Modification of adverse inflammation is required to cure new-onset type 1 diabetic hosts

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In nonobese diabetic (NOD) mice with overt new-onset type 1 diabetes mellitus (T1DM), short-term treatment with a ''triple-therapy'' regimen [rapamycin plus agonist IL-2-related and antagonist-type, mutant IL-15-related Ig fusion proteins (IL-2.Ig and mutIL-15.Ig)] halts autoimmune destruction of insulin-producing beta cells and restores both euglycemia and immune tolerance to beta cells. Increases in the mass of insulin-producing beta cells or circulating insulin levels were not linked to the restoration of euglycemia. Instead, the restoration of euglycemia was linked to relief from an inflammatory state that impaired the host's response to insulin. Both restoration of immune tolerance to beta cells and relief from the adverse metabolic effects of an inflammatory state in insulin-sensitive tissues appear essential for permanent restoration of normoglycemia in this T1DM model. Thus, this triple-therapy regimen, possessing both tolerance-inducing and select antiinflammatory properties, may represent a prototype for therapies able to restore euglycemia and self-tolerance in T1DM.

autoimmunity | diabetes | NOD mice | tolerance | transplantation

In humans and mice with type 1 diabetes mellitus (T1DM), autoimmune T cells infiltrate and destroy insulin-producing beta cells (1). Numerous treatments that prevent the development of diabetes and progression of the autoimmune "invasive insulitis" in prediabetic nonobese diabetic (NOD) mice have been identified (2). Far more challenging is the restoration of a euglycemic state in mice with frank T1DM (2). To date, only short-term treatments with anti-CD3 mAb (3–5), polyclonal antilymphocyte serum plus exendin-4 (6), or infusion of islet-specific regulatory T cells (7) has proven effective in restoring, in the long term, a euglycemic state in at least 50% of treated new-onset diabetic NOD mice in the absence of islet transplantation. Yet, the excellent results achieved with anti-CD3 treatment in diabetic NOD mice have served as the basis for initiating successful clinical trials of anti-CD3 mAb in humans with T1DM (8, 9). Therefore, treatments able to reverse established autoimmunity in NOD mice may identify treatment regimens appropriate for clinical testing (2, 8, 9). Recently, evidence that diabetic NOD mice express insulin resistance, a frequent feature of type 2 diabetes mellitus (5) has been established. Nonetheless, the etiology of insulin resistance and its significance for the restoration of euglycemia has not been explored.

Application of a short-term triple-therapy regimen [rapamycin (RPM) plus agonist IL-2-related and antagonist-type mutant IL-15-related Ig cytolytic fusion proteins (IL-2.Ig and mutIL-15.Ig)] that powerfully promotes long-term allograft survival/tolerance in daunting transplant models provides for enduring drug-free remission from overt diabetes through ablation of insulitis, restoration of immune tolerance to beta cells, and relief from an unforeseen inflammatory state residing in insulin-responsive tissues that impairs the ability of these tissues to respond to insulin. Hence, the paucity of success reported to date in creating enduring drug-free remissions of T1DM in NOD mice with narrowly targeted, T cell-directed therapies may relate to an unattended need to ablate inflammation-induced insulin resistance. The triple-therapy regimen, possessing both immune-tolerizing (10) and select antiinflammatory activities (11, 12), may serve as a prototype for regimens that are well suited to restore euglycemia in humans with a modest residual beta cell mass and new-onset T1DM.

Results

Short-Term Triple (RPM + IL-2.Ig + mutIL-15.Ig) Therapy Restores an Enduring Euglycemic State in Recent-Onset Diabetic NOD Mice. We tested the efficacy of a 14- or 28-day course of the triple-therapy regimen in new-onset (>10 days) T1DM NOD mice. All non-treated diabetic NOD mice remained hyperglycemic without spontaneous remissions (Table 1, group A), and most died within 7 weeks despite insulin treatment. In contrast, euglycemia was achieved within 5–7 weeks and maintained throughout a follow-up period of >300 days in 55 of 60 triple-therapy-treated diabetic NOD mice (Table 1, groups B and C). By comparison with triple therapy, remissions were less frequent (Table 1, groups D–F) and delayed (data not shown) if elements of the triple-therapy protocol were eliminated.

To determine whether the presence of the T cell regulatory-enriched $CD25^+$ T cell population is crucial to the beneficial therapeutic effects, we treated new-onset diabetic NOD mice with an anti-CD25 mAb regimen known to delete $CD4^+CD25^+$ T cells (12, 13). Seven days after the initiation of anti-CD25 mAb treatment, these $CD25^+$ T cell-depleted mice received triple-therapy treatment for 28 days (Table 1, group G). None of the diabetic NOD mice receiving triple therapy after anti-CD25 administration was rendered euglycemic (mean blood-glucose level, 335 mg/dl) by day 200 (Table 1, group G). As a control for the delay in instituting triple therapy imposed by the anti-CD25 pretreatment regimen, we delayed institution of triple therapy for 7 days in four NOD mice with new-onset diabetes (Table 1, group H). Each diabetic NOD mouse in the group started on the triple therapy on day 7 without prior administration of anti-CD25 was rendered normoglycemic by day 56 (mean blood-glucose level, 129 mg/dl) and remained euglycemic for >200 days of follow up (Table 1, group H). The full beneficial effects of triple therapy clearly require the presence of the regulatory-rich $CD25^+$ cell population. Note that administration of

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Abbreviations: NOD, nonobese diabetic; NOD/SCID, NOD/severe combined immunodeficiency; NOD-sp, spontaneous new-onset diabetic NOD mice; RPM, rapamycin; T1DM: type 1 diabetes mellitus; stz, streptozotocin; IR, insulin receptor; IRS-1, insulin response substrate-1.

