0.007	1.4	0.0004	1.6	0.00007	1.1	NS	2.4	1.13E-07
RpL26 ribosomal protein L26	0.6	0.003	1.4	0.0008	1.7	0.0006	1.1	NS	2.4	0.000006
Rps6 ribosomal protein S6	0.6	0.003	1.7	0.0002	2.1	<1E-07	1.1	NS	2.9	<1E-07
RpL23 ribosomal protein L23	0.5	0.0002	1.6	0.002	1.9	<1E-07	1.2	NS	2.9	<1E-07
Rps5 ribosomal protein S5	0.5	0.001	1.0	NS	1.5	0.002	1.1	NS	2.0	0.00003
Rpl3 ribosomal protein L3	0.5	0.009	1.0	NS	1.5	0.0002	1.0	NS	1.9	0.0002
Rps7 ribosomal protein S7	0.5	0.007	1.3	0.04	1.6	0.00006	1.1	NS	2.5	9.13E-07
RpL10 ribosomal protein L10	0.5	0.0002	1.4	0.03	2.2	0.00006	1.0	NS	2.9	<1E-07
RpL31 ribosomal protein L31	0.5	0.00002	1.1	NS	1.6	0.00006	1.2	NS	2.4	<1E-07
RpL12 ribosomal protein L12	0.5	0.003	1.4	0.006	2.0	1.1E-07	1.1	NS	3.0	<1E-07

**TABLE 1. (continued) Gene expression changes in NOD, congenic NOD, and B9-23-treated NOD mice**

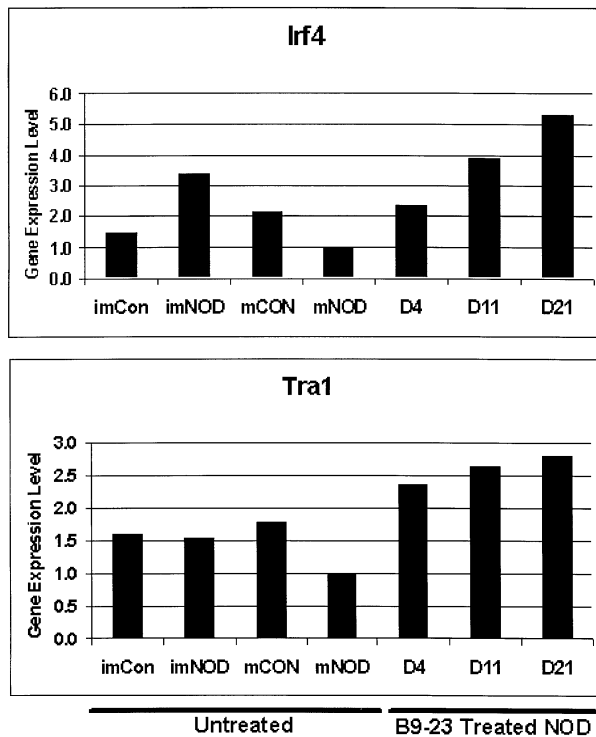
Name	B9-23/mNOD		B9-23/imNOD		mCON/imNOD		imCON/imNOD		mNOD/imNOD	
	ratio	p-value	ratio	p-value	ratio	p-value	ratio	p-value	ratio	p-value
Rps25	0.5	0.001	1.4	0.0	2.0	0.00001	1.1	NS	2.9	5E-07
Normal cell functions										
Tpm3	0.4	0.0009	1.2	NS	1.8	2.3E-07	0.9	NS	2.6	1.51E-07
Mor2	0.7	0.008	1.4	0.01	2.1	0.0002	1.0	NS	2.1	0.00009
NAD (soluble)										
Up-regulated by B9-23 treatment										
Lymphocyte development and function										
Irf4	4.2	0.002	1.2	NS	0.6	NS	0.4	0.001	0.3	0.00004
Tra1	2.5	0.0005	1.6	0.00002	1.2	NS	1.0	NS	0.6	0.0001
Syk	1.8	0.001	1.9	0.001	1.2	NS	0.7	NS	1.1	NS
Apoptosis										
Prdx2	1.6	0.008	0.8	NS	0.5	<1E-07	0.9	NS	0.5	0.00002
Bnip3l	1.5	0.008	0.8	0.003	0.6	0.00007	1.1	NS	0.5	<1E-07
Cell motility										
Arcp5	1.4	0.002	1.5	0.04	1.4	0.04	0.8	NS	1.1	NS
Transcription/ translation										
Pam	3.1	0.004	2.1	NS	1.2	NS	0.4	NS	0.7	NS
Hps1	2.7	0.002	0.9	NS	0.9	NS	1.6	0.02	0.3	3.94E-06
Rnf14	2.4	0.00005	1.3	NS	0.9	NS	0.8	NS	0.6	0.02

