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Nonmitogenic CD3 Antibody Reverses Virally Induced (Rat Insulin Promoter-Lymphocytic Choriomeningitis Virus) Autoimmune Diabetes Without Impeding Viral Clearance[1]

Matthias G. von Herrath,[2]* Bryan Coon,* Tom Wolfe,* and Lucienne Chatenoud†

Treatment with nonmitogenic CD3 Ab reverses established autoimmune diabetes in nonobese diabetic mice by restoring self-tolerance, and is currently under clinical evaluation in patients presenting recent onset type I diabetes. Due to the immunosuppressive potential of this strategy, it was relevant to explore how this treatment would influence the outcome of concomitant viral infections. In this study, we used a transgenic model of virally induced autoimmune diabetes (rat insulin promoter-lymphocytic choriomeningitis virus) that allows for more precise tracking of the autoaggressive response and choice of the time point for initiation of autoimmunity. CD3 was most effective during a clearly defined prediabetic phase and prevented up to 100% of diabetes by drastically lowering activation of autoaggressive CD8 lymphocytes and their production of inflammatory cytokines. Interestingly, reversion of established disease could be achieved as well, when nonmitogenic CD3 was administered late during pathogenesis to overtly diabetic recipients. Most importantly, competence to clear viral infections was maintained. Thus, administration of nonmitogenic CD3 prevents diabetes by sufficient systemic reduction of (auto)aggressive lymphocytes, but without compromising antiviral immune competence. ***The Journal of Immunology*****, 2002, 168: 933–941.**

CD3 mAbs are potent immunosuppressants that have been extensively used in the clinical and experimental transplantation and more recently also in autoimmunity (1–11). Most CD3 Abs are now non-FcR-binding monoclonals such as $F(ab')_2$ in mice (6–8, 12) and humanized Fc-mutated monoclonals in humans (13–16), which are devoid of the massive cytokine-releasing potential regularly observed with conventional FcR-binding CD3 Abs (i.e., 145 2C11 in mice (17–20); OKT3 in humans (21–23)). Both FcR- and non-FcR-binding CD3 Abs do possess tolerogenic properties, as best exemplified by their capacity to induce durable regression of recently diagnosed autoimmune type 1 diabetes (T1D)[3] in nonobese diabetic (NOD) mice by restoring self-tolerance following short-term treatment (9, 12). This result has led to clinical trials in recent onset type 1 diabetic patients that are presently ongoing. Extensive investigations attempted to dissect the mode of action of CD3 Abs in autoimmune diabetes, and also evaluated potential risks related to its well-known immunosuppressive capacity. Concerning these mechanistic aspects, data from the NOD mouse model indicate that CD3 Abs work in two consecutive phases. First, immediate clearance of insulitis occurs, explaining the rapid reversal of ongoing disease (9, 12). This is followed by resetting of $CD4^+$ T cells, leading to active tolerance mechanisms involving a $CD25^+CD62L^+$ subset that does not express a Th2 cytokine profile (24, 25).

One problem in the previous studies in NOD mice was that autoaggressive lymphocytes and their Ag specificities were difficult to precisely define, quantify, and track (26). We therefore elected to evaluate this intervention in the RIP-LCMV model for T1D. These transgenic mice express a defined protein from lymphocytic choriomeningitis virus (LCMV) (i.e., the gp) as target autoantigen under control of the rat insulin promoter (RIP) (27–29). In these RIP-LCMV-gp mice, the expression of the transgene, which is restricted to the β cells, does not lead spontaneously to islet destruction or infiltration (indifference or ignorance). Only upon LCMV infection self-tolerance is broken and T1D rapidly develops within 10–14 days in over 95% of such mice (27–29). Destruction of islet cells is initiated by virus (self) Ag-specific T lymphocytes that attack β cells via perforin and inflammatory cytokines (mainly IFN-γ) (30). $CD8^+$ T cells play a major role in this effector phase since their in vivo depletion prevents disease, whereas depletion of $CD4^+$ T cells does not (28).

The model offers four main advantages for our present goal: first, the time of initiation of autoimmunity can be chosen experimentally, and therefore the issue of timing of CD3 Ab therapy in relation to the autoimmune process can be addressed. Second, autoaggressive (LCMV-specific) T cells can be precisely tracked and quantified in RIP-LCMV mice because the primary autoantigen is known and most target epitopes have been mapped (31, 32). Third, one may analyze the effect of CD3 Ab treatment in a T1D model, in which it has been well demonstrated that $CD8^+$ T lymphocytes play a major effector role (28, 30). Finally, this model provides a unique opportunity to evaluate the fate of the LCMV viral infection to which the host is exposed at the time of the CD3 Ab treatment. This is of major importance in the context of the presently ongoing clinical application of this strategy in patients presenting with recent onset T1D.

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[3] Abbreviations used in this paper: T1D, type 1 diabetes; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; LT, lymphotoxin; NOD, nonobese diabetic; NP, nucleoprotein; RIP, rat insulin promoter.

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We report in this work that complete prevention of diabetes occurs when non-FcR-binding CD3 Ab is given from days 8 to 12 after LCMV infection, which is right before onset of diabetes in untreated control animals. Long-term diabetes protection is also obtained when giving the Ab earlier (days 0–5 after infection), but in this case, surprisingly, with a transient disease appearance and its secondary regression. Similar regression is observed when giving the Ab at the very onset of overt diabetes (days 15–20 after infection). Importantly, in mice treated with non-FcR-binding CD3, these significant protective effects were obtained without impeding viral clearance.

Materials and Methods

Transgenic mice and diabetes induction

RIP-gp H-2^b transgenic mice were described previously and express the LCMV-gp only in their islets, but not any other organs (28). These mice have been backcrossed to the C57BL/6 background for over 12 generations and develop diabetes independent of CD4 lymphocytes. The presence of the RIP-gp construct was determined by slot-blot hybridization or PCR using gene-specific probes or primer sets, respectively, as described previously (27, 28, 33).

