Original Article

Novel Leptin Receptor Mutation in NOD/LtJ Mice Suppresses Type 1 Diabetes Progression

II. Immunologic Analysis

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Recently, we identified in normally type 1 diabetes–prone NOD/LtJ mice a spontaneous new leptin receptor (LEPR) mutation (designated $Lepr^{db-5J}$) producing juvenile obesity, hyperglycemia, hyperinsulinemia, and hyperleptinemia. This early type 2 diabetes syndrome suppressed intra-islet insulitis and permitted spontaneous diabetes remission. No significant differences in plasma corticosterone, splenic CD4$^+$ or CD8$^+$ T-cell percentages, or functions of CD3$^+$ T-cells in vitro distinguished NOD wild-type from mutant mice. Yet splenocytes from hyperglycemic mutant donors failed to transfer type 1 diabetes into NOD.$Rag1^{-/-}$ recipients over a 13-week period, whereas wild-type donor cells did so. This correlated with significantly reduced ($P < 0.01$) frequencies of insulin and islet-specific glucose-6-phosphatase catalytic subunit–related protein–reactive CD8$^+$ T-effector clonotypes in mutant mice. Intra-islet insulitis was also significantly suppressed in lethally irradiated NOD-$Lepr^{db-5J}$/Lt recipients reconstituted with wild-type bone marrow ($P < 0.001$). In contrast, type 1 diabetes eventually developed when mutant marrow was transplanted into irradiated wild-type recipients. Mitogen-induced T-cell blastogenesis was significantly suppressed when splenic T-cells from both NOD/Lt and NOD-$Lepr^{db-5J}$/Lt donors were incubated with irradiated mutant peritoneal exudate cells ($P < 0.005$). In conclusion, metabolic disturbances elicited by a type 2 diabetes syndrome (insulin and/or leptin resistance, but not hypercorticism) appear to suppress type 1 diabetes development in NOD-$Lepr^{db-5J}$/Lt by inhibiting activation of T-effector cells. *Diabetes* 55:171–178, 2006

Leptin, mainly secreted by white adipose tissue, is well known as a hormone associated with the regulation of food intake and energy expenditure (1). The leptin receptor (LEPR) is structurally and functionally related to interleukin (IL)-6 cytokine receptor family (2,3), and the effects of leptin signaling through its receptor on immune responses have been reported (4). Leptin can upregulate alveolar macrophage phagocytic function (5) and proinflammatory cytokine secretion by peritoneal macrophages, including tumor necrosis factor-α, IL-6, and IL-12 (6). Moreover, leptin induces proliferation of the naïve CD4$^+$CD45RA$^+$ T-cells, but not CD4$^+$CD45RO$^+$ T-cells (7). Leptin can also polarize immune responses toward T helper-1 (Th1) cytokine production (8).

In relation to autoimmune diseases, it has been reported that both leptin-deficient (*ob/ob*) and LEPR-deficient (*db/db*) mice were resistant to experimental autoimmune encephalomyelitis (EAE) (9–11), experimentally induced arthritis (12), and hepatitis (13). However, leptin treatment restored EAE susceptibility in *ob/ob* mice with the Th1-cytokine promoting activity of the hormone suggested as the mechanism (9). Evidence strongly supporting a role for leptin to modulate autoimmune type 1 diabetes was the finding that early leptin treatment of postnatal NOD female mice drastically accelerated disease onset, with a Th1 deviation again proposed as the mechanism (14).

Recently, we identified in NOD/LtJ mice a spontaneous mutation in the extracellular domain of the LEPR (designated $Lepr^{db-5J}$), which produced a type 2 diabetes-like syndrome characterized by obesity, juvenile hyperglycemia, hyperinsulinemia, and hyperleptinemia (15). This new mutation produced a Gly640Val transition at a highly conserved glycine residue. Unlike the $Lepr^{db-1J}$ mutation, which because of a truncated mRNA, does not produce the full-length receptor (termed Rb), the $Lepr^{db-5J}$ Rb gene product is not truncated and contains intracellular JAK/STAT signal recruitment domains. Interestingly, when the $Lepr^{db-1J}$ mutation was congenically transferred from the C57BLKS/J strain of origin onto the NOD background, the obesity and insulin resistance syndrome produced by this truncated mutant receptor failed to circumvent the autoimmune type 1 diabetes syndrome associated with insulitic destruction of pancreatic β-cells (16). Unlike this congenic NOD.BKS-$Lepr^{db-1J}$ stock, the coisogenic NOD-

TABLE 1
No significant differences in relative proportions and total numbers $\times 10^6$ (in parenthesis) of splenic leukocyte subsets in NOD/Lt and NOD-$Lepr^{db-5J}$/Lt mice

Mice	CD4 T-cells	CD8 T-cells	B-cells	MØ/DC	Granulocytes
NOD/Lt	36.01 ± 0.09	14.97 ± 0.89	34.95 ± 2.89	3.89 ± 0.20	4.26 ± 0.09
(total 88.0 ± 12.8)	(31.7 ± 5.0)	(13.2 ± 1.7)	(30.7 ± 0.6)	(3.3 ± 0.4)	3.8 ± 0.3
NOD-$Lepr^{db-5J}$/Lt	37.55 ± 1.30	15.63 ± 0.63	32.22 ± 3.24	4.35 ± 0.09	3.92 ± 0.28
(total 86.9 ± 7.5)	(33.1 ± 2.2)	(13.6 ± 1.2)	(28.0 ± 0.5)	(3.8 ± 0.3)	3.4 ± 0.2

Data are mean % $\pm$ SE ($n = 5$) of each splenic leukocyte subset assessed in 9- to 10-week-old females by flow cytometry as described in RESEARCH DESIGN AND METHODS. MØ/DC, macrophages/dendritic cells.

$Lepr^{db-5J}$/Lt mice exhibited an unexpected suppression of intra-islet insulitis, allowing through β-cell expansion a full remission of the early onset hyperglycemia in mutant females and in some male mice (15). In the present study, we analyzed alterations in the immunologic phenotypes of NOD-$Lepr^{db-5J}$/Lt mice to understand how induction of a type 2 diabetes syndrome associated with both leptin and insulin resistance suppressed the autoimmune syndrome that normally develops in NOD/Lt mice.