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Table 1. Short-term treatment of T1DM NOD mice with RPM + IL-2.Ig + mutIL-15.Ig triple-therapy treatment permanently restores euglycemia

Group	Treated host	Treatment	Duration of treatment, days	Restored to normoglycemia, no./total treated
A	NOD-sp	None	None	0/150
B	NOD-sp	Triple therapy	28	37/40
C	NOD-sp	Triple therapy	14	18/20
D	NOD-sp	mutIL-15.Ig + RPM	28	9/19
E	NOD-sp	IL-2.Ig + RPM	28	10/20
F	NOD-sp	RPM	28	5/19
G	NOD-sp; CD25 + depleted	Triple therapy	28	0/4
H	NOD-sp; non-CD25 + depleted controls	Triple therapy	28	4/4

anti-CD25 mAb, but not IL-2/Fc, destroys $CD4^+CD25^+$ regulatory T cells (10).

RPM + IL-2.Ig + mutIL-15.Ig Therapy Aborts Autoimmunity and Induces Specific Immune Tolerance to Beta Cells in NOD Mice with New-Onset T1DM. The capacity of the triple-therapy regimen to destroy or inactivate diabetogenic T cells and/or tilt the balance of antiislet immunity toward tolerance was affirmed through experiments in which syngeneic islets were placed into new-onset diabetic hosts that were given triple therapy and thus rendered euglycemic. As shown in Table 2, untreated new-onset T1DM NOD recipients of syngeneic islets became diabetic 4–21 days posttransplantation (Table 2, group A), whereas treatment with a 28-day course of RPM + IL-2.Ig + mutIL-15.Ig started on the day of transplantation enabled permanent acceptance of syngeneic islet grafts (Table 2, group B).

To determine whether euglycemic, triple-therapy treated NOD mice were rendered tolerant to their islets, we chemically destroyed their beta cells through administration of streptozotocin (stz) long after (230–330 days) cessation of therapy (Table 2, groups C and D). Subsequently, syngeneic islet grafts were transplanted into these successfully treated NOD mice whose diabetic state was rekindled with stz administration. Without reinstitution of immunosuppressive therapy, all stz-treated recipients of syngeneic islets became normoglycemic within 24 h and remained normoglycemic thereafter (Table 2, group C). In contrast to the ready acceptance of syngeneic islet transplants, allogeneic islets are uniformly rejected within 4 weeks of transplantation (Table 2, group D). Hence, the triple-therapy regimen created a specific, drug-free tolerant state to syngeneic insulin-producing beta cells (Table 2, group D).

Table 2. Short-term treatment of T1DM NOD mice with triple RPM + IL-2.Ig + mutIL-15.Ig therapy treatment specifically restores immune tolerance to self-beta cells

Group	Donor	Recipient	Treatment	Graft survival, days*
A	NOD/SCID	NOD-sp	None	4, 7, 8, 10, 12, 21
B	NOD/SCID	NOD-sp	Triple therapy	>150†, >200 × 4
C	NOD/SCID	NOD-sp/stz	None	>100 × 5
D	C57BL/6	NOD-sp/stz	None	29, 29, 30, 30, 34

A streptozotocin-induced diabetic state (NOD-sp/stz) was induced in NOD recipients. Spontaneously diabetic NOD mice (NOD-sp; groups B and C) were previously restored to a euglycemic state by triple-therapy treatment. These mice remained euglycemic for 230–330 days after the cessation of treatment. Syngeneic NOD/SCID islet (groups A, B, and C) or allogeneic C57BL/6 (group D) islet grafts were transplanted into NOD recipients.

*Numbers indicate survival data for individual animals in each group (six in A, five each in B, C, and D).

†Islet graft removed at >150 days.

Expression of Cytotoxic T Lymphocyte (CTL) Genes, Th1 Genes, and Proinflammatory Cytokine Genes Within the Pancreatic Lymph Node Was Grossly Reduced in Triple-Therapy-Treated (RPM + IL-2.Ig + mutIL-15.Ig) Hosts. By using RT-PCR, expression for the CTL-type granzyme B, Th1-type IFNγ, and the proinflammatory IL-1β- and TNFα-cytokine genes was grossly reduced in pancreatic lymph nodes in successfully treated T1DM NOD mice 50 days after initiation of the triple-therapy regimen as compared with untreated controls (Fig. 1). These data indicate an inhibitory effect of triple therapy on local inflammation and islet-directed cytopathic Th1- and CTL-type immunity. Because proinflammatory cytokines can exert detrimental effects on beta cells (14–16), the marked inhibition of TNFα and IL-1β gene expression, even in the pancreatic node, in triple-therapy-treated NOD mice was of considerable interest.