Virus stocks consisted of LCMV strain ARMSTRONG (ARM; clone 53b) and Pichinde and vaccinia viruses. LCMV was plaque purified three times on Vero cells, and stocks were prepared by a single passage on BHK-21 cells. A dose of 10^5 PFU in 0.2 ml PBS was routinely given i.p. to induce diabetes.

Abs and immunization schedules

The hybridoma-producing 145 2C11 (hamster Ig anti-murine CD3) was obtained from J. A. Bluestone (34). The purified endotoxin-free Ab used for in vivo treatment was produced by CellTech (Berkshire, U.K.). Anti-CD3 F(ab′)$_2$ was prepared by conventional pepsin digestion of the entire Ab molecule (2-h digestion at 37°C in pH 3 buffer, pepsin at 2% (w/v) final concentration). Digested CD3-F(ab′)$_2$ were purified using a Sepharose CL-4-B protein A (Pharmacia, Piscataway, NJ) affinity chromatography column, followed by an UltroGel AcA54 column (Pharmacia). Ab to systemically block IL-4 was generated from the 11B11 hybridoma (BD PharMingen, San Diego, CA). Both Abs were resuspended at a final concentration of 1 mg/ml, and 100 μg was injected in 100 μl i.v. into the tail vein. For CD3-F(ab′)$_2$, injections were either given before or after LCMV infection of RIP-LCMV-gp mice daily on 5 consecutive days. For the IL-4 Ab, injections (500 μg i.v.) were administered every other day for 2 wk following LCMV infection of RIP-gp mice. The precise immunization schedules are indicated in the legend for each figure or table, respectively.

CTL assays and precursor analysis

CTL activity was measured in a 5- to 6-h in vitro ^{51}Cr release assay. Briefly, to judge CTL recognition and lysis, syngeneic or allogeneic target cells were either infected with LCMV-ARM (multiplicity of infection = 1), or with recombinant vaccinia virus expressing the full-length LCMV-ARM gp or nucleoprotein (NP) (multiplicity of infection = 3). Uninfected target cells coated with LCMV peptides gp aa 33–41, 276–286 or NP aa 396–404, or NP aa 118–127 were also used as targets. Epitopes gp aa 33–41, gp aa 276–286, and NP aa 396–404 of LCMV are all H-2^b (D^b) restricted for CTL recognition, while NP aa 118–127 is restricted by the L^d haplotype. Assays used splenic lymphocytes at E:T ratios of 50:1, 25:1, and 12.5:1, or CTL clones and secondary CTL lines at ratios of 10:1, 5:1, 2.5:1, and 1:1. To determine CTL activity after secondary stimulation, spleen cells harvested from mice 30–120 days after primary inoculation with 1×10^5 PFU LCMV i.p. were incubated with MHC-matched, irradiated, LCMV-infected, or peptide-coated macrophages in the presence of T cell growth factor containing IL-2 and irradiated syngeneic spleen feeder cells for 5–12 days. MC57 (H-$2K^bD^b$) and BALB/Cl7 (H-2^d) cells used as CTL targets were grown as reported. Precursor frequencies of LCMV-specific CTL were determined as described.

Histologic and immunochemical analysis of tissues

Tissues taken for histologic analysis were fixed in 10% zinc Formalin and stained with H&E. Immunochemical studies were conducted on 6- to 10-μm freshly frozen cryomicrotome sections for immunostaining of islets to detect expression of MHC class I and II, D^b, insulin, CD4, CD8, B220, and F4/80. Primary Abs were applied for 1 h. These consisted of rat anti-mouse CD4 (clone RM 4-5), anti-CD8 (clone 53-6.7), anti-B220 (clone RA3 6B2), anti-F4/80 (clone A3-1), anti-MAC-1 (clone M 1/70), anti-MHC class I (clone M 1/42), and anti-class II (clone M5/114) (BD PharMingen, and Boehringer Mannheim, Indianapolis, IN). After washing in PBS, the secondary Ab (biotinylated goat anti-rat (or anti-mouse) IgG; Vector Laboratories, Burlingham, CA) was applied for 1 h. Color reaction was developed with sequential treatment using avidin-HRP conjugate (Boehringer Mannheim) and diaminobenzidine-hydrogen peroxide.

Blood glucose monitoring

Blood samples were obtained from the retroorbital plexus of mice, and plasma glucose concentration was determined using Accucheck III (Boehringer Mannheim). Mice with blood glucose values higher than 300 mg/dl were considered to be diabetic.

Cytokine assays: ELISPOTs

Quantitation of cytokines in cell cultures with LCMV Ag-specific or generalized (PMA/ionomycin) in vitro stimulation was performed in sandwich ELISPOT assays, as described elsewhere (35, 36). Briefly, 96-well plates were precoated overnight at 4°C with 2 μg/ml purified capture Abs. After washing and blocking with 10% FCS in PBS, serial dilutions of live cells were added and were incubated overnight in a humidified cell incubator at 37°C in the presence of Ag (LCMV peptides) and Ag presenting (irradiated splenocytes). Washing and a 1-h incubation with 1 μg/ml matched biotinylated detection Abs (BD PharMingen) overnight at 4°C were followed by a 30-min incubation with a streptavidin-peroxidase conjugate (1:1000; Boehringer Mannheim). For color reaction, H_2O_2-activated ABTS (Sigma-Aldrich, St. Louis, MO) solution in 0.1 M citric acid (pH 4.35) was added. Plates were quantified blinded by two different investigators under a cell counting microscope.