RESEARCH DESIGN AND METHODS

Mice and reagents. NOD/Lt and obese NOD-$Lepr^{db-5J}$/Lt female mice were maintained in a specific pathogen-free research animal facility and allowed free access to NIH-31 pellet diet (6% fat; PMI, St. Louis, MO) and acidified drinking water. Currently, type 1 diabetes frequency is 100% in NOD/Lt females by 28 weeks and 55% in males by 37 weeks. NOD.$Rag1^{-/-}$ females were used as recipients of adoptively transferred splenic leukocytes, and CBA/J females were used as donors of allogenic stimulator cells for mixed lymphocyte reactions. All mice were housed in a specific pathogen-free research animal facility, Monoclonal antibodies (mAbs; BD Pharmingen, San Jose, CA) used for flow cytometric analyses of splenic leukocyte subsets were fluorochrome-conjugated anti-CD4 (GK 1.5), CD8 (53-6.7), B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB68C5), and CD25 (PC61).

Flow cytometry. Single cell suspensions were prepared from the indicated mice for splenic leukocyte subset and regulatory T-cell (T-reg) assessments. Antibody mixtures for staining CD4, CD8, and B-cells or macrophage/dendritic cells and granulocytes were used in leukocyte subset analyses, and antibody combinations of anti-CD4 or -CD8 and anti-CD25 were used for T-reg quantifications. The stained cells were washed and analyzed by multicolor flow cytometry and Cell Quest software (FACScalibur; BD Biosciences, San Jose, CA).

Mitogen-stimulated T-cell blastogenesis. Triplicate aliquots of 10^5 splenic T-cells/well purified (>95%) by a streptoavidin magnetic bead system (Miltenyl Biotec, Auburn, CA) (17) were seeded into a round-bottomed 96-well plate with ^{137}Cs-irradiated (2 Gy) peritoneal exudate cells (PECs; 5×10^3/well) from either NOD/Lt or NOD-$Lepr^{db-5J}$/Lt mice and RPMI 1640 medium with or without concanavalin A (ConA) (final 2.5 μg/ml) was added. After a 3-day incubation, 1 μCi/well [^{3}H]thymidine (Perkin-Elmer Life Science, Boston, MA) was added for the final 14 h of culture, and the cells were harvested and counted with a LKB Betaplate 1205 system (LKB Instruments, Gaithersburg, MD).

CD3/CD28-driven CD4$^+$ T-cell proliferation assay and mixed lymphocyte reaction. Splenic CD4$^+$ cells (purity >95%) were prepared as described above (17), and 5×10^5 cells/well were seeded into flat-bottomed 96-well plates precoated with anti-CD3 (145–2C11)/anti-CD28 (37.51) mAb. For mixed lymphocyte reactions, 5×10^5 responder T-cells/well were seeded into a 96-well plate with the same number of stimulator cells (2 Gy-irradiated splenic leukocytes) from NOD/Lt, NOD-$Lepr^{db-5J}$/Lt, or CBA/J female donors. After 4 days, [^{3}H]thymidine incorporation was analyzed as above.

Tetramer staining of autoreactive CD8$^+$ T-cells. Splenic β-cell autoreactive CD8$^+$ T-cell clonotypes were quantified by flow cytometry using previously reported tetramers (18,19). Phycoerythrin-conjugated islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) (KYNKAN VFL), insulin B-chain peptide 15–23 (LYLVCGERL), and irrelevant control TUM (KYQAVTTTL) peptide tetramers were provided by Dr. Pere Santamaria (University of Calgary, Alberta, Canada). The staining results were analyzed with >10^6 live cell events gated.

Adoptive transfer of splenic leukocytes into NOD.$Rag1^{-/-}$ recipients and cyclophosphamide treatment. Aliquots of 10^7 splenic leukocytes from hyperglycemic NOD/Lt (17-week-old) and NOD-$Lepr^{db-5J}$/Lt (15-week-old)

female donors were injected intravenously into 4- to 6-week-old NOD.$Rag1^{-/-}$ female recipients. The recipients were then monitored for diabetes development over a 13-week follow-up period. After this, surviving nondiabetic NOD.$Rag1^{-/-}$ recipients of NOD-$Lepr^{db-5J}$/Lt splenic leukocytes received an intraperitoneal injection of either cyclophosphamide (200 mg/kg body wt) or PBS and were then monitored for diabetes development over the following 2 weeks.

Reciprocal bone marrow transfer. Mice were lethally irradiated with 1.2 Gy (2×0.6 Gy, 4 h apart) at 4–6 weeks of age and reciprocally reconstituted with bone marrow as previously described (20). Body weight and diabetes development were monitored for up to 24 weeks after reconstitution. At diabetes onset or the end of the observation period, leukocytic reconstitution of spleen was analyzed by flow cytometry, and pancreases were removed for histologic scoring of insulitis as previously described (15).

Plasma hormone radioimmunoassay to assess diabetes type in bone marrow transfer studies. Type 1 diabetes development in NOD is reflected by insulinopenia, whereas type 2 diabetes is reflected by hyperinsulinemia and hyperleptinemia. At 8, 16, and 20 weeks after reciprocal bone marrow transfer, plasma samples were collected from all recipients, and insulin and leptin levels were determined using a rat insulin radioimmunoassay kit and a mouse leptin radioimmunoassay kit (Linco, St. Charles, MO), respectively.

Plasma corticosterone measurement. Plasma corticosterone (10 A.M. sampling) from untreated 12-week-old mice of both sexes and genotypes was determined by rat corticosterone radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX).

Statistical analysis. Mann-Whitney U test was performed to assess significance of experimental differences. A P value <0.05 was considered statistically significant in group comparisons.

RESULTS

Flow cytometric comparison of splenic leukoctye subsets. No significant differences were observed between wild type and mutants in thymus or spleen wet weights. As shown in Table 1, the percentage of the macrophage/dendritic cell subset was slightly increased in NOD-$Lepr^{db-5J}$/Lt spleens (4.35 ± 0.09 vs. $3.89 \pm 0.2\%$ in wild type), but the difference was not statistically significant. Interestingly, despite the repressed insulitis previously observed in NOD-$Lepr^{db-5J}$/Lt mice (15), wild-type NOD/Lt females actually had a slightly higher percentage of potentially regulatory CD4$^+$CD25$^+$ T-cells compared with NOD-$Lepr^{db-5J}$/Lt females (9.5 ± 0.7 vs. $8.0 \pm 0.2\%$). There were no differences between wild-type and mutant mice in proportions or numbers of any other leukocyte subset.