Islet Histology, Beta Cell Mass, and Circulating Insulin Levels. Histologic analysis of islets from new-onset diabetic NOD mice indicates that (*i*) most islets are atrophic with few beta cells remaining (unstained central cells in Fig. 2 *A* and *B*); (*ii*) a minority of islets retain a near-normal proportion of beta cells; (*iii*) leukocytes invade the islets (invasive insulitis); and (*iv*) beta cells are partially degranulated (Fig. 2*C*). In contrast, islet histology of diabetic NOD mice rendered euglycemic by treatment and analyzed at least 70 days after cessation of therapy (Fig. 2 *D–F*) reveals that atrophic islets predominate normal islets (Fig. 2*D*). Nevertheless, residual islets with a significant number of beta cells are surrounded, but no longer invaded, by lymphocytes. Moreover, a higher proportion of remaining beta cells are granulated.

Despite signs of improvement, the morphometric analysis revealed an equivalent beta cell mass in recent-onset TIDM ($n = 7$; beta cell mass, 0.32 ± 0.21 mg) and in RPM + IL-2.Ig + mutIL-15.Ig-treated normoglycemic mice ($n = 7$; beta cell mass, 0.25 ± 0.15 mg) 70 days or more after onset of TIDM (see Table 3). For comparison, NOD/SCID mice of 13–18 weeks of age had a beta cell mass of 1.36 ± 0.12 mg ($n = 26$) (17). Thus, triple therapy arrests the loss of beta cells, but does not promote an increase in beta cell mass. In humans and in some rodent models, a reduction of the beta cell mass to ≈50% results in diabetes (18). Despite the restoration of euglycemia, circulating insulin levels did not rise in successfully treated NOD mice (Table 4). Perhaps unaccounted factors are involved in the return to normoglycemia in successfully treated hosts bearing only 20% of the normal beta cell mass.

RPM + IL-2.Ig + mutIL-15.Ig Treatment Ablates Insulin Resistance in Diabetic NOD Mice. Next, we sought to determine via insulin tolerance tests whether successful therapy influences the sensitivity of NOD mice to insulin-driven disposal of glucose. Blood glucose levels in 10-week-old new-onset diabetic mice fell by only 37% over a 1-h period after an i.p. injection of insulin, but dropped by 81–87% in (*i*) triple-therapy treated and (*ii*) age-matched control nondiabetic NOD mice (Fig. 3). Clearly, triple therapy ablates insulin

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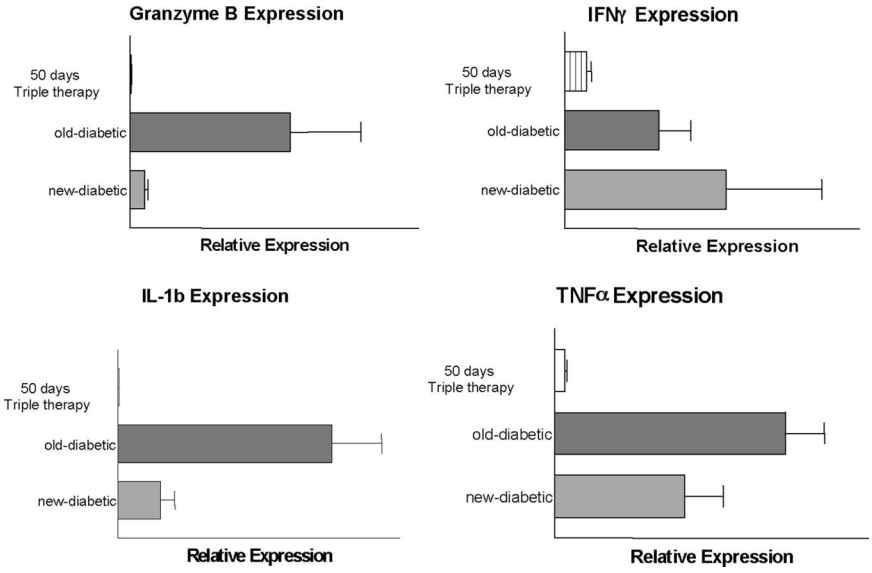

Fig. 1. Expression of cytotoxic T lymphocyte, Th1, and proinflammatory cytokine genes were grossly reduced in RPM + IL-2.Ig + mutIL-15.Ig triple-therapy-treated hosts. To quantitatively analyze gene-expression profiles, pancreatic-draining lymph nodes were harvested from newly diabetic (n = 4, onset of T1DM within 1 week), old-diabetic (n = 4, diabetic >30 days), and RPM + IL-2.Ig + mutIL-15.Ig-treated new-onset diabetic mice (n = 4, at day 50 after initiation of treatment). Expression of granzyme B, IFNγ, and the IL-1β and TNFα proinflammatory cytokine genes were analyzed on a linear scale and expressed relative to GAPDH expression.

resistance, thereby normalizing the response of host tissues to insulin.