Flow cytometry

Staining of cell surface Ag and intracellular Ags was performed as described previously (36). For staining of intracellular cytokines, cells were stimulated for 5–10 h by either of the following methods using PE-conjugated anti-cytokine Abs (PE-IFN-γ, 18115A; PE-TNF-α, 18135A; PE-IL-2, 18005A; PE-IL-4, 18035A): Polyclonal stimulation was provided by 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) or, alternatively, plate-bound CD3 and soluble CD28 Ab (37). Virus-specific stimulation was provided by addition of 10^{-6} M LCMV-gp33–42 peptide in the presence of 50 U/ml human rIL-2. All stimulation cultures contained 1 μg/ml brefeldin A (B7651; Sigma-Aldrich) to block protein transport into post-Golgi compartments and allow cytokines to accumulate within cells. In some experiments, LCMV-infected, irradiated peritoneal exudate cells provided Ag-specific stimulation, and brefeldin A was added for 5–10 h after transfer to fresh peritoneal exudate cells. Negative controls were stained with cytokine-specific PE-conjugated Abs preincubated for 30 min at 4°C with an excess of recombinant cytokine. Cells were acquired and analyzed on a FACSort or FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software.

RNA analysis/RNase protection assays

Whole pancreata were harvested on days 2, 7, 17, and 21 after LCMV infection and immediately homogenized in 2 ml of Tri-reagent (Molecular Research Center, Cincinnati, OH) using a Polytron homogenizer. Total RNA was extracted with chloroform, followed by isopropanol precipitation and washing with ethanol. Twenty micrograms of total pancreatic RNA was used for hybridization with a [^{32}P]UTP-labeled multitemplate set containing specific probes for IFN-γ and TNF-α provided by a commercial kit (Riboquant, mCK-3b; BD PharMingen). The RNase protection assay was conducted according to the manufacturer's guidelines. The resulting analytical acrylamide gel was scanned using a STORM-860 PhosphorImager System (Molecular Dynamics, Sunnyvale, CA), and the intensity of bands corresponding to protected mRNAs was quantified using the ImageQuant image analysis software (Molecular Dynamics) (38).

Viruses

Virus stocks consisted of LCMV-ARM (clone 53b), Pichinde virus, and vaccinia virus/LCMV gp and NP recombinants that expressed LCMV-gp aa 1–398 and LCMV-NP aa 1–558 (39). Virus was plaque purified three times on Vero cells, and virus stock was prepared by a single passage on BHK-21 cells. Stocks of recombinant vaccinia viruses were prepared by infection of 143 thymidine kinase$^-$ cells in media containing bromodeoxyuridine (39).

Adaptive transfers

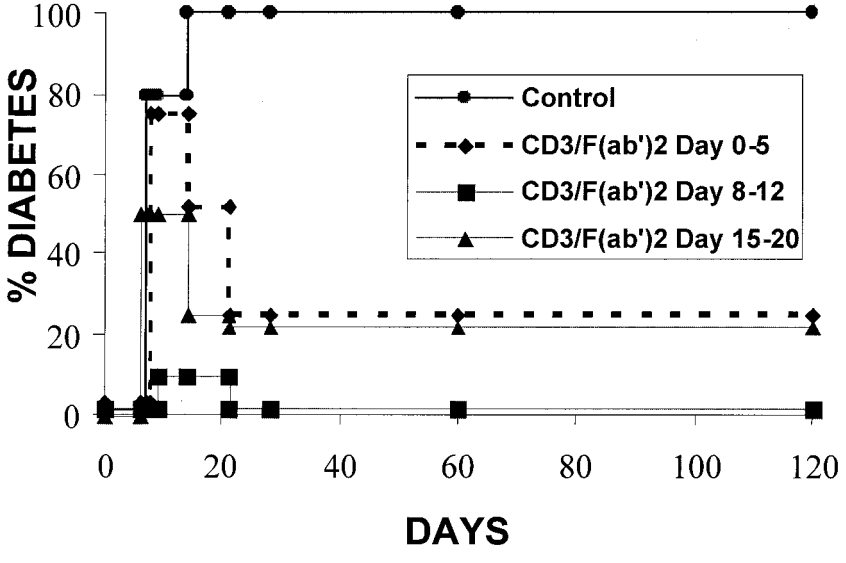
For adaptive transfers, splenocytes from each donor mouse were obtained by gently pressing the spleens through a mesh and lysing the RBCs, as described previously (28). One donor spleen was used per recipient, and splenocytes were injected i.p. in 1 ml of PBS at day 12 post-LCMV infection. CD8 depletion was conducted before transfer using magnetic bead technology (33, 37).

Results

Non-FcR-binding CD3 Ab prevents and reverses diabetes in RIP-gp mice

The natural course of diabetes in LCMV virus-infected RIP-LCMV-gp mice evolves in three phases (30). First, activation of APCs, production of chemokines such as IFN-γ-inducible protein, and secretion of IFN-γ occurs between days 2 and 5 after viral infection and is directly due to the presence of LCMV in the pancreas. Second, the specific autoimmune process is initiated on day 7 after LCMV infection, reflected in the arrival of autoaggressive (anti-LCMV) lymphocytes in pancreas and islets. Finally, destruction of most β cells is seen between days 10 and 18 after LCMV infection and is mediated by inflammatory cytokines secreted from islet-infiltrating lymphocytes (30).

Thus, it appeared important to assess the ability of CD3 Ab to affect diabetes development when applied during each of these stages. Groups of 8–10 RP-LCMV-gp mice were treated for 5 days daily with 100 μg of CD3-F(ab′)$_2$ as shown in Fig. 1. When the Ab was given in the first phase, long-term protection from diabetes was observed. However, diabetes initially appeared with the same incidence and rate seen in untreated controls. Interestingly, the Ab induced regression of established diabetes that took place 7–10 days after cessation of Ab administration. Thus, early administration of the Ab during diabetes pathogenesis did not induce primary prevention, but modified the late course of the disease (Fig. 1). In contrast, when administered during the second phase, the Ab completely prevented diabetes development, and only 10% of the mice