NOD-$Lepr^{db-5J}$/Lt PECs suppress ConA–stimulated T-cell activation. We next assessed the ability of mutant and wild-type macrophages to serve as accessory cells in supporting ConA-stimulated T-cell proliferation. Data in Fig. 1A show that PECs from hyperleptinemic and hyperinsulinemic mutant mice significantly blunted blastogenic T-cell responses ($P < 0.05$). This suppressive effect was observed regardless of whether the purified T-cells were from wild-type or mutant donors. Furthermore, the same suppressive effect was observed when thioglycollate-elicited PECs were used (data not shown). Because leptin
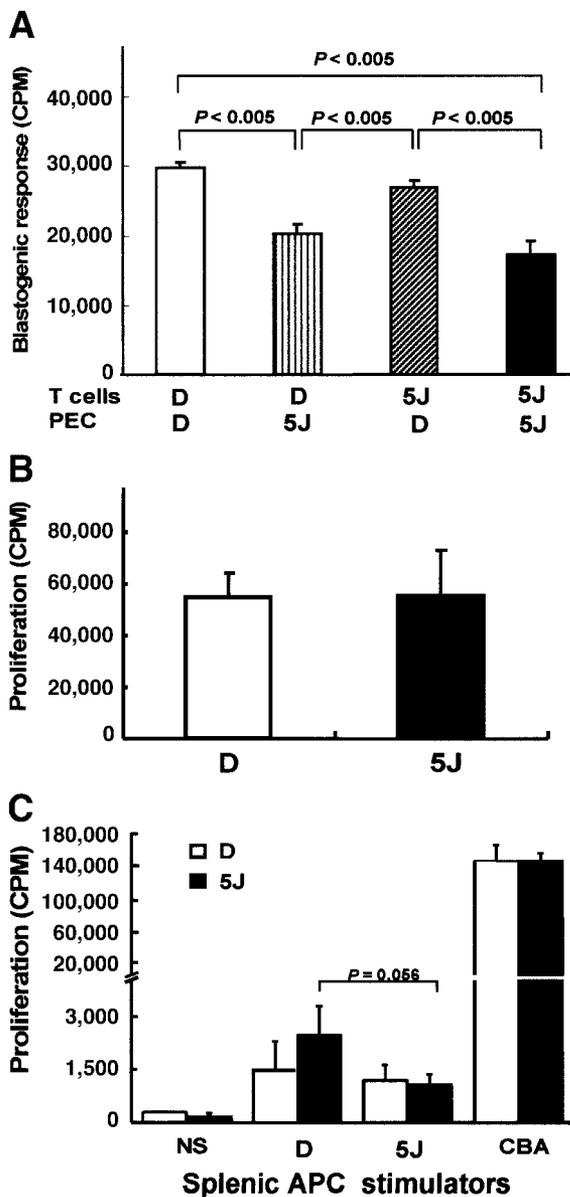
FIG. 1. *A*: Blastogenic responses of purified splenic T-cells (10^5/ml) to ConA (2.5 µg/ml) are significantly ($P < 0.05$) suppressed by presence of irradiated PECs from NOD-*Lepr*$^{db-5J}$/Lt origin as accessory cells. D and 5J, respectively, denote NOD/Lt or NOD-*Lepr*$^{db-5J}$/Lt source of T-cells or PECs. Data are obtained from 10-week-old NOD/Lt ($n = 3$) and NOD-*Lepr*$^{db-5J}$/Lt ($n = 3$) females. *B*: No significant proliferation response differences of purified splenic CD4$^+$ T-cells (5 × 10^5/ml) stimulated by mAbs against CD3/CD28 in 11-week-old NOD/Lt ($n = 3$) and NOD-*Lepr*$^{db-5J}$/Lt ($n = 3$) females. *C*: Suppressed SMLR by purified splenic T-cells (5 × 10^5/ml) from both 10-week-old NOD/Lt ($n = 3$) and NOD-*Lepr*$^{db-5J}$/Lt ($n = 3$) in response to mutant APC stimulators. Unfilled and filled boxes denote wild-type and mutant donors of purified T-cell responders, respectively. Both strains showed equivalent strong responses to allogeneic CBA/J APCs. NS, no stimulator cells added.

signaling reportedly suppresses anti-CD3–driven proliferation of human memory T-cells while stimulating that of naive human T-cells (7), we compared wild-type versus mutant CD4 T-cell activation in vitro via signaling through the T-cell receptor. Purified CD4$^+$ T-cells from both genotypes exhibited equal proliferative responses to anti-CD3/CD28 stimulation in vitro (Fig. 1*B*). In contrast, purified T-cell activation in a syngeneic mixed lymphocyte response (SMLR) was reduced when irradiated mutant splenocytes served as the antigen-presenting cell (APC)
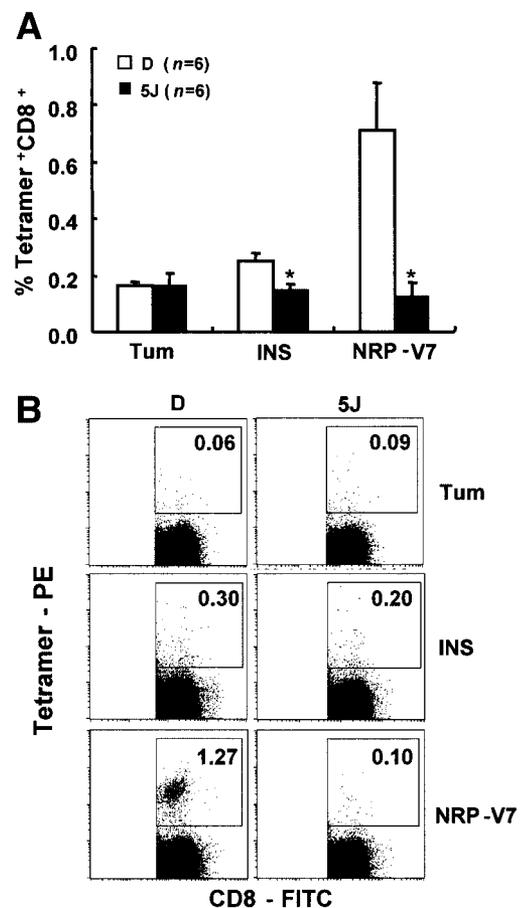
FIG. 2. Autoreactive CD8$^+$ T-cells detected by high-avidity peptide/major histocompatibility complex class I tetramers. *A*: Significantly reduced insulin (INS) and islet-specific glucose-6-phosphatase catalytic subunit (NRP-V7) tetramer-positive CD8$^+$ T-cells were observed in spleens from 11- to 12-week-old NOD-*Lepr*$^{db-5J}$/Lt females compared with age-matched standard NOD/Lt controls. Data are mean percentages ± SE. *Significantly different from NOD/Lt at $P < 0.01$. *B*: Representative examples are illustrated of TUM (control), INS, and NRP-V7 tetramer staining determined by flow cytometry. Percentages of gated cells are displayed in the *top right box* (tetramer-binding CD8$^+$ T-cells).

source for self-antigen stimulation, regardless of whether responder T-cells were from wild-type or mutant donors (Fig. 1*C*, difference nearing significance at $P = 0.056$). No T-cell response differences were noted when allogeneic (CBA/J) stimulators were used (Fig. 1*C*), indicating that the SMLR differences initiated at the APC and not the T-cell level.