Triple Therapy Restores *in Vivo* Insulin Signaling in Diabetic NOD Mice. We examined the effects of short-term RPM + IL-2.Ig + mutIL-15.Ig treatment on insulin signaling in skeletal muscle of new-onset diabetic NOD mice *in vivo* (19). *In vivo* insulin signaling was monitored by Western blot analysis of muscle protein extracts by using antibodies specific to (*i*) tyrosine-phosphorylated insulin receptor (IR), (*ii*) tyrosine-phosphorylated insulin response substrate-1 (IRS-1), and (*iii*) PKB/Akt proteins (Fig. 4). In this analysis, each blot represents an individual mouse. Insulin-stimulated tyrosine phosphorylation of IR was markedly diminished in new-onset T1DM NOD mice, with a 90% reduction in blot densitometry, compared with age-matched control nondiabetic NOD mice (Fig. 4). Impaired insulin signaling was also evident with respect to insulin-stimulated tyrosine phosphorylation of IRS-1 and PKB/Akt, molecules that normally transmit the downstream signals of the insulin-activated IR (Fig. 4). Thus, application of this therapeutic regimen ablates insulin resistance (see Fig. 3) via restoration of insulin signaling (Fig. 4) in T1DM NOD mice.

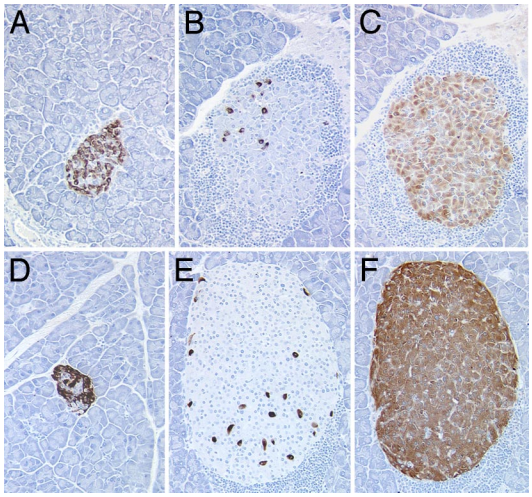

Fig. 2. Islet histology of spontaneous diabetic NOD mice at recent onset of disease and 52 days after ending treatment with RPM + IL-2.Ig + mutIL-15.Ig triple-therapy treatment (at least 70 days after onset). In recent onset, most islets are atrophic (*A*), with mainly glucagon-positive cells remaining after destruction of beta cells; a few residual islets (same islet in *B* and *C*) with many beta cells are present but manifest invasive insulitis. In these hyperglycemic mice, beta cells are partially degranulated, as seen in *C*. After treatment and restoration of a euglycemic state, here seen as 237 days after onset, atrophic islets are most prevalent (*D*); the islets with significant numbers of beta cells (same islet *E* and *F*) are surrounded by, but no longer invaded by, mononuclear leukocytes, have a more defined boundary of endocrine cells, and are no longer degranulated. Glucagon immunostaining was used in *A*, *B*, *D*, and *E*; insulin immunostaining was used in *C* and *F*.

RPM + IL-2.Ig + mutIL-15.Ig Triple Therapy Dampens Expression of Inflammatory Genes in Insulin-Responsive Tissue. By using RT-PCR, a targeted transcriptional profile for select inflammation-associated gene-expression events within muscle and fat, key tissues for insulin-driven disposal of glucose, was compiled in NOD mice [Table 5; supporting information (SI) Fig. 5]. Transcriptional profiles in new-onset T1DM mice rendered euglycemic by RPM + IL-2.Ig + mutIL-15.Ig triple therapy were compared with profiles obtained from mice rendered euglycemic from the time of diagnosis of T1DM with intense insulin therapy delivered with osmotic pumps. Triple therapy, unlike insulin-pump therapy, does not immediately render the treated mice euglycemic. Because triple-therapy treated mice remain hyperglycemic for 5–7 weeks, we temporarily used nonintensive, conventional insulin therapy delivered (i.p.) in triple-therapy-treated hosts to prevent extreme hyperglycemia until the advent of euglycemia (at which time insulin therapy is discontinued). Hence, we also analyzed insulin-sensitive tissues by RT-PCR in new-onset T1DM mice treated by conventional insulin treatment for 5–7 weeks (chronic diabetic group). As compared with both control groups (chronic diabetic and osmotic insulin-pump-treated NODs), expression of several proinflammatory cytokine-, acute phase- and other inflammation-associated genes were markedly decreased in fat (Table 5 and SI Fig. 5) (n = 5 for each data point) and muscle (data not shown) of triple-

Table 3. Morphological analysis of spontaneously diabetic NOD mice after treatment with triple therapy

NOD mice	n	Body weight, g	BGL, mg/dl	BCM, mg
New onset	7	29.7 ± 1.3	447 ± 48	0.32 ± 0.21
Triple therapy	7	29.8 ± 0.6	127 ± 7	0.25 ± 0.15

BGL, blood glucose level; BCM, beta cell mass.