Treatment with αCD3 (days post LCMV)	Diabetes incidence after LCMV infection (diabetic mice/total mice group)													
	Day 0		Day 7		Day 14		Day 21		Day 28		2 mo		4 mo	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
None (or control antibody)	0/8	0/12	0/8	0/12	6/8	8/12	8/8	12/12	8/8	12/12	---	---	---	---
Days 0-5	0/4	0/8	0/4	0/8	3/8	8/8	2/4	4/8	1/4	2/8	1/4	2/8	1/4	2/8
Days 8-12	0/10	0/10	0/10	0/10	1/10	1/10	1/10	1/10	0/10	0/10	0/10	0/10	---	---
Days 15-20	0/4	0/8	0/4	0/8	7/4	4/8	1/4	2/8	1/4	2/8	1/4	2/8	1/4	2/8
Prior to d0 or after d20	0/8	0/5	0/5	0/5	5/5	4/5	5/5	5/5	5/5	5/5	---	---	---	---

FIGURE 1. Nonmitogenic CD3 prevents and reverses ongoing diabetes in RIP-LCMV mice when administered during a crucial prediabetic time period. Groups of RIP-LCMV-GP mice were infected with 1×10^5 PFU LCMV i.p. and treated daily with 100 μg of anti-CD3 F(ab′)$_2$ i.v. during the time intervals indicated. Ab had to be given after induction of autoimmunity and before clinical diabetes development to be optimally effective. Best success was obtained, if CD3 F(ab′)$_2$ were administered during the prediabetic phase, and while autoaggressive lymphocytes usually expand, which is between days 8 and 12 after viral infection. Blood glucose was assessed by Accuckeck III (see *Materials and Methods*), and mice with blood sugar values exceeding 300 mg/dl were considered diabetic. The experiment was performed twice with very similar results. The table shows the number of diabetic mice for each time point (*left column*, Expt. 1; *right column*, Expt. 2). The figure displays the data from both experiments taken together.

developed diabetes transiently 2–3 wk after LCMV infection (Fig. 1). Finally, late administration (day 15–20) of CD3-F(ab′)$_2$ to recently diabetic RIP-gp mice resulted in reversion from diabetes, and 75% of the animals were permanently protected. Thus, CD3 has the ability to not only protect RIP-LCMV-gp mice from diabetes, but also to reverse clinical disease in this mouse strain, which we had never observed previously with any of the clinical interventions we evaluated.

Non-FcR-binding CD3-F(ab′)$_2$ prevents expansion and activation of autoaggressive lymphocytes and augments systemic production of IL-4 by CD4$^+$ cells

Numbers of autoaggressive LCMV CTL were determined at 7 and 14 days post-LCMV infection in RIP-LCMV mice treated successfully with FcR-binding CD3 and non-FcR-binding CD3-F(ab′)$_2$ (Table I). In the first experiment, autoaggressive LCMV-specific lytic CTL activities on day 7 post-LCMV infection were significantly reduced in CD3-F(ab′)$_2$-treated mice (Table II). For these studies, intact CD3 or CD3-F(ab′)$_2$ were given early (days 0 through 5) to complete one full treatment course before harvesting splenocytes for CTL assays on day 7. LCMV-gp$_{33}$-specific lytic CTL killing was 55 ± 8% in untreated mice, 20 ± 6% in CD3-F(ab′)$_2$-treated mice, and 4 ± 1% in FcR-binding CD3-treated mice. Thus, both the intact FcR-binding CD3 and the non-FcR-binding CD3-F(ab′)$_2$ statistically significantly reduced LCMV CTL activities in RIP-gp mice, but CD3-F(ab′)$_2$ treatment left some sufficient residual CTL activity intact, whereas intact CD3 completely abolished LCMV CTL, which fits with the lack of viral clearance observed in this group.

Next, inflammatory cytokine production by LCMV-responsive CD8, but not CD4 cells on day 14 was reduced significantly by a factor 5 (Tables I and II). For these studies, treatment with CD3-F(ab′)$_2$ was given daily during the optimal time frame between days 5 and 12 post-LCMV infection of RIP-gp mice. Furthermore, overall generation of IFN-γ (Tables I and II) and TNF-α (data not shown) by CD8 lymphocytes after Ag-nonspecific stimulation with CD3/CD28 was reduced 3- to 4-fold, indicating that overall systemic loss of inflammatory effector functions was most likely responsible for the specific reduction of autoaggressive (LCMV-responsive) lymphocytes (Tables I and II). At the same time, IL-4 production by CD4 lymphocytes was increased by a factor of >3, as assessed by ELISPOT analysis after CD3/CD28 stimulation (Tables I and II). Thus, anti-CD3 treatment decreases lysis and cytokine production by LCMV-specific responsive autoaggressive lymphocytes in RIP-LCMV-gp mice, a finding that fits well with the observed prevention of T1D (Fig. 1).

Non-FcR-binding CD3-F(ab′)$_2$ Ab treatment does not impede viral clearance

Interestingly, despite this systemic reduction of antiviral CTL and an immune deviation reflected by a Th1-Th2 shift in cytokine production, the ability to clear LCMV infection was not impaired and occurred on day 14 postinfection, in CD3-F(ab′)$_2$-treated mice with a similar efficiency as in untreated mice (Tables I and II). In addition, the capacity to eliminate other viral infections was unaffected (clearance of Pichinde and vaccinia viruses, as assessed plaque assays described in *Materials and Methods*; data not shown). These observations are of particular importance, because T cells predominantly mediate clearance of these viruses. Thus, administration of CD3-F(ab′)$_2$ appears to be a safe and effective way to prevent autoimmunity by systemically modulating the immune system to a sufficient, but not excessive degree, which is reflected in the ability to still clear viral infections. In contrast, treatment with intact FcR-binding CD3 Ab had major side effects in that it severely affected viral clearance (Tables I and II) as well as survival, as illustrated by the 75% mortality rate scored at 4 wk following LCMV infection in FcR-binding CD3-treated RIP-LCMV-gp mice.