Reduced frequency of diabetogenic CD8$^+$ T-cell clonotypes in NOD-*Lepr*$^{db-5J}$/Lt spleen. β-Cell autoreactive CD8$^+$ T-cell clonotypes detected by NRP-V7 (identifying the IGRP$_{206-214}$-reactive clonotypes) and insulin tetramers were significantly decreased ($P < 0.01$) in spleens from NOD-*Lepr*$^{db-5J}$/Lt females compared with standard NOD/Lt controls (Fig. 2*A*). These two tetramers enumerate two of the most prevalent CD8 clonotypes documented in the insulitic infiltrates (19). The mean numbers of IGRP and insulin-reactive CD8$^+$ clonotypes were 5.7- and 1.7-fold lower in NOD-*Lepr*$^{db-5J}$/Lt spleens compared with standard NOD/Lt females. Representative flow profiles are shown in Fig. 2*B*.

Splenic leukocytes from NOD-*Lepr*$^{db-5J}$/Lt adoptively transfer insulitis, but not type 1 diabetes, into

NOD.$Rag^{-/-}$ recipients. To assess the diabetogenic potency of splenic leukocytes from NOD-$Lepr^{db-5J}$/Lt donors when removed from their abnormal metabolic milieu in situ, splenic leukocytes were collected from young, hyperglycemic NOD-$Lepr^{db-5J}$/Lt females and adoptively transferred into NOD.$Rag^{-/-}$ recipients. As expected, splenic leukocytes from diabetic NOD/Lt wild-type donors transferred type 1 diabetes to six of six recipients within 5 weeks. In contrast, NOD-$Lepr^{db-5J}$/Lt splenocytes failed to transfer overt type 1 diabetes to any of seven recipients by 13 weeks after transfer. At necropsy after an additional 2 weeks, a moderate level of intra-islet insulitis (mean insulitis score = 1.7 ± 0.5) was observed in three of these nonhyperglycemic mutant females injected with PBS at week 13. Cyclophosphamide treatment of the remaining four mice at week 13 rendered one diabetic within 2 weeks and markedly increased intra-islet insulitis to a mean score of 3.6 ± 0.3, indicative of incipient diabetes. These results show that the $Lepr^{db-5J}$ mutation had not deleted potential diabetogenic T-effectors but rather created an environment limiting their frequency before transfer so that homeostatic expansion of T-effectors to a critical mass was markedly prolonged.

Type 1 diabetes developed in NOD/Lt radiation chimeras transplanted with NOD-$Lepr^{db-5J}$/Lt marrow, but reciprocal NOD/Lt bone marrow failed to transfer type 1 diabetes to NOD-$Lepr^{db-5J}$/Lt recipients. Lethally irradiated NOD/Lt and NOD-$Lepr^{db-5J}$/Lt recipients were reconstituted with reciprocal or syngeneic sex-matched bone marrow. At 24 weeks after bone marrow transfer, final glycosuria frequencies were comparable in NOD-$Lepr^{db-5J}$/Lt recipients of NOD/Lt bone marrow and NOD/Lt recipients of NOD-$Lepr^{db-5J}$/Lt marrow (Fig. 3A). As anticipated, NOD/Lt marrow into lean NOD/Lt recipients produced the typical adult onset type 1 diabetes expected for the NOD model, whereas marrow from obese NOD-$Lepr^{db-5J}$/Lt donors transplanted into obese syngeneic recipients produced type 2 diabetes development in these mutants (15,21). Therefore, not surprisingly, hyperglycemia developed considerably earlier in NOD-$Lepr^{db-5J}$/Lt recipients of NOD/Lt bone marrow compared with wild-type NOD/Lt recipients of $Lepr^{db-5}$ marrow (Fig. 3A). Longitudinal changes in body weight (Fig. 3B) clearly distinguished these two reciprocal transfer sets. NOD-$Lepr^{db-5J}$/Lt recipients developing obesity at the start of the experiment maintained elevated body weight throughout the study period, and no spontaneous mortality was encountered. However, we elected to necropsy 3 of the 12 NOD-$Lepr^{db-5J}$/Lt recipient females at 17 and 19 weeks of age because of delayed-onset declines in body weight (9–11.6 g). Nevertheless the finding of low mean insulitis scores (1.5, 1.6, and 1.1) for these three individuals indicated primarily a type 2 diabetes pathogenesis of gradual β-cell failure and islet atrophy rather than a type 1 diabetes end-stage insulitis associated with rapid weight loss after hyperglycemia diagnosis. In contrast, lean NOD/Lt recipients of NOD-$Lepr^{db-5J}$/Lt marrow resembled NOD/Lt recipients of marrow from lean NOD donors. They remained lean and as shown below, had much more extensive insulitis.

That the NOD-$Lepr^{db-5J}$/Lt recipients of NOD/Lt marrow were developing the less-devastating, chronic type 2 diabetes syndrome whereas NOD/Lt recipients of NOD-$Lepr^{db-5J}$/Lt marrow were developing the lethal type 1 diabetes syndrome was reinforced by tracking temporal changes in plasma endocrine concentrations (Fig. 4).
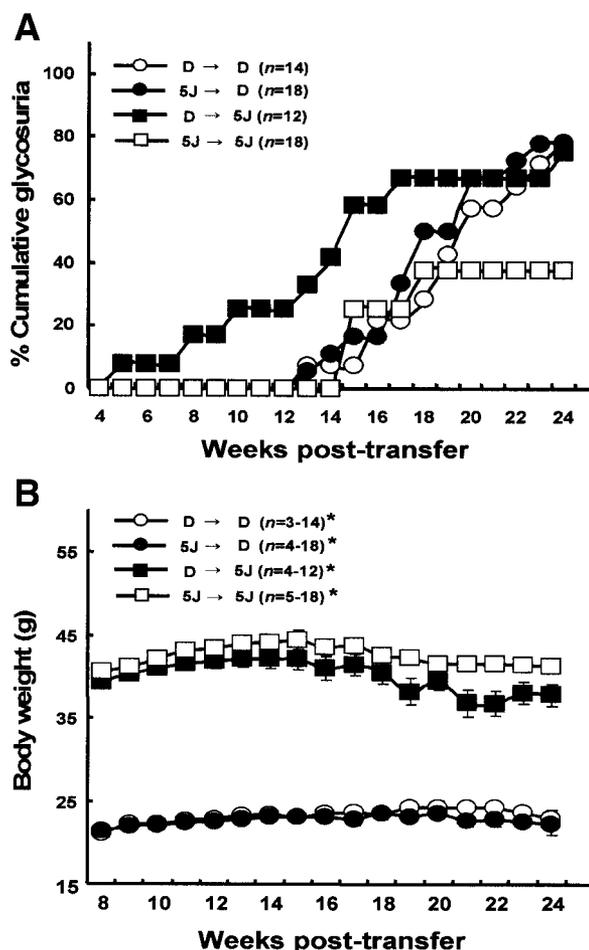
FIG. 3. Cumulative glycosuria development (**A**) and body weight changes (**B**) monitored for 24 weeks after transfer in lethally irradiated recipients reciprocally reconstituted with bone marrow from NOD/Lt (**D**) or NOD-$Lepr^{db-5J}$/Lt (**5J**) females. *Number range for the group denotes declining numbers of glycosuria-free survivors as lean diabetic mice are culled soon after diagnosis. Larger number is initial group size; smaller number is group size at experiment termination.