Table 4. Circulating insulin levels in successfully treated new-onset T1DM NOD mice

Animal no.	Day*	Insulin, ng/ml	Day†	Insulin, ng/ml
1	0	1.275	51	0.644
2	0	2.451	51	1.109
3	0	9.368	65	3.940
4	0	2.390	51	1.355
5	0	2.2297	41	1.690

*Day 0, at which time RPM + IL-2.Ig + mutIL-15.Ig treatment commenced in new-onset T1DM NOD mice.
†The number of days after initiation of therapy at which time restoration of normoglycemia was evident.

therapy-treated new-onset T1DM NODs (Table 5 and SI Fig. 5). Whereas osmotic insulin-pump therapy as compared with conventional insulin-treated chronic diabetic NOD mice led to reduced expression of some inflammation-associated genes (e.g.,TNFα, SOCS2), the effects were not as broad or as potent as those produced by triple therapy (Table 5 and SI Fig. 5). Interestingly, expression of the TGFβ antiinflammatory gene was not dampened by triple-treatment therapy. Expression of IL-6 and IFN-γ were low in all samples and not influenced by therapy.

Discussion

We have studied the effects of the RPM + IL-2.Ig + mutIL-15.Ig regimen in the NOD model of T cell-dependent new-onset T1DM. This regimen consists of: (*i*) an agonist (wild type) IL-2.Ig fusion protein to enhance activation-induced cell death of effector, but not regulatory, T cells (12, 20) and provide IL-2-mediated "nonredundant function in the differentiation of (Foxp3+) regulatory T cells" (21); (*ii*) a high-affinity IL-15Rα antagonist, mutant IL-15.Ig fusion protein (mutIL-15.Ig) (22, 23) to block proliferation and promote passive cell death of activated effector T cells by aborting proliferative and antiapoptotic IL-15 signals (10, 20, 24) and to block the ability of IL-15 to induce expression of proinflammatory cytokines by activated mononuclear inflammatory cells (12); and (*iii*) RPM to blunt the proliferative response of activated T cells to T cell growth factors without inhibiting the activation-induced cell death signal imparted by IL-2 (25). Moreover, the agonist IL-2- and antagonist IL-15-related proteins were designed as IgG2a-derived Fc fusion proteins to ensure a prolonged circulating half-life and a potential means to kill activated effector, but not regulatory, IL-2R$^+$ and IL-15R$^+$ target cells via the activation of complement and FcR$^+$ leukocytes (12). Hence, the IgG2a-based complement-dependent and antibody-dependent cell-cytotoxicity-activating Ig-related fusion proteins are potentially cytotoxic proteins that target certain vulnerable activated IL-2R$^+$/ IL-15R$^+$, not IL-2R$^-$/ IL-15R$^-$ resting, mononuclear leukocytes (22, 26). This regimen has proven effective in extremely stringent transplant models (12).

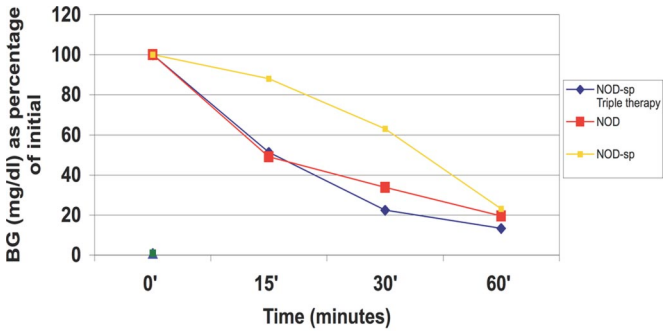

Fig. 3. Insulin tolerance test in NOD mice. The RPM + IL-2.Ig + mutIL-15.Ig triple therapy ablates insulin resistance in diabetic NOD mice. Insulin tolerance tests were performed in (*i*) age-matched spontaneous new-onset diabetic NOD mice, (*ii*) the RPM + IL-2.Ig + mutIL-15.Ig-treated spontaneous new-inset NOD mice, and (*iii*) nondiabetic NOD mice. Food was withheld 3 h before testing. Animals were weighed and blood samples were collected at 0 min; animals were injected i.p. with 0.75 units/kg of regular human insulin (Novolin; Novo Nordisk). Blood samples were then collected at 15, 30, and 60 min. The results were expressed as the percentage of the initial blood-glucose concentration. NOD-sp triple therapy, triple-therapy-treated NOD-sp mice.

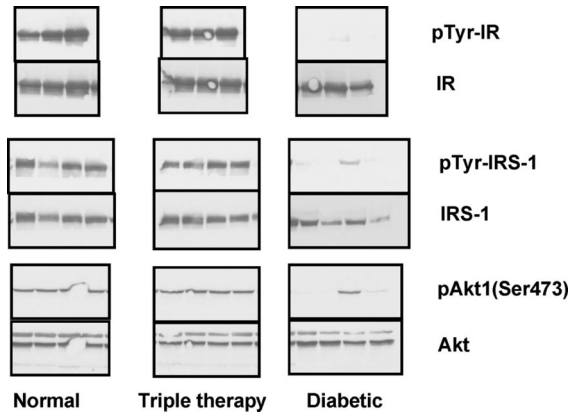

Fig. 4. Triple-therapy (RPM + IL-2.Ig + mutIL-15.Ig) treatment restores insulin signaling in new-onset diabetic NOD mice. Mice were fasted overnight and injected with human insulin (20 units per kg of body weight i.p.) to acutely stimulate insulin signaling. Skeletal muscle (gastrocnemius) was dissected and frozen in liquid nitrogen for immunoblotting analysis of insulin-signaling proteins. Each blot represents a different mouse.