Reduction of TNF-α, lymphotoxin (LT)β, and overall lymphocyte numbers in spleens of CD3-F(ab′)$_2$-treated RIP-LCMV-gp mice

We extended our cytokine analysis by performing RNase protection analyses on spleens comparing CD3-F(ab′)$_2$-treated and untreated control mice on day 10 post-LCMV infection. The amounts of LTβ and TNF-α were significantly reduced, and overall expression of CD4, CD8, and TCRα was lowered as well (Fig. 2). This confirms our previous observations (Tables I and II) showing lack of expansion of CD4 and CD8 lymphocytes in CD3-F(ab′)$_2$-treated mice and reduction of their inflammatory cytokines. It is interesting to note that no change in IL-2 production was detected. In parallel, there was a lack of CD8 infiltration in islets of RIP-gp mice protected from diabetes after receiving CD3-F(ab′)$_2$ treatment (Fig. 3).

Protection from autoimmune diabetes cannot be transferred

Since we had observed an increased systemic number of IL-4-producing CD4 lymphocytes after CD3 administration (Tables I and II) in ELISPOT assays, we evaluated the possibility that active

Table I. *Nonmitogenic CD3 treatment lowers production of IFN-γ by CD4 and CD8 lymphocytes, and increases overall IL-4 production, without affecting viral clearance*[a]

	% IFN-γ-Producing Lymphocytes							
	CD3/anti-CD28		LCMV GP peptides		IL-4 Anti-CD3/28	LCMV(PFU/g tissue) Average 3 Mice		Pichinde Titer (PFU/g tissue)
	CD4	CD8	CD4	CD8	CD4	Day 14	Day 7	Day 14
No treatment	9 ± 2	25 ± 3[b]	4 ± 1.3	11 ± 3[b]	18 ± 4/10^5[b]	<10	4×10^5	<10
CD3 F(ab′)$_2$ (days 8–12)	2 ± 1	6 ± 2[b]	3 ± 1.2	2 ± 1[b]	60 ± 8/10^5[b]	<10	3×10^5	<10
Intact CD3 (days 8–13)	1 ± 1	3 ± 1	1 ± 1	1 ± 1	ND	$10^5 \pm 10^3$[b]	3.8×10^5	5×10^4

[a] Two to three RIP-LCMV-GP mice were infected with 10^5 PFU of LCMV on day 0, treated with CD3 i.v. from days 5 to 12, and splenocytes were harvested at day 14 after infection. For enumeration of IFN-γ (shown) and TNF-α (not shown, exhibited parallel changes compared to IFN-γ)-producing lymphocytes by intracellular cytokine FACS cells were stimulated for 5 h with anti-CD3/CD28 (overall cytokine production, not Ag specific) or 10^{-6} M LCMV gp33 (MHC class I-restricted) or gp61 (MHC class II-restricted) peptides in the presence of IL-2. Unspecific background cytokine production in the presence of IL-2 (<1.13% cells positive) was subtracted from all mean values (±SE) shown. IL-4-producing cells were enumerated by ELISPOT analysis after 24-h stimulation of magnetic bead-purified CD4 lymphocytes from spleens in the presence of anti-CD3/CD28 and irradiated syngenic feeder splenocytes (unspecific background = 8 spots/10^6 cells). Viral titers were assessed in spleen homogenates by plaque assays as described in *Materials and Methods*.

[b] Statistically (*t* test) significant differences <0.05 are indicated.

Table II. *Nonmitogenic CD3 treatment reduces the autoaggressive (LCMV-specific) response*[a]

	% ^{51}Cr Release from Targets				
	H-2^b				H-2^d
	No. Peptide		gp33		LCMV infected
	50:1	25:1	60:1	25:1	50:1
No treatment	2 ± 1	0	55 ± 8	20 ± 4	3 ± 1
CD3 F(ab′)$_2$ (days 0–5)	2 ± 1	0	20 ± 6	10 ± 2	0
Intact CD3 (days 0–5)	0	0	4 ± 1	0	0

[a] Three RIP gp mice were infected with 10^5 PFU of LCMV and treated with anti-CD3 F(ab′)$_2$ or intact CD3 from days 0 to 5. On day 7, spleens were harvested and in vivo CTL activity was tested on syngenic (H-2^b) targets coated with LCMV CTL peptides (see *Materials and Methods*) at E:T ratios of 50:1 and 25:1. Means ± SE are displayed.

regulatory cells had been induced. In these studies, 2×10^7 splenocytes were isolated on day 12 after CD3-F(ab′)$_2$ treatment (days 8 through 12) of RIP-LCMV-gp mice and transferred i.p. into synchronously infected, but not CD3-treated RIP-gp recipients. No reduction of T1D was observed in recipients of such transfers, and all mice developed T1D within 3 wk post-LCMV infection (Fig. 4). To exclude the possibility that cotransfer of pathogenic CD8 lymphocytes would inhibit the function of regulatory T cells, i.e., CD25^{high} (24, 40) or CD4$^+$ IL-4$^+$ T cells (36, 41), we depleted CD8 lymphocytes in donor spleens from CD3-F(ab′)$_2$-treated mice before injection into syngeneic RIP-gp recipients, but no protection was observed (Fig. 4). The lack of transferable protection fits with the finding that we did not observe any changes in numbers of CD25^{high} lymphocytes (data not shown) and their cytokine production (10% IFN-γ positive), making the induction of CD25$^+$ regulatory cells less likely in the RIP-LCMV model, although we had observed their generation in CD3-F(ab′)$_2$-treated NOD mice (24) (L. Chatenoud, unpublished observation). One must take into account that the rapid onset RIP-GP model is less prone to immune regulation (28), that CD25$^+$ regulatory cells might only occur in high enough numbers in the pancreatic draining node, and that induction of this type of regulation requires more time before it can be assessed in transfer experiments. Finally, it was possible that the systemic increase in IL-4 production could still have a beneficial effect, despite the fact that immune regulation could not be transferred, indicating that no significant numbers of active regulatory lymphocytes were induced after CD3-F(ab′)$_2$ treatment. However, systemic blockade of IL-4 by injection of 11B11 Ab had no effect on the degree of protection achieved by CD3-F(ab′)$_2$ treatment (Fig. 4).