Sustained hyperinsulinemia typical of unmanipulated NOD-$Lepr^{db-5J}$/Lt mice was observed in NOD-$Lepr^{db-5J}$/Lt recipients of syngeneic marrow (Fig. 4A). Although less pronounced, stable hyperinsulinemia was maintained in NOD-$Lepr^{db-5J}$/Lt recipients of nominally diabetogenic NOD/Lt marrow. This contrasted with the wild-type (e.g., normal) levels of plasma insulin maintained by diabetes-free mice in both lean transplant groups. As expected, elevated plasma leptin tracked with NOD-$Lepr^{db-5J}$/Lt recipients regardless of the source of bone marrow (Fig. 4B). The age-related decline in plasma leptin in obese recipients of NOD/Lt marrow correlated with a gradual decrease in mean body weight of this cohort (Fig. 3B).

To further address whether the diabetes development in NOD-$Lepr^{db-5J}$/Lt recipients of NOD/Lt marrow was type 1 diabetes, the extent of intra-islet insulitis was analyzed in each group (Fig. 5). This analysis clearly showed a significant suppression of insulitis progression in NOD-$Lepr^{db-5J}$/Lt recipients of either syngeneic or NOD/Lt bone marrow (mean score 0.6 ± 0.1 and 1.3 ± 0.2, respectively) compared with that in NOD/Lt recipients of NOD marrow or $Lepr^{db-5J}$ marrow (mean score = 3.9 ± 0.1 and 3.5 ± 0.2, respectively). This suppression of intra-islet insulitis when bone marrow from either genotype was reconstituting in
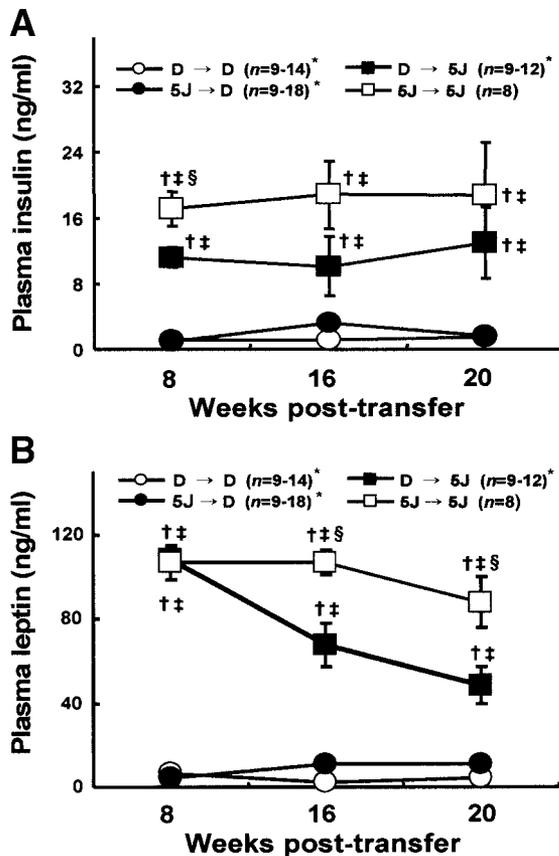
FIG. 4. Comparisons of insulin (_A_) and leptin (_B_) levels in recipients of reciprocal bone marrow transfer between NOD/Lt and NOD-_Lepr_[db-5J]/Lt females. Data were obtained at 8, 16, and 20 weeks after transfer and expressed as means ± SE. D, NOD/Lt donor or recipient; 5J, NOD-_Lepr_[db-5J]/Lt donor or recipient. *Variable number for the group denotes declining numbers of glycosuria-free survivors as stated in Fig. 3. †Significantly different from age-matched D → D recipients at _P_ < 0.01. ‡Significantly different from age-matched 5J → D recipients at _P_ < 0.01. §Significantly different from age-matched D → 5J recipients at _P_ < 0.01.

mutant recipients indicated that the host environment rather than the marrow donor was the primary determinant of the course and nature of the diabetes syndrome. **Plasma corticosterone levels were comparable in NOD/Lt and in NOD-_Lepr_[db-5J]/Lt.** Plasma corticosterone levels are significantly increased above normal in obese C57BLKS/J-_Lepr_[db-1J] (_db/db_) as well as C57BL/6J-_Lep_[ob] (_ob/ob_) mutant mice (22–24). Female mice typically show higher corticosterone levels than males, a pattern repeated on the NOD strain background. No significant differences distinguished mutants (female, _n_ = 3, 344 ± 76 ng/ml; male, _n_ = 3, 143 ± 81 ng/ml) from wild type (female, _n_ = 3, 358 ± 26 ng/ml; male, _n_ = 3, 126 ± 76 ng/ml). Hence, hypercorticism cannot be invoked as the endocrine perturbation that is repressing activation of immune effectors.