The effects of therapy were tested in the clinically relevant new-onset T1DM NOD model (2). A 14- or 28-day course of RPM + IL-2.Ig + mutIL-15.Ig restored an enduring euglycemic state in 55 of 60 treated, spontaneously and acutely diabetic NOD mice. In parallel, the T cell-rich autoimmune islet-destructive invasive insulitis process was aborted, and a discriminating state of immune tolerance to "self"-islet beta cells was restored. Several other lines of evidence demonstrate that aggressive, beta cell-directed autoimmunity was markedly curtailed as a consequence of triple therapy even in the few NODs that failed to become euglycemic (see SI Table 6 and *SI Text*). Whereas triple therapy destroys or inactivates beta cell-destructive T cell populations, deletion of the regulatory T cell-rich population of CD25$^+$ T cells before treatment precludes restoration of a euglycemic state in RPM + IL-2.Ig + mutIL-15.Ig-treated new-onset diabetic NOD mice. The importance of preservation of the regulatory T cell-rich CD25$^+$ T cell populations after RPM + IL-2.Ig + mutIL-15.Ig therapy was also evident in the NOD passive transfer model (SI Table 6 and *SI Text*). Overall, the RPM + IL-2.Ig + mutIL-15.Ig regimen induces specific tolerance and tips the immune balance from diabetogenic toward beta cell-protective immunity.

Although triple therapy halted the progressive and destructive autoimmune-invasive insulitis, morphometric analysis showed no apparent increase in beta cell mass or in circulating insulin levels between recent-onset TIDM and successfully treated normoglycemic NOD mice. Hence, we sought to determine whether new-onset T1DM NOD mice exhibit insulin resistance as well as the well known destruction of insulin-producing beta cells.

Infiltration of activated macrophages or expression of proinflammatory cytokines and proteins within critical insulin-sensitive tissue hampers insulin responsiveness and insulin signaling in obesity-linked type 2 diabetes mellitus (14, 27). Chaparro *et al.* (5) recently reported that new-onset T1DM NOD mice do indeed manifest an insulin-resistant state. We tested the hypothesis that resolution of an inflammation-associated insulin-resistant state and of faulty insulin-triggered tyrosine phosphorylation of insulin-signaling molecules may be linked to restoration of euglycemia.

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Table 5. RPM + IL-2.Ig + mutIL-15.Ig triple therapy reduces intraadipose expression of inflammation-associated genes

Groups compared	SOCS1	SOCS2	TNFα	C3	Cp	CRP	GBP1	IL-1β	PAI-1	SAA-1	TGFβ	IL-6	IFNγ
Triple therapy vs. osmotic pump	*	NS	NS	**	**	NS	**	NS	**	**	*	NS	NS
Triple therapy vs. chronic diabetic NOD	**	NS	**	**	NS	**	*	NS	NS	NS	NS	NS	NS
Triple therapy vs. normal NOD	***	*	**	***	*	***	NS	*	NS	NS	NS	NS	NS

Mann–Whitney test was used for data analysis. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Indeed, successful treatment ablates insulin resistance and restores normal tyrosine phosphorylation linked insulin signaling in new-onset T1DM NOD mice. It is particularly pertinent that triple-therapy-induced relief of insulin resistance occurs in concert with a gross reduction of inflammation-related gene-expression events within fat and muscle, because expression of these molecules is known to cause insulin resistance in certain forms of type 2 diabetes mellitus (14). Inflammatory signals are known to disrupt insulin-stimulated tyrosyl phosphorylation of the insulin receptor and other downstream signaling molecules, a necessary action for insulin-triggered signal transduction (28). That triple therapy restored insulin-stimulated tyrosyl phosphorylation of the insulin receptor, IRS-1, and other downstream signaling molecules provides a mechanism by which triple therapy may resolve the insulin resistance. Other inflammatory proteins can also impair insulin signals albeit by mechanisms other than faulty insulin-triggered tyrosine phosphorylation (14, 29). In short, RPM + IL-2.Ig + mutIL-15.Ig therapy grossly dampens the pattern of inflammation-associated insulin resistance and faulty tyrosine phosphorylation of critical insulin-signaling cascade proteins but does not lead to an increase in circulating insulin or beta cell mass. Therefore, it seems likely that in addition to beneficial effects on the T cell-dependent autoimmune state, relief from insulin resistance is a critical factor in the restoration of euglycemia induced by successful therapy. In this respect, it is notable that the RPM + IL-2.Ig + mutIL-15.Ig regimen includes an IL-15R antagonist, because IL-15 is known to trigger the expression of proinflammatory cytokines (11, 12). It is notable that few T cell-directed therapies tested to date have proven successful in restoring euglycemia in the new-onset NOD model. Unless inflammation-induced insulin resistance and faulty insulin-signaling are restored, immune system-targeted therapies may fail to restore euglycemia.