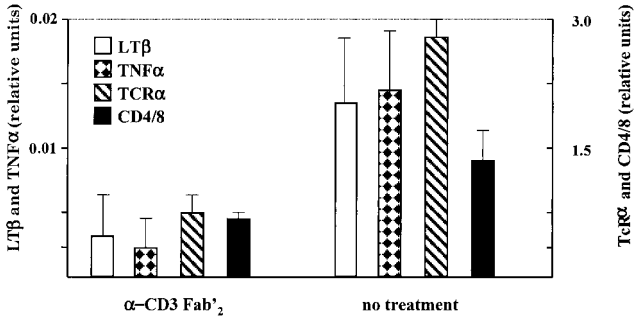


FIGURE 2. RNase protection analysis of cytokines in spleens after nonmitogenic CD3 treatment. Spleens were harvested from groups of two to three RIP-LCMV-GP mice 10 days after LCMV infection. Treatment with CD3 F(ab′)$_2$ was done daily from days 8 to 10; 100 μg of i.v. RNA was prepared as described in *Materials and Methods*, and RNase protection analysis was performed using each sample in duplicates. Relative units are determined after normalization to housekeeping genes (GAPDH) and quantitation by PhosphorImager (see *Materials and Methods*). Only cytokines with significant changes are on display; other cytokines without significant alteration were IL-2, LTα, IL-6, IFNα, and IL-13. RNase protection analysis was performed twice, and the mean of all groups and analysis ±SD is shown (at least 4, maximum 6 datapoints).

Reversion of clinical diabetes by non-FcR-binding CD3-F(ab′)$_2$ results in nonaggressive islet infiltration

Histological examination of RIP-GP transgenic mice that had received CD3-F(ab′)$_2$ treatment after onset of clinical signs of T1D (days 15–20 after LCMV infection) revealed a minor loss of infiltration in most islets (Fig. 3). Thus, permanent reversion from clinically apparent T1D occurs by preserving just enough β cell mass to maintain normoglycemia, as already observed in the NOD model (12). Islets are not free of infiltration, but insulitis (Table III) remains nonprogressive. This is clinically important, because it demonstrates that this type of intervention is: 1) one of the few treatments capable of reverting clinical T1D, and 2) effective in doing so not only in the NOD, but also in another animal model for T1D, the RIP-LCMV model. Importantly, overall immune competence remains intact, which makes this strategy attractive for the use in humans at high risk for T1D.

Discussion

The CD3 complex is expressed on each T cell. Consequently, one should expect that the immunosuppression invariably associated with a CD3 Ab treatment is essentially nonspecific and shows no selectivity for a particular T cell subset. Thus, despite the demonstrated beneficial tolerogenic properties of this strategy, CD3-mediated protection from autoimmune diabetes has the inherent potential risk associated with the immunosuppression induced at the time of Ab administration. This is exemplified by the unfavorable outcome of LCMV infection, as assessed by the high mortality rate in CD3-treated mice given the intact FcR-binding Ab molecule at the peak of the antiviral immune response. In this context, it is quite remarkable that CD3-F(ab′)$_2$ retain enough of the therapeutic capabilities of the entire Ab molecule to prevent and reverse autoimmune diabetes while preserving a sufficient immune capacity to deal with concomitant viral infections. It is important to stress in this work that CD3-F(ab′)$_2$-treated mice retained an intact capacity to clear not only LCMV, but also Pichinde and vaccinia virus infections.

This observation is crucial from the therapeutic viewpoint and provides a major additional argument for the usage of non-FcR-binding CD3 Abs in the clinic. The clinical application of such Abs was already strongly driven by their lack of mitogenicity, explaining their inability to induce, upon first injection, the massive systemic cytokine release syndrome typically observed with intact FcR-binding CD3 Abs (15, 16, 21–23). Our present results show

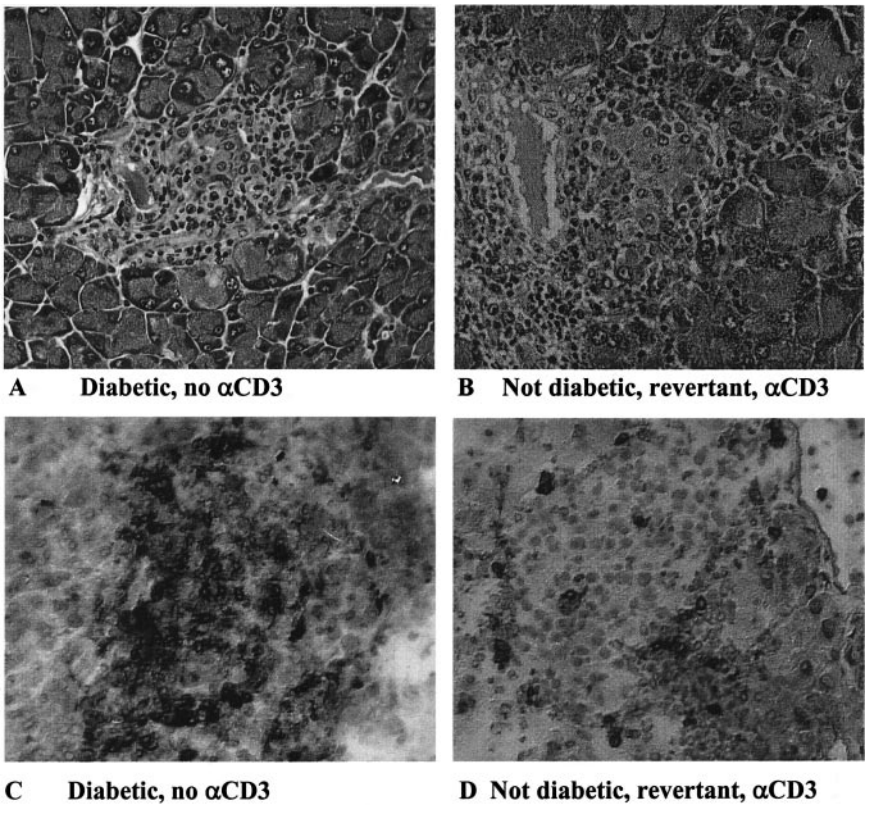


FIGURE 3. Infiltration into islets of CD3 $F(ab')_2$-treated RIP-GP mice that reverted from clinically onset diabetes. *A*, H&E stain, untreated RIP-GP control, diabetic, day 14 post-LCMV infection. *B*, H&E, CD3 $F(ab')_2$-treated (days 15–20), previously diabetic RIP-GP mouse, no diabetes, day 21 post-LCMV infection. *C*, CD8 stain, untreated RIP-GP control, diabetic, day 14 post-LCMV infection. *D*, CD8, CD3 $F(ab')_2$-treated (days 15–20), previously diabetic RIP-GP mouse, no diabetes, day 21 post-LCMV infection. ts were analyzed per pancreas.