DISCUSSION

An expanding body of literature is now focusing attention on the role of leptin as a regulator of proinflammatory responses and as a key player in maintenance of lymphocyte homeostasis (4,25). In contrast to human purified CD4^{+}CD45RA ("naive") T-cells whose anti-CD3–stimulated proliferation is stimulated by leptin (7,8), we failed to see a direct effect of leptin in vitro on proliferation of NOD T-cells in response to anti-CD3 stimulation (data not

shown), possibly because of contaminating leptin present in fetal bovine serum in culture medium. The report of repressed anti-CD3 blastogenic response in B6 splenocytes treated with leptin in vitro (7) may have reflected a direct effect of leptin on APCs rather than T-cells. Our in vitro studies show that when either irradiated PECs (primarily macrophage) or irradiated splenic leukocytes from NOD-_Lepr_[db-5J]/Lt donors were coincubated with purified T-cells, they actually suppressed both mitogen-stimulated blastogenesis (Fig. 1_A_) and SMLR (Fig. 1_C_). In human and murine bone marrow cultures, leptin did not stimulate cell proliferation or survival, but rather enhanced phagocytotic function and cytokine responses of PECs to parasites in vitro (26). Macrophage phagocytotic activity has been reported to be markedly decreased in both leptin-deficient B6-_Lep_[ob] mice and LEPR-Rb–deficient C57BLKS/J-_Lepr_[db-1J] mice (6). Leptin treatment normalizes defective macrophage phagocytosis in _Lep_[ob] but not in leptin-resistant _Lepr_[db-1J] mice (6). Because reciprocal bone marrow transfer experiments did not indicate a primary defect in a hematopoietic lineage (Figs. 3 and 4), we infer that a component of the disturbed metabolic milieu suppressing autoimmune activation of T-effectors is the leptin resistance generated by the mutation in the extracellular (Ra) domain of the _Lepr_[db-5J] receptor. Preliminary transfection studies in COS7 cells indicate reduced leptin-binding affinity of both short form (LEPR-Ra) and long (signaling) form (Rb) of the _Lepr_[db-5J] receptor. Therefore, we infer that either the suppressive effects observed are an incomplete function of long-form receptor by low binding affinity of leptin due to the mutation (glycine to valine residue at 640 residue) in the extracellular domain or else represents a receptor-independent action of leptin.

There are several phenotypic features of NOD-_Lepr_[db-5J]/Lt mice that set them apart from the well-characterized C57BLKS/J-_Lepr_[db-1J] mice. Specifically, the former retain reproductive competence and do not develop hypertriglyceridemia (15). Furthermore, we now demonstrate that NOD-_Lepr_[db-5J]/Lt mice do not develop hypercorticism, a phenotype characterizing C57BLKS/J-_Lepr_[db-1J] and C57BL/6J-_Lep_[ob] mice (23). These differences presumably devolve from the site of the mutations (e.g., the extracellular
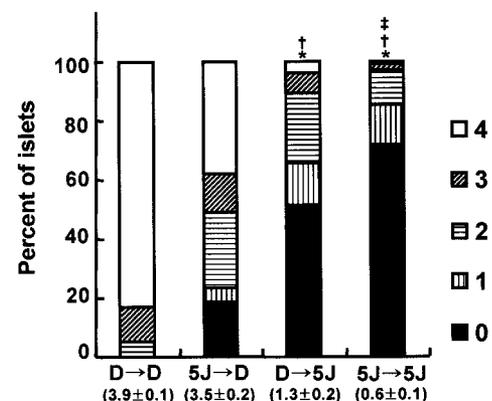

FIG. 5. Comparisons of pancreatic insulitis in recipients of reciprocal bone marrow transfer between NOD/Lt (_D_) and NOD-_Lepr_[db-5J]/Lt (5J) females. The insulitis of each individual islet is graded into 0 (normal), 1 (peri-insulitis), 2 (infiltration ≤25% of islet mass), 3 (infiltration between 25 and 75% of islet mass), and 4 (islet destruction), and the graded scores were compared for statistical analysis among groups. Insulitis scores were represented as means ± SE in parentheses. *Significantly different from D → D recipients at _P_ < 0.001. †Significantly different from 5J → D recipients at _P_ < 0.001. ‡Significantly different from D → 5J recipients at _P_ < 0.01.

domain of NOD-*Lepr*[db-5J]/Lt receptor that does not prevent transcription of a full-length receptor with an intracellular JAK/STAT signaling domain (15) that is missing in C57BLKS/J-*Lepr*[db-1J] mice. Recently, a LEPR Rb mutation has been generated in the murine STAT3 recruitment domain by site-specific mutagenesis (24,27). Like NOD-*Lepr*[db-5J]/Lt mice, these mice retain reproductive function and exhibit a less severe insulin resistance syndrome than C57BL6/J-*Lepr*[db-1J] mice, suggesting that certain effects of leptin on glucose homeostasis may be mediated by a route other than through Rb/STAT3 signaling. It remains to be demonstrated whether the immune suppressive actions of the *Lepr*[db-5J] mutation in NOD mice are mediated by receptor-dependent (presumably decreased function of leptin via the long Rb isoform?) or -independent mechanisms. The finding that the effect of the *Lepr*[db-5J] mutation on the course of diabetogenesis in NOD mice is seemingly quite different from that reported for the *Lepr*[db-1J] mutation (16) might suggest that some degree of Rb signaling is the salient feature distinguishing the *Lepr*[db-5J] from *Lepr*[db-1J] mutation. Female mice exhibit higher circulating leptin concentrations in plasma than do males. That the increased leptin concentrations naturally present in NOD females compared with females of other strains was a contributor to the female bias in type 1 diabetes development in this model was supported by the finding that postnatal administration of recombinant leptin accelerated type 1 diabetes onset in NOD females, but not males (14). At the present time, it remains unclear whether loss of leptin sensitivity conferred by the *Lepr*[db-5J] mutation or some other disturbed metabolic parameter (chronic hyperinsulinemia?) underlies the suppressed insulitis development and autoimmune destruction of *Lepr*[db-5J] β-cells. Nevertheless, the present study demonstrates an important contribution of an intact LEPR in the normal course of type 1 diabetes progression in NOD mouse model.

In standard NOD/Lt mice, CD8 T-cells reactive against either IGRP or insulin B chain peptide represent a major proportion of islet-infiltrating T-cells as insulitis progresses (19). The significant decrease in these diabetogenic clonotypes in spleens of NOD-*Lepr*[db-5J]/Lt is an excellent indicator of downregulated autoimmunity (Fig. 2). Early administration of insulin to young prediabetic NOD mice retards onset of insulitis and diabetes (28). Conceivably, the spontaneous hyperinsulinemia produced by the *Lepr*[db-5J] mutation could have produced central deletion or peripheral anergy of insulin-reactive clones. In the case of CD8 T-cells specific for insulin B chain 15–23, tetramer staining showed they were present in mutant spleens but in reduced numbers (Fig. 2). This was also the case of IGRP-reactive CD8 clonotypes (Fig. 2). Hence, T-cell tolerance mediated through central deletion did not appear to be occurring. Moreover, insulin autoantibodies were detected in some wild-type and mutant mice (data not shown), indicating absence of humoral tolerance, at least to insulin. The finding that splenic leukocytes from obese donors adoptively transferred insulitis, albeit at a retarded rate, showed that diabetogenic clonotypes were present but were not at levels comparable with normoinsulinemic, normoleptinemic wild-type controls. In part, this decreased level of diabetogenic T-cells might be attributed to the suppressive effects of Mac-1–positive/Gr-1–negative cells (both macrophage and dendritic cells) in peripheral lymphoid tissues. Another aspect of the hyperinsulinemic environment that might contribute to repressed immune effector activation is a previous demonstration in vitro that insulin is

a potent macrophage chemoattractant (29). Possibly, the systemic hyperinsulinemia destroys what otherwise would be an insulin gradient that macrophages follow to extravasate into the islets during the earliest phase of insulitis initiation (30).