Materials and Methods

Mice. Female NOD (NOD/LtJx) mice and NOD/SCID (NOD.CB17-Prkdc^{scid}/J) were purchased from Jackson Laboratories (Bar Harbor, ME) at 4 weeks of age and maintained under pathogen-free conditions at the Massachusetts General Hospital. All animal studies were approved by our institutional review board.

Blood glucose levels of NOD mice were monitored twice weekly with the Accu-Check blood glucose monitor system (Roche, Indianapolis, IN). When nonfasting blood-glucose levels were in excess of 300 mg/dl on three consecutive measurements, a diagnosis of new-onset diabetes was made. For syngeneic islet transplant recipients, blood glucose levels were checked at the time of transplantation, then daily for 2 weeks, and then two to three times per week afterward.

Induction and Management of Diabetes. Successfully treated and now-euglycemic NOD mice were rendered hyperglycemic with stz (275 mg/kg i.p.) treatment 230–300 days after the original spontaneous onset of diabetes. With the reemergence of hyperglycemia after stz administration, these diabetic NOD mice were used as syngeneic or allogeneic islet graft recipients. Graft failure was defined as the first day of 3 consecutive days of blood glucose levels >250 mg/dl.

Islet Transplantation. NOD/SCID mice and C57BL/6 mice (10–12 weeks old) were used as islet donors by using a modification of the method of Gotoh *et al.* (30). After Histopaque gradient (Histopaque-1077; Sigma, St. Louis, MO) purification, islets with diameters between 75 and 250 μm were hand-picked and transplanted under the renal capsule. Each recipient received 600–800 NOD/SCID or C57BL/6 islets.

Reagents and Treatment Protocols. The mutant IL-15.Ig and IL-2.Ig proteins were expressed and purified as previously described (22, 26). A rat anti-mouse CD25- (PC61 5.3, IgG1, ATCC TB222) producing hybridoma was purchased from American Type Culture Collection (Rockville, MD) and grown in serum-free hydridoma media (Invitrogen, Carlsbad, CA). The anti-CD25 mAb was purified by protein-G-affinity chromatography. RPM was purchased from the Massachusetts General Hospital pharmacy.

The triple-therapy regimen for mice includes antagonist-type mutant IL-15.Ig, wild-type IL-2.Ig proteins, and RPM. RPM was given i.p. at a dose of 3 mg/kg daily for the first 7 days, and every other day thereafter for a total of 14 or 28 days. IL-2.Ig and mutIL-15.Ig proteins were administered (5 μg i.p. daily) for 14 or 28 days. In some experiments, RPM alone or RPM plus one, but not both fusion proteins, were administered by using the aforementioned dosing regimen.

To deplete CD25$^+$ T cells, new-onset NOD mice were treated with three doses of anti-CD25 mAb (PC 61) at days 7, 5, and 3 before initiation of RPM + IL-2.Ig + mutIL-15.Ig therapy (13).

Insulin Tolerance Test. Insulin tolerance tests (28) were performed in age-matched NODs including (*i*) spontaneous new-onset diabetic NOD mice; (*ii*) RPM + IL-2.Ig + mutIL-15.Ig treated new-onset NOD mice; and (*iii*) nondiabetic NOD mice. Food was withheld 3 h before testing. Animals were weighed and blood samples collected just before the injecting the animals with 0.75 units per kg of regular human insulin (i.p.) (Novolin; Novo Nordisk, Clayton, NC). Blood samples were then collected at 15, 30, and 60 min after the insulin injection. The results were expressed as the percentage of initial blood-glucose concentration (28).

Morphometric Analysis of Beta Cell Mass. Animals were anesthetized by Nembutal (Sigma–Aldrich, St. Louis, MO), pancreases were excised, weighed, fixed in Bouin's solution, and embedded in paraffin. Islet sections (5 μm) were immunostained (peroxidase-antiperoxidase) by using rabbit anti-bovine glucagon (1:3,000; gift of M. Appel, University of Massachusetts) or anti-insulin (1:200; Millipore, Billerica, MA). Beta cell mass was measured by point-counting morphometry (31): one full footprint section of each pancreas was scored systematically at a magnification of ×420 by using a 90-point grid to obtain the number of intercepts over beta cell, alpha cell, exocrine pancreatic tissue, and nonpancreatic tissue; 200–500 fields per animal were counted. The beta cell relative volume (intercepts over beta cells divided by intercepts over total pancreatic tissue) was multiplied by the pancreas weight to calculate the beta cell mass (31).