that nonmitogenic CD3 Abs also avoid the harmful consequences of this therapy on responses to viral infections even when these occur simultaneously in relation to the Ab treatment.

On the mechanistic side, intact FcR-binding CD3 may affect the antiviral immune response through massive T cell depletion. It is in fact well established that T cell depletion is more profound and long-lasting following administration of intact CD3 Abs with a functional Fc fragment as compared with non-FcR-binding CD3 (9, 12, 42). This fits well with the present data showing that LCMV-specific CTLs are undetectable in mice treated with FcR-binding CD3. Another nonmutually exclusive possibility is that the high mortality occurring with intact FcR-binding CD3 is due to the cytokine release syndrome that develops at the time of ongoing LCMV infection. In this case, some antiviral immunity could persist for a prolonged period of time (43). These data also provide an important example for the differential threshold in terms of the frequency of T lymphocytes required for induction of autoimmunity, which appears to be higher than that required for antiviral immune competence. Indeed, our earlier studies using an MHC class I-restricted blocking peptide had defined that >1/5000 LCMV-specific T cells are needed to destroy LCMV-GP transgenic β cells, but that this number is still sufficient to eliminate the virus (44). Our present data support this concept, since intact CD3 completely abrogates the LCMV-specific lytic CTL activities and their IFN-γ production, whereas CD3-$F(ab')_2$ leaves residual lytic CTL activity and cytokine production intact. It is interesting to note that the putative additional modes of action elicited by CD3-$F(ab')_2$ (i.e., antigenic modulation (12, 45), systemic immune deviation, induction of regulatory T cells (24)) appear to be important for protection from diabetes, but do not severely affect clearance of viral infections. In conclusion, it will be vital that immune-based interventions with systemic activity respect this line between abrogating the pathogenic response, yet retain a sufficient immune capacity to warrant antiviral immune competence. Our experimental data demonstrate that this is a real possibility.

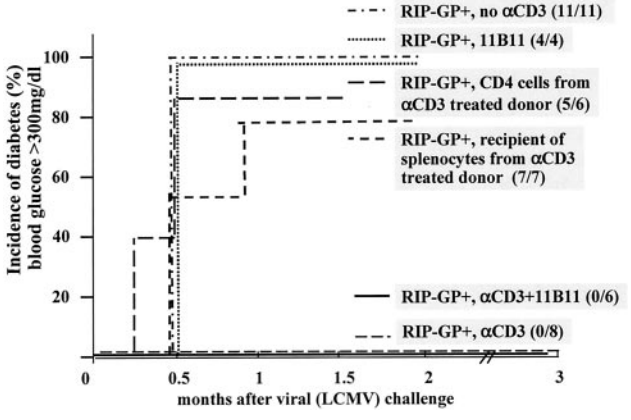


FIGURE 4. Protection from diabetes after nonmitogenic CD3 $F(ab')_2$ treatment is not transferable. Several groups of RIP-LCMV-GP mice were injected with LCMV and treated with CD3 $F(ab')_2$ from days 5 to 12 postinfection (optimal protocol). Adoptive transfers were done at day 12 postinfection, and recipients received one donor spleen equivalent i.p. "CD4 cells" in the above figure means that CD8 cells were depleted from the donor spleen using magnetic bead technology, but CD4 cells were not purified separately. The 11B11 IL-4-blocking Ab was given i.v. every other day, as described in *Materials and Methods*. The number of mice in each group are indicated.

Another impressive feature of the data we present is the ability of non-FcR-binding CD3 to not only protect RIP-LCMV-GP mice from diabetes, but also to reverse established disease in this mouse strain, which we had never observed previously with any of the clinical interventions we evaluated. The natural course of the disease in RIP-LCMV-GP mice is rather acute, most β cells being eliminated as soon as 18 days after LCMV infection. The acuteness of the disease in this model relies on the characteristics of the

Table III. *Quantitation of insulitis*[a]

	% Insulitis							
	None[b]		Peri		Intra		Full	
Treatment	∅	CD3	∅	CD3	∅	CD3	∅	CD3
Day 2	0	0	0	0	0	0	0	0
Day 7	80	80	18	16	2	4	0	0
Day 14	6	10	6	10	10	70	78	10
Day 20	*	10	*	10	*	75	*	5

[a] Three mice were analyzed (pancreata) per time point, at least 15 islets were analyzed per pancreas.
[b] None, no lymphocytes in or around islets, *, more islets present; Peri, lymphocytes solely in periphery; Intra, lymphocytes in and around islets; Full, islet appears to be completely filled with lymphocytes.

major effector population, i.e., mostly LCMV-specific high-affinity CD8 T cells that colonize the periphery because of lack of expression of the transgene in the thymus (28, 30). Indeed, this is clearly demonstrated through studies in another transgenic line of mice, RIP-LCMV-NP, that express the transgene (the NP of LCMV) in both the β cells and the thymus. Accordingly, LCMV infection triggers a slow onset T1D in these mice that appears within 1–6 mo depending on the host's MHC background (27, 28, 46). In this model, high affinity autoreactive CD8 T cells are deleted in the thymus, and the autoimmune process is dependent on low affinity autoreactive CD8 that escapes selection, and on a major CD4 component that plays an important modulatory role (28, 33). Thus, in preclinical terms and focusing on disease kinetics, the RIP-LCMV-NP model, as the NOD model, displays more similarities with slow onset adulthood diabetes. In contrast, RIP-LCMV-GP mice may represent a better model for rapid onset T1D typical of childhood.

The finding that the non-FcR-binding CD3 Ab can reverse disease when given to overtly diabetic RIP-LCMV mice was unexpected. However, it fits well with the notion, supported by in vitro and in vivo data from the NOD model, that the physical destruction of β cells is to a large extent preceded by a phase of reversible T cell-mediated inflammation that results in a significant impairment in their capacity to release insulin in response to conventional stimulations. Thus, heavily infiltrated pancreatic islets from old, but still nondiabetic female NOD fail to release insulin upon stimulation with high glucose concentrations when examined immediately after the isolation. However, this inhibition of β cell function is fully reversible upon clearing of the immune cell infiltrate after culturing the islets for 7 days in vitro (47). Moreover, Sreenan et al. (48) showed that at the time of diabetes onset there is a significant residual β cell mass of ~30%, and that in old nondiabetic NOD female mice the in vivo insulin secretion is reduced to a greater degree than the actual β cell mass (48). Thus, the diabetes regression we observed strongly indicates that in the RIP-LCMV-GP model as well as in the NOD, a large component of the diabetogenic effect of aggressive infiltrating T lymphocytes is due to an inflammation that impairs β cell function. This may be effectively reversed using agents such as CD3 Abs that are effective at rapidly and completely clearing the insulitis (9, 12).

The results obtained when the Ab was administered in the early, first phase (day 0–5) appear more paradoxical, since such treatment proved to be unable to prevent diabetes onset, but finally led to disease regression similar to that obtained after late Ab administration. In such early treated mice, one cannot invoke a solely antiinflammatory effect, as suggested above for mice treated late, since diabetes regression occurred 6 days after the end of CD3 Ab treatment. It is more tempting in this setting to suspect an additional immunoregulatory mechanism.

Complete and durable prevention of disease was observed when the non-FcR-binding CD3 was applied from days 8 to 12 after infection once the autoimmune process is actively engaged, as assessed by the islet invasion (30). A marked decrease in the cytotoxic and cytokine (IFN-γ and TNF-α)-producing capacity of LCMV-specific autoaggressive CD8 lymphocytes was observed in treated as compared with control mice. Given the key role of IFN-γ as an effector mechanism in the RIP-LCMV-GP model (by directly acting on β cells in synergy with other cytokines and by sensitizing β cells to CTL lysis through up-regulation of class I molecules (30)), its reduction fits well with the observed prevention of type 1 diabetes. Moreover, CD4 cells from treated mice displayed a typical Th2 immune deviation with an excess of IL-4 production upon polyclonal stimulation. This could have been of potential relevance to explain the therapeutic effect, because immune intervention strategies such as autoantigen (insulin) administration, which shift the intraislet infiltrate from a Th1 to a Th2 phenotype (33), have a beneficial effect in the RIP-LCMV model, and because destructive insulitis in the final effector phase of T1D in RIP-LCMV mice is characterized by a predominance of IFN-γ over IL-4 (30, 49). However, the CD3 Ab-induced protection was not abrogated by IL-4 Ab therapy.

Thus, taken together, our data indicate that non-FcR-binding CD3 Ab may act in two nonmutually exclusive ways: 1) A direct action on effector T cells by partially reducing their numbers (i.e., partial depletion, as already demonstrated in other models including the NOD mice (12)) and affecting their lytic as well as inflammatory functions; and 2) induction of an immunomodulatory effect.

These two effects have been shown to coexist in a chronological fashion in the CD3-protected NOD mice (12, 24). It is interesting that neither in RIP-LCMV nor in NOD mice immunomodulation appears to involve Th2-type cells, since the Ab-induced protection was not abrogated by IL-4 Ab therapy. In contrast, studies in the NOD mouse model had demonstrated that protection from diabetes after CD3 $F(ab')_2$ treatment can be transferred to NOD-SCID recipients by mixing $CD25^+$ or $CD62L^+$ regulatory cells with diabetogenic lymphocytes in coadoptive transfers (24, 50–51) (L. Chatenoud, personal communication). These studies indicated the induction of an active regulatory cell population among the $CD25^+$ lymphocytes of yet unknown Ag specificity. It is possible that we were unable to find evidence for transferable protection in our present investigation, because the numbers of $CD25^+$ are too low to be effective in direct transfers or even CD8-depleted spleens (Fig. 4). These studies do not preclude the existence of a regulatory population that could affect diabetes in a model with slower disease onset, but our data show that down-modulation of a Th1 response can occur in the absence of IL-4-producing immunoregulatory cells.

In conclusion, treatment with nonmitogenic CD3-F(ab′)$_2$, but not with intact FcR-binding CD3, appears to be a safe and efficacious way to prevent autoimmune diabetes. We find that the main mechanism underlying this effect is reduced expansion of overall inflammatory T lymphocytes, which results in a drastic reduction of lytic activity as well as IFN-γ and TNF-α generation by autoaggressive CD8 lymphocytes. Importantly, general immune competence is not affected by this intervention, whereas intact FcR-binding CD3 treatment abrogates the ability to clear LCMV infection. We find no direct evidence for regulatory cells in our RIP-LCMV model with rather rapid disease onset, and the protection from T1D cannot be transferred. It will be advisable to quantify primarily autoaggressive T cells during clinical trials that should be reduced in all successfully treated individuals. Augmentation of certain regulatory lymphocytes (CD25$^+$ cells or IL-4 producers) might vary depending on the individual situation and kinetics of disease. Finally, nonmitogenic CD3 appears to be one of the few interventions capable of reverting clinical T1D, which occurs in NOD as well as RIP-LCMV mouse transgenic lines. These observations open new perspectives for the use of this intervention not only in patients presenting with recent onset diabetes, but also in prediabetic subjects and in recipients of islet allografts.

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