TABLE 1. (*continued*) Gene expression changes in NOD, congenic NOD, and B9-23-treated NOD mice

Name	B9-23/mNOD		B9-23/imNOD		mCON/imNOD		imCON/imNOD		mNOD/imNOD	
	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value	ratio	<i>p</i> -value
Siat7b	2.0	0.002	1.7	NS	0.8	NS	0.5	NS	0.9	NS
Imp13	1.8	0.005	1.0	NS	0.7	0.0004	1.0	NS	0.6	0.0002
Normal cell functions										
Aqp1	2.1	0.0007	1.3	NS	0.7	0.03	1.1	NS	0.6	0.0007
Hemgn	2.0	0.002	1.8	8.5E-05	0.8	NS	0.9	NS	0.9	NS
Spna1	1.9	0.006	1.0	NS	0.9	NS	1.2	NS	0.5	0.00005
Alas	1.8	0.007	0.7	0.03	0.5	0.00009	1.4	NS	0.4	0.00008
Gypa	1.8	0.003	1.0	NS	0.5	0.0004	1.0	NS	0.5	0.00003
Ppplcb	1.7	0.0002	1.0	NS	0.6	9E-06	1.0	NS	0.6	0.00001

B9-23: insulin B9-23-treated NOD (8–10 wks), mNOD: mature NOD (8–10 wks), imNOD: immature NOD (1–4 wks), mCON: mature congenic NOD (8–10 wk), imCON: immature congenic NOD (3–4 wks).

RT-PCR confirmation.<sup>7</sup> In contrast, the expression of the gene was increased 4.2-fold in the combined group of B9-23-treated NOD mice compared to the age-matched NOD controls (TABLE 1). Further analysis of the longitudinal expression data for the B9-23-treated NOD mice demonstrates a continuous increase in *Irf4* expression after the booster immunization (days 4, 11, and 21) (FIG. 1). The expression of *Irf4* was increased 2.4-, 3.9-, and 5.2-fold after 4, 11, and 21 days post immunization, respectively. This gradual increase provides additional support for the validity of our expression data. The increase in *Irf4* expression is mirrored to a lesser extent in the protective NOD congenic mice supporting the direct role of *Irf4* in the protective phenotype. *Irf4* is a lymphoid-specific transcription factor expressed in B cells and T cells.<sup>25,26</sup> Several recent studies have shown that the expression of *Irf4*



**FIGURE 1.** Gene expression levels of the two genes (*Irf4* and *Tra1*) involved in the Th2 response induced by B9-23 treatment. The x-axis consists of two categories of mice indicated by the two *solid bars*—the untreated mice and the NOD treated with insulin B9-23. The untreated category contains four groups: imCon, immature congenic NOD (3–4 weeks); imNOD, immature NOD (1–4 weeks); mCON, mature congenic NOD (8–10 weeks); and mNOD, mature NOD (8–10 weeks). The B9-23-treated NOD is broken down into three groups: D4, D11, and D21. This indicates the mice that were sacrificed 4, 11, and 21 days after the booster injection. The y-axis indicates the relative level of gene expression of each group of mice; the expression for NOD was artificially set to 1. The specific gene is indicated at the top of each graph.

is important for T cell differentiation. In fact, *Irf4*<sup>-/-</sup> mice have an impairment in the production of Th2 cells.<sup>27</sup> It is likely that increased expression of *Irf4* may explain the increased production of Th2 cytokines in B9-23-treated mice.