When type 1 diabetes is retarded by experimental manipulation of the NOD model, $CD4^+CD25^+$ T-regs are often implicated in the protective mechanism (31–33). It is especially tempting to invoke upregulation of T-reg function as the mechanism underlying reduced insulitis and suppression of type 1 diabetes in NOD-*Lepr*[db-5J]/Lt mice in light of a recent study showing that serum leptin inversely correlates with T-reg proliferation in relapsing-remitting multiple sclerosis patients and in an EAE mouse disease model (34). This latter study reported a higher percentage of T-regs in lymphoid organs of leptin-deficient *Lep*[ob] or leptin-resistant *Lepr*[db] mice than in controls (34). Disease suppressive contributions of this T-cell subset are usually demonstrated in the context of homeostatic expansion after adoptive co-transfer with T-effectors into immunodeficient NOD-*Prkdc*[scid] or NOD-*Rag* recipients (31,35). In contrast to the slight increase of splenic macrophage/dendritic cell percentages and total numbers in NOD-*Lepr*[db-5J]/Lt mice, the $CD4^+CD25^+$ splenic T-cell subset was actually decreased in mutant mice of both sexes. We have previously shown that defects in immune suppressor function in NOD/Lt mice was associated with defects in APC co-stimulation (36). In that earlier study, the SMLR was used as a measure of self–major histocompatibility complex–restricted T-suppressor function. Our finding that the SMLR of NOD-*Lepr*[db-5J]/Lt mice is even lower than that of NOD/Lt wild-type mice (Fig. 1C) strongly suggests that T-reg activation is not the mechanism underlying retarded type 1 diabetes in this model. Our finding that purified mutant T-cells activated in vitro in the absence of mutant APCs are indistinguishable in proliferative response to wild type (Fig. 1B), whereas their blastogenic responses are suppressed by syngeneic APCs (Fig. 1C), suggests that an APC rather than a T-cell elaborated factor or factors is involved. Interestingly, "viable motheaten" mice with a defect in their SHP-1 hemopoietic stem cell phosphatase gene show a similar suppressive macrophage phenotype (37). Treatment of cultures with anti–transforming growth factor (TGF)-β alleviated their suppressive potency (38). However, TGF-β1 is unlikely to be a factor for the type 1 diabetes suppression of NOD-*Lepr*[db-5J]/Lt because we observed comparable TGF-β1 (by ELISA) in lipopolysaccharide-stimulated supernatants from both NOD/Lt and NOD-*Lepr*[db-5J]/Lt bone marrow-derived macrophage cultures (data not shown). Likewise, multiplex bead assay for IL-1β, IL-6, IL-10, tumor necrosis factor-α, IL-12p70, and interferon-γ concentrations in the same supernatants indicated no significant differences (data not shown), such that a Th1 to Th2 deviation often associated with suppression of NOD diabetogenesis was not indicated. Similarly, no differences in secreted eicosanoids (prostaglandin E2, leukotriene B4, 15S-HETE, and lipoxin A4) in the same supernatants were detectable (data not shown). T-cell activation and hepatocyte killing in a model of ConA-induced hepatitis was reduced by adiponectin, raising the possibility that this or other adipokines elevated in the obese NOD stock may have been immunoprotective (39)

In summary, we have found that superimposition of a leptin-resistant obesity syndrome in NOD/Lt mice that normally develop type 1 diabetes at high frequency pro-

duces a type 2 diabetes syndrome that suppresses levels of autoimmune T-effectors. Decreases in numbers of diabetogenic T-cells in obese mutant mice correlated with failure of their splenic leukocytes to adoptively transfer type 1 diabetes into immunodeficient recipients within a standard time frame, although immune effectors were clearly present. Reciprocal bone marrow reconstitution experiments demonstrated the primary role of mutation-induced disturbance of the endocrine/metabolic milieu rather than defects expressed at the level of hemopoietic stem cell derivatives. APCs isolated from these leptin- and insulin-resistant obese mutant mice suppressed T-cell proliferative responses in vitro. Although type 1 diabetes is considered primarily a consequence of an imbalanced immune system and type 2 diabetes, an imbalanced endocrine/metabolic system, the endocrine system (including sex steroids, glucocorticoids, leptin, and insulin) shapes immune responsiveness in type 1 diabetes; and reciprocally, proinflammatory reactants released by the immune system contribute to the metabolic defects associated with type 2 diabetes. The present study emphasizes the importance of gaining a better understanding of the interplay between the endocrine and immune systems in both healthy and disease states.