RT-PCR Methods. To quantitatively analyze gene-expression profiles, pancreatic-draining lymph nodes were harvested from prediabetic, newly diabetic (onset of T1DM within 1 week), old diabetic (diabetic >30 days), and successfully treated formerly new-onset diabetic mice (at day 50 after initiation of treatment). Messenger RNA was extracted by using an RNeasy Mini Kit (Qiagen, Valen-

cia, CA). Reverse transcription to cDNA was performed by using TaqMan Reverse Transcription reagents obtained from Applied Biosystems (Foster City, CA). Specific message levels were quantified by real-time PCR by using the ABI 7700 Sequence Detection System (Applied Biosystems). Amplification was performed for a total of 40 cycles, and target gene products were detected by using gene-specific primers and FAM-labeled probes designed by Applied Biosystems. A GAPDH primer and VIC-labeled probe were used as the internal control (Applied Biosystems). Quantification of all target genes was based on a standard comparative threshold cycle (Ct) method.

To quantitatively analyze gene-expression profiles, fat ($n = 5$ for each data point) and muscle ($n = 3$ for each data point) were harvested from prediabetic, newly diabetic (onset of T1DM within 1 week), chronic diabetic (diabetic >30 days), and RPM + IL-2.Ig + mutIL-15.Ig-treated diabetic mice (at day 50 after initiation of treatment). Messenger RNA was extracted from fat and muscle by using Invitrogen Micro to Midi kits (Invitrogen) according to the manufacturer's instructions. Reverse transcription to cDNA was performed by using TaqMan Reverse Transcription reagents (Applied Biosystems). Oligonucleotide primers and fluorogenic probes were designed and synthesized and tested for efficiency and validity for the measurement of mRNA levels of Suppressor of cytokine signaling1 (SOCS1), Suppressor of cytokine signaling2 (SOCS2), tissue necrosis factor α (TNFα), Complement 3 (C3), Ceruloplasmin (Cp), C-reactive protein (CRP), Guanylate nucleotide binding protein-1 (GBP1), interleukin-1β (IL-1β), plasminogen activator inhibitor type-1 (PAI-1), Serum amyloid A-1 (SAA-), and transforming growth factor-β (TGFβ) (32). To measure mRNA levels of the internal control GAPDH transcript, a commercially available probe and primer mix (Applied Biosystems) were used. PCR analysis was performed by a two-step process. In the first step, a preamplification reaction was set up by using the ABI Biosystems Thermocycler (Applied Biosystems) with 3 μl of cDNA and 7 μl of dNTP, 10× PCR buffer, TaqDNA polymerase, and gene-specific oligonucleotide primer pairs. This step was followed by measurement of mRNA expression with an ABI Prism 7900HT sequence detection system (Applied Biosystems). PCRs for all of the samples were set up in duplicates as a 25-μl reaction volume by using 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 2.5 μl of preamplified template cDNA, 300 nM primers, and 200 nM probe. PCR amplification protocol included 40 cycles of denaturing at 95°C for 15 sec and primer annealing and extension at 60°C for 1 min. Transcript levels were calculated by using the standard curve method (32). The PCR amplicon for 18S rRNA was kindly provided by M. Suthanthiran (Weill Medical College, Cornell University, New York, NY). 18S rRNA amplicon was quantified and used for developing standard curves. The standard curves were based on the principle that a plot of the log of the initial target copy number of a standard versus threshold cycle results in a straight line. Messenger RNA levels in the samples were expressed as the number of copies per microgram of total RNA isolated from fat and muscle. Messenger RNA copy numbers were normalized with the use of GAPDH copy numbers (the number of mRNA copies in 1 μg of RNA divided by the number of GAPDH mRNA copies in 1 μg of RNA). In the absence of detectable level of a transcript, a value equal to half the minimum observed GAPDH-normalized level was assigned (33).

***In Vivo* Insulin-Signaling Studies.** *In vivo* insulin signaling experiments were performed on mice after a 16-h fast. Mice were injected i.p. with 20 units per kg of human insulin (Eli Lilly, Indianapolis, IN) or saline and killed 10 min later. Fat and skeletal muscle (gastrocnemius) were immediately dissected and frozen in liquid nitrogen for immunoblotting analysis of insulin-signaling proteins.

Immunoblotting. Fat and skeletal muscle (gastrocnemius) from the *in vivo* insulin-signaling studies were homogenized in a modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris·HCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 200 μM Na_3VO_3, supplemented with 1% protease inhibitor mixture (Sigma), and 1% tyrosine phosphatase inhibitor mixture (Sigma). Cell homogenates were incubated on ice for 45 min to solubilize all proteins, and insoluble portions were removed by centrifugation at 14,000 × g at 4°C for 15 min. Whole-cell lysates were separated by SDS/PAGE. Proteins on the gels were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The transferred membranes were blocked, washed, incubated with various primary antibodies, and followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Rabbit polyclonal anti-IR (pY1162/1163) and anti-IRS-1 (pY612) antibodies were purchased from BioSource (Camarillo, CA). Rabbit polyclonal anti-IR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-IRS-1 was obtained from Upstate Biotechnology (Lake Placid, NY). Visualization was performed with a chemiluminescence reagent, by using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech). The blots were quantified by using densitometry (Molecular Dynamics, Sunnyvale, CA).

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