Tumor rejection antigen gp96 (Tra1) is upregulated 2.5-fold in the combined group of B9-23-treated NOD compared to untreated NOD (TABLE 1). The expression level of Tra1 is lower in the mature NOD mice with extensive autoimmunity than in all other groups of mice analyzed in the study (FIG. 1), suggesting that the reduced level of Tra1 is associated with autoimmunity. Similar to *Irf4*, B9-23 treatment gradually increases Tra1 expression post booster injection (FIG. 1). Tra1 is a member of the heat-shock family of proteins and is upregulated by a number of factors including stress signals. The introduction of B9-23 in the presence of IFA may act as a stress signal that induces B cells to express the protein gp96 on their cell surface. This protein acts as a costimulatory molecule that preferentially stimulates a Th2 response in the engaged T cells.<sup>28</sup>

## CONCLUSION

Our expression profiling has shown that insulin B9-23 immunization may prevent diabetes in the NOD mice by preventing pro-inflammatory activation of lymphocytes that normally occurs during the natural progression of the disease in NOD mice. The decreased expression of these genes may serve as biomarkers for the effectiveness of therapeutic regimens or other interventions. Our studies also discovered two genes that may explain the shift from a Th1 response in the untreated NOD mice to the Th2 response observed in the insulin B9-23-treated mice. Such immune deviation is believed to be the immunological mechanism associated with the protection conferred by insulin immunization. The discovery of the genes that promote a protective Th2 response may suggest novel ways for preventing the disease.

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## REFERENCES

1. SERREZE, D.V., H.D. CHAPMAN, D.S. VARNUM, *et al.* 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice *J. Exp. Med.* **184**: 2049–2053.
2. AKASHI, T., S. NAGAFUCHI, K. ANZAI, *et al.* 1997. Direct evidence for the contribution of B cells to the progression of insulinitis and the development of diabetes in non-obese diabetic mice. *Int. Immunol.* **9**: 1159–1164.
3. NOORCHASHM, H., N. NOORCHASHM, J. KERN, *et al.* 1997. B-cells are required for the initiation of insulinitis and sialitis in nonobese diabetic mice. *Diabetes* **46**: 941–946.
4. CHRISTIANSON, S.W., L.D. SHULTZ & E.H. LEITER. 1993. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and

- CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. *Diabetes* **42**: 44–55.
5. BENDELAC, A., C. CARNAUD, C. BOITARD & J.F. BACH. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *J. Exp. Med.* **166**: 823–832.
  6. YOON, J.W. & H.S. JUN. 1999. Cellular and molecular roles of beta cell autoantigens, macrophages and T cells in the pathogenesis of autoimmune diabetes. *Arch. Pharmacol. Res.* **22**: 437–447.
  7. ECKENRODE, S.E., Q. RUAN, P. YANG, *et al.* 2004. Gene expression profiles define a key checkpoint for type 1 diabetes in NOD mice. *Diabetes* **53**: 366–375.
  8. ATKINSON, M.A., N.K. MACLAREN & R. LUCHETTA. 1990. Insulinitis and diabetes in NOD mice reduced by prophylactic insulin therapy. *Diabetes* **39**: 933–937.
  9. DANIEL, D. & D.R. WEGMANN. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc. Natl. Acad. Sci. USA* **93**: 956–960.
  10. KAROUNOS, D.G., J.S. BRYSON & D.A. COHEN. 1997. Metabolically inactive insulin analog prevents type I diabetes in prediabetic NOD mice. *J. Clin. Invest.* **100**: 1344–1348.
  11. ZHANG, Z.J., L. DAVIDSON, G. EISENBARTH & H.L. WEINER. 1991. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc. Natl. Acad. Sci. USA* **88**: 10252–10256.
  12. MUIR, A., A. PECK, M. CLARE-SALZLER, *et al.* 1995. Insulin immunization of nonobese diabetic mice induces a protective insulinitis characterized by diminished intraslet interferon-gamma transcription. *J. Clin. Invest.* **95**: 628–634.
  13. HUTCHINGS, P.R. & A. COOKE. 1995. Comparative study of the protective effect afforded by intravenous administration of bovine or ovine insulin to young NOD mice. *Diabetes* **44**: 906–910.
  14. HARRISON, L.C., M. DEMPSEY-COLLIER, D.R. KRAMER & K. TAKAHASHI. 1996. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. *J. Exp. Med.* **184**: 2167–2174.
  15. MUKHERJEE, R., P. CHATURVEDI, H.Y. QIN & B. SINGH. 2003. CD4+CD25+ regulatory T cells generated in response to insulin B:9-23 peptide prevent adoptive transfer of diabetes by diabetogenic T cells. *J. Autoimmun.* **21**: 221–237.
  16. MCMURRAY, R.W. 1996. Adhesion molecules in autoimmune disease. *Semin. Arthritis Rheum.* **25**: 215–233.
  17. PECK, S.R. & H.E. RULEY. 2000. Ly108: a new member of the mouse CD2 family of cell surface proteins. *Immunogenetics* **52**: 63–72.
  18. VALDEZ, P.A., H. WANG, D. SESHASAYEE, *et al.* 2004. NTB-A, a new activating receptor in T cells that regulates autoimmune disease. *J. Biol. Chem.* **279**: 18662–18669.
  19. XU, K., T. YEN & C.L. GECZY. 2001. Il-10 up-regulates macrophage expression of the S100 protein S100A8. *J. Immunol.* **166**: 6358–6366.
  20. CRAMER, T., Y. YAMANISHI, B.E. CLAUSEN, *et al.* 2003. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* **112**: 645–657.
  21. PLOIX, C., I. BERGEROT, N. FABIEN, *et al.* 1998. Protection against autoimmune diabetes with oral insulin is associated with the presence of IL-4 type 2 T-cells in the pancreas and pancreatic lymph nodes. *Diabetes* **47**: 39–44.
  22. RAMIYA, V.K., X.Z. SHANG, P.G. PHARIS, *et al.* 1996. Antigen based therapies to prevent diabetes in NOD mice. *J. Autoimmun.* **9**: 349–356.
  23. BOT, A., D. SMITH, S. BOT, *et al.* 2001. Plasmid vaccination with insulin B chain prevents autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* **167**: 2950–2955.
  24. TIAN, J., C. CHAU & D.L. KAUFMAN. 1998. Insulin selectively primes Th2 responses and induces regulatory tolerance to insulin in pre-diabetic mice. *Diabetologia* **41**: 237–240.
  25. RENGARAJAN, J., K.A. MOWEN, K.D. MCBRIDE, *et al.* 2002. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J. Exp. Med.* **195**: 1003–1012.
  26. NISHIYA, N., K. YAMAMOTO, Y. IMAIZUMI, *et al.* 2004. Identification of a novel GC-rich binding protein that binds to an indispensable element for constitutive IRF-4 promoter activity in B cells. *Mol. Immunol.* **41**: 855–861.

27. LOHOFF, M., H.W. MITTRUCKER, S. PRECHTL, *et al.* 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc. Natl. Acad. Sci. USA* **99**: 11808–11812.
28. BANERJEE, P.P., D.S. VINAY, A. MATHEW, *et al.* 2002. Evidence that glycoprotein 96 (B2), a stress protein, functions as a Th2-specific costimulatory molecule. *J. Immunol.* **169**: 3507–3518.