ACKNOWLEDGMENTS

REFERENCES

1. Friedman JM: Leptin, leptin receptors, and the control of body weight. *Nutr Rev* 56:s38–s46; discussion s54–s75, 1998
2. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI: Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263–1271, 1995
3. Madej T, Boguski MS, Bryant SH: Threading analysis suggests that the obese gene product may be a helical cytokine. *FEBS Lett* 373:13–18, 1995
4. Matarese G, Moschos S, Mantzoros CS: Leptin in immunology. *J Immunol* 174:3137–3142, 2005
5. Mancuso P, Gottschalk A, Phare SM, Peters-Golden M, Lukacs NW, Huffnagle GB: Leptin-deficient mice exhibit impaired host defense in Gram-negative pneumonia. *J Immunol* 168:4018–4024, 2002
6. Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, Klein AS, Bulkley GB, Bao C, Noble PW, Lane MD, Diehl AM: Leptin regulates proinflammatory immune responses. *FASEB J* 12:57–65, 1998
7. Lord GM, Matarese G, Howard JK, Bloom SR, Lechler RI: Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T-cells but enhances the production of proinflammatory cytokines. *J Leukoc Biol* 72:330–338, 2002
8. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI: Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394:897–901, 1998
9. Matarese G, Di Giacomo A, Sanna V, Lord GM, Howard JK, Di Tuoro A, Bloom SR, Lechler RI, Zappacosta S, Fontana S: Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J Immunol* 166:5909–5916, 2001
10. Matarese G, Sanna V, Di Giacomo A, Lord GM, Howard JK, Bloom SR, Lechler RI, Fontana S, Zappacosta S: Leptin potentiates experimental autoimmune encephalomyelitis in SJL female mice and confers susceptibility to males. *Eur J Immunol* 31:1324–1332, 2001
11. Sanna V, Di Giacomo A, La Cava A, Lechler RI, Fontana S, Zappacosta S, Matarese G: Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T-cell responses. *J Clin Invest* 111:241–250, 2003
12. Busso N, So A, Chobaz-Peclat V, Morard C, Martinez-Soria E, Talabot-Ayer D, Gabay C: Leptin signaling deficiency impairs humoral and cellular immune responses and attenuates experimental arthritis. *J Immunol* 168:875–882, 2002
13. Faggioni R, Jones-Carson J, Reed DA, Dinarello CA, Feingold KR, Grunfeld C, Fantuzzi G: Leptin-deficient (ob/ob) mice are protected from T-cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18. *Proc Natl Acad Sci U S A* 97:2367–2372, 2000
14. Matarese G, Sanna V, Lechler RI, Sarvetnick N, Fontana S, Zappacosta S, La Cava A: Leptin accelerates autoimmune diabetes in female NOD mice. *Diabetes* 51:1356–1361, 2002
15. Lee CH, Reifsnder PC, Naggert JK, Wasserfall C, Atkinson MA, Chen J, Leiter EH: Novel leptin receptor mutation in NOD/LtJ mice suppresses type 1 diabetes progression: I. Pathophysiologic analysis. *Diabetes* 54:2525–2532, 2005
16. Nishimura M, Miyamoto H: Immunopathological influence of the Ay, db, ob and nu genes placed on the inbred NOD background as murine models for human type I diabetes. *J Immunogenet* 14:127–130, 1987
17. Serreze DV, Johnson EA, Chapman HD, Graser RT, Marron MP, DiLorenzo TP, Silveira P, Yoshimura Y, Nathenson SG, Joyce S: Autoreactive diabetogenic T-cells in NOD mice can efficiently expand from a greatly reduced precursor pool. *Diabetes* 50:1992–2000, 2001
18. Trudeau JD, Kelly-Smith C, Verchere CB, Elliott JF, Dutz JP, Finegood DT, Santamaria P, Tan R: Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T-cells in peripheral blood. *J Clin Invest* 111:217–223, 2003
19. Lieberman SM, Takaki T, Han B, Santamaria P, Serreze D, DiLorenzo TP: Individual NOD mice exhibit unique patterns of CD8 T-cell reactivity to three islet antigens, including the newly identified widely expressed dystrophia myotonica kinase. *J Immunol* 173:6727–6734, 2004
20. Serreze DV, Leiter EH: Development of diabetogenic T-cells from NOD/Lt marrow is blocked when an allo-H-2 haplotype is expressed on cells of hematopoietic origin but not on thymic epithelium. *J Immunol* 147:1222–1229, 1991
21. Leiter EH, Lee CH: Mouse models and the genetics of diabetes: is there evidence for genetic overlap between type 1 and type 2 diabetes? *Diabetes* 54 (Suppl. 1):S151–S158, 2005
22. Shimomura Y, Bray G, Lee M: Adrenalectomy and steroid treatment in obese (ob/ob) and diabetic (db/db) mice. *Horm Metabol Res* 19:295–299, 1987
23. Leiter EH: Obesity genes and diabetes induction in the mouse. *Crit Rev Food Sci Nutr* 33:333–338, 1993
24. Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwarzt MW, Myers MG Jr: Roles for leptin receptor/STAT3-dependent and -independent signals in the regulation of glucose homeostasis. *Cell Metab* 1:169, 2005
25. Matarese G, La Cava A, Sanna V, Lord GM, Lechler RI, Fontana S, Zappacosta S: Balancing susceptibility to infection and autoimmunity: a role for leptin? *Trends Immunol* 23:182–187, 2002
26. Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, Ng A, Nicola NA, Alexander WS, Hilton DJ: Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc Natl Acad Sci U S A* 93:14564–14568, 1996
27. Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwartz MW, Myers MG Jr: STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* 421:856–859, 2003
28. Atkinson MA, Maclaren NK, Luchetta R, Burr I: Prophylactic insulin therapy prevents insulitis and insulin dependent diabetes (IDD) in NOD mice. *Diabetes* 38:87A, 1989
29. Leiter EH: Murine macrophages and pancreatic β cells: chemotactic properties of insulin and β-cytostatic action of interleukin 1. *J Exp Med* 166:1174–1179, 1987
30. Fox C, Danska J: IL-4 expression at the onset of islet inflammation predicts nondestructive insulitis in nonobese diabetic mice. *J Immunol* 158:2414–2424, 1997
31. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA: B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T-cells that control autoimmune diabetes [In Process Citation]. *Immunity* 12:431–440, 2000
32. Chatenoud L, Salomon B, Bluestone JA: Suppressor T-cells: they're back and critical for regulation of autoimmunity! *Immunol Rev* 182:149–163, 2001

33. Setoguchi R, Hori S, Takahashi T, Sakaguchi S: Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T-cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201:723–735, 2005

34. Matarese G, Carrieri PB, La Cava A, Perna F, Sanna V, De Rosa V, Aufiero D, Fontana S, Zappacosta S: Leptin increase in multiple sclerosis associates with reduced number of CD4+CD25+ regulatory T-cells. *Proc Natl Acad Sci U S A* 102:5150–5155, 2005

35. Lejon K, Fathman CG: Isolation of self antigen-reactive cells from inflamed islets of nonobese diabetic mice using CD4(high) expression as a marker. *J Immunol* 163:5708–5714, 1999

36. Serreze DV, Leiter EH: Defective activation of T suppressor cell function in nonobese diabetic mice: potential relation to cytokine deficiencies. *J Immunol* 140:3801–3807, 1988

37. Hayashi S, Witte PL, Shultz LD, Kincade PW: Lymphohemopoiesis in culture is prevented by interaction with adherent bone marrow cells from mutant viable motheaten mice. *J Immunol* 140:2139–2147, 1988

38. Koo GC, Manyak CL, Dasch J, Ellingsworth L, Shultz LD: Suppressive effects of monocytic cells and transforming growth factor-beta on natural killer cell differentiation in autoimmune viable motheaten mutant mice. *J Immunol* 147:1194–1200, 1991

39. Sennello JA, Fayad R, Morris AM, Eckel RH, Asilmaz E, Montez J, Friedman JM, Dinarello CA, Fantuzzi G: Regulation of T-cell-mediated hepatic inflammation by adiponectin and leptin. *Endocrinology* 146:2157–2164, 2005