Variable Effects of Transgenic c-Maf on Autoimmune Diabetes

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Autoimmune diabetes is associated with T helper 1 polarization, but protection from disease can be provided by the application of T helper 2 (Th2) cytokines. To test whether genetic manipulation of T-cells can provide protective Th2 responses, we developed transgenic mice in which T-cells express the interleukin-4–specific transcription factor c-Maf. When crossed with a transgenic model that combines a class II restricted T-cell receptor specific for influenza hemagglutinin with islet β-cell expression of hemagglutinin, the c-Maf transgene provided significant protection from spontaneous autoimmunity but not from adoptively transferred diabetes. In a second transgenic model in which islet cells express the lymphocytic choriomeningitis virus nucleoprotein, the virus infection triggers autoimmune diabetes within a few weeks involving both CD4 and CD8 T-cells; here too transgenic c-Maf provided significant protection. Surprisingly, when the c-Maf transgene was backcrossed with the NOD model of spontaneous disease, no protection was evident. Thus, transgenic c-Maf can strongly influence autoimmune disease development in some models, but additional factors, such as background genetic differences, can influence the potency of its effect. ***Diabetes*** **50:39–46, 2001**

Several rodent models of autoimmune diabetes have been developed in which lymphocytic infiltration of pancreatic islets of Langerhans results in specific destruction of insulin-producing β-cells and concomitant hyperglycemia. In NOD mice, diabetes occurs spontaneously and is dependent on both CD4 and CD8 T-cells (1–5). In another case, transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) on β-cells (rat insulin promoter [RIP]-LCMV-NP) develop diabetes only after infection with the virus (6,7). As with NOD mice, diabetes development requires both CD4 and CD8 cells. Still other mouse models have been developed in which diabetes occurs spontaneously but only requires CD4 or CD8 cells. For example, in transgenic mice simultaneously expressing influenza hemagglutinin (HA) on islet β-cells and a class II restricted T-cell receptor (TCR)-SFE specific for HA diabetes develops spontaneously within 6 weeks of life (8). Models dependent on CD8 T-cells have also been developed in both LCMV and HA systems (9,10).

Whereas these disease models may differ in kinetics, T-cell subset involvement, and inducing factors, other aspects remain similar. These include the activation of autoreactive T-cells and the development of highly structured islet infiltrates before clinical hyperglycemia (8,11–14). These characteristics are also shared with human diabetes, making them useful models for uncovering disease mechanisms and helpful for the evaluation of potential treatments, such as cytokine and gene therapies (15,16).

Acceleration of autoimmune diabetes has been associated with T helper 1 (Th1) cells (17–20), whereas resistance has been associated with increased T helper 2 (Th2) cytokines (21–27). Thus, one immunotherapeutic approach for diabetes is to introduce genetic manipulations that skew T-cell responses toward Th2 cytokine production. For example, recent work suggests that transgenic interleukin (IL)-10 overproduction by islet-specific T-cells can provide limited protection from diabetes (28). Alternatively, genetic manipulation of key transcription factors may provide a more efficient way of controlling cytokine production. Because IL-4 is pivotal for Th2 cell development (29,30) and its increase is also associated with diabetes inhibition (31,32), transcription factors that control IL-4 transactivation, such as c-Maf (33–35), are of particular interest. Fortunately, c-Maf transgenic T-cells do not constitutively produce IL-4, but instead overexpress IL-4 rapidly after T-cell receptor–mediated stimulation (34). So in effect, antigen-inducible expression of IL-4 may be accomplished through constitutive production of the transcription factor c-Maf.

Here we test the ability of transgenic c-Maf to attenuate autoimmune diabetes using three different models of disease. In two of these models (TCR-SFE/Ins-HA and RIP-LCMV-NP), constitutive T-cell expression of c-Maf leads to significant inhibition of disease. This corresponds to c-Maf–mediated early increases in type 2 cytokine production by activated T-cells. Interestingly, diabetes was not attenuated in c-Maf transgenic NOD mice, suggesting that additional complex genetic factors influence the ability of c-Maf to affect disease.

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CTL, cytotoxic T-lymphocyte; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; IFN-γ, γ-interferon; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; NP, nucleoprotein; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; RIP, rat insulin promoter; TCR, T-cell receptor; Th1, T helper 1; Th2, T helper 2; TSRI, the Scripps Research Institute.

RESEARCH DESIGN AND METHODS

Mice. Mice bearing a murine c-Maf cDNA transgene have been previously described (34). c-Maf transgenic mice were crossed with B10.D2 mice (The Scripps Research Institute [TSRI] rodent breeding colony, La Jolla, CA) until homozygosity was achieved at the major histocompatibility locus for H-2^d. Mice expressing a TCR-SFE specific for the HA peptide 110–119 (SFERFEIFPK) presented by I-E^d have been previously characterized (8) and were maintained on a B10.D2 background. Likewise, Ins-HA transgenic mice expressing hemagglutinin on islet β-cells under the control of the rat insulin promoter have been previously described (36). RIP-LCMV-NP 25–3 mice have been previously described (7) and were maintained on a B10.D2 background. c-Maf mice were bred with TCR-SFE or RIP-LCMV-NP mice to generate double transgenic c-Maf/TCR-SFE or c-Maf/RIP-LCMV-NP mice. To study the influence of c-Maf on spontaneous diabetes c-Maf/TCR-SFE/Ins-HA mice and TCR-SFE/Ins-HA littermates (age- and sex-matched) were monitored for hyperglycemia weekly. In experiments involving NOD mice, c-Maf transgenic mice were backcrossed for five generations with NOD/Shi mice (TSRI rodent breeding colony) before analysis to minimize the influence of any diabetes resistance genes potentially linked to the transgene. Randomly chosen fifth generation female NOD/c-Maf and nontransgenic littermate mice were monitored for hyperglycemia once every 2 weeks. Transgene integration was determined by polymerase chain reaction (PCR), except for RIP-LCMV-NP mice. All mice were maintained in a virus-free environment at TSRI rodent colony in accordance with National Institutes of Health and TSRI institutional guidelines.

Mouse genotyping. Standard PCR analysis of tail DNA was used to determine mouse genotypes. To identify homozygous H-2^d (B10.D2) mice, the microsatellite marker MM23 primers (sense: 5′-GTTTCAGTTCTCAGGGTCCTA-3′ and anti-sense: 5′-CAGGATTCTGTGGCAATCTGG-3′) were used. The following primers were used to identify integration of the indicated transgenes: c-Maf, sense: 5′- TGTTGTGGTGCAGAACTGGAT-3′ and anti-sense: 5′-GTTTCA GGTTCAGGGGGAGGT-3′; TCR-SFE, sense: 5′-GAACTGCTCAGCATAACT CCC-3′ and anti-sense: 5′-GAGGCTGCAGTCACCCAAAG-3′; Ins-HA, sense: 5′-CAATTGGGGAAATGTAACATCGCCG-3′ and anti-sense: 5′-AGCTTTGGGTAT GAGCCCTCCTTC-3′. Genotyping of RIP-LCMV-NP mice was performed by hybridization of tail DNA with NP-specific probes as previously described (7).

Adoptive transfer and viral induction models. For adoptive transfer models of disease, spleen and lymph node mononuclear cells were pooled from c-Maf/TCR-SFE or TCR-SFE mice. The total number of $CD4^+$ T-cells in each pool was determined by multiplying the total cell yield with the percent $CD4^+$ cells as determined by flow cytometry using anti-CD4-phycoerythrin (PharMingen). Adoptive transfers of 1×10^7 $CD4^+$ T-cells were performed by intravenous injections into irradiated (700 rad) Ins-HA recipients. The total cell numbers varied for each donor group, but CD4 T-cell numbers were kept constant for each recipient injection. Blood glucose levels were monitored weekly after cell transfer. In a second adoptive transfer model, 5×10^6 purified $CD4^+$ cells from spleen and lymph nodes of age-matched diabetic TCR-SFE/Ins-HA or nondiabetic c-Maf/TCR-SFE/Ins-HA mice were injected intravenously into nonirradiated RAG-$1^{-/-}$/Ins-HA recipients. Blood glucose levels were monitored every 3–4 days posttransfer. Diabetes induction of 6- to 8-week-old c-Maf^{+or}/RIP-LCMV-NP mice was accomplished by intraperitoneal injection of 10^5 PFU of LCMV Armstrong, clone 53b (37). Blood glucose levels were monitored weekly after infection.

Blood glucose monitoring and statistics. Onset of diabetes (>16.6 mmol/l) was determined by monitoring blood glucose levels using Chemstrip bG test strips with an Accu-Chek III blood glucose monitor (Boehringer Mannheim). Incidence of diabetes was graphed on Kaplan-Meier cumulative survival plots, and the log-rank (Mantel-Cox) test for statistical significance was performed using StatView software.

Histology. Pancreas tissue was harvested, fixed in zinc formalin, and paraffin embedded before sectioning. Sections were stained with periodic acid-Schiff (PAS) by standard methodology.

T-cell purification. $CD4^+$ or $CD8^+$ T-cells were purified from lymph nodes using negative selection with magnetic beads. Briefly, lymph nodes were dissociated into single-cell suspensions, passed through 70-μm cell strainers, and washed with RPMI-1640 plus 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine, 25mmol/l HEPES, and 5×10^{-5} mmol/l β-mercaptoethanol (complete medium). Cells were incubated for 30 min at 4°C in phosphate-buffered saline containing 2% serum and 2 μg/ml rat anti–mouse B220 (PharMingen) plus either 2 μg/ml rat anti–mouse CD8 (PharMingen) for CD4 cell purification or 2 μg/ml rat anti–mouse CD4 (L3T4, PharMingen) and 2 μg/ml rat anti–mouse CD4 (YTS.177) for CD8 cell purification. Then cells were washed and undesired cell populations were removed using BioMag goat anti–rat IgG (H+L)–conjugated magnetic beads (Polysciences) according to the manufacturer's instructions.

Proliferation assays and enzyme-linked immunosorbent assay. Lymph node mononuclear cells were isolated from 6- to 8-week-old c-Maf, TCR-SFE, NOD/c-Maf, or NOD mice. For proliferation assays, 3×10^5 lymph node CD4 or CD8 cells/well (purified by negative selection) were stimulated with plate bound anti–mouse CD3ε (10 μg/ml; PharMingen) plus anti-mouse CD28 (1 μg/ml; PharMingen) for 24 h and pulsed with 1 μCi/well for another 24 h before harvest. Triplicate samples were counted on a Microbeta Trilux liquid scintillation counter (Wallac).

For cytokine analysis, 3×10^5 $CD4^+$ or $CD8^+$ lymph node cells from 6- to 8-week-old mice were stimulated as described above in 200 μl/well complete medium. Supernatants were collected after 48 or 72 h and assayed for IL-4, IL-5, IL-10, or γ-interferon (IFN-γ) by sandwich enzyme-linked immunosorbent assay (ELISA) using the appropriate antibody pairs (PharMingen) and peroxidase-conjugated streptavidin plus 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). All incubations were for 30 min at 37°C. Plates were washed with phosphate-buffered saline containing 0.05% Tween 20 after each incubation. Cytokine concentrations were interpolated from standard curves obtained from spectrophotometer (Spectra 2.06) readings using DeltaSoft 3 software. For analysis of secondary responses, cells were stimulated 72 h then rested in complete medium with 20 U/ml rhIL-2 (Pepro Tech, Rocky Hill, NJ) for 4–5 days. Cells were then restimulated at 3×10^5 cells/well (200 μl/well) and supernatants were harvested after 24 h and assayed for cytokine levels as described above.

Cytotoxic T-lymphocyte (CTL) assays and pCTL frequency. For analysis of primary CTL activity, c-Maf transgenic or nontransgenic littermate mice were injected intraperitoneally with 10^5 PFU of LCMV. Effector cells were obtained from spleens harvested 7 days postinfection. CTL activity was assessed by standard ^{51}Cr release assays using uninfected or LCMV-infected H-2^d fibroblasts (BALB C17) as target cells at 100:1 and 50:1 effector/target ratios. Triplicate cultures were incubated for 5 h at 37°C with 5% CO_2 as previously described (7). For analyses of CTL activity after secondary stimulation, splenocytes were harvested 30–60 days postinfection (as described above) and cultured in vitro for 1 week with syngeneic LCMV-infected macrophages and 50 U/ml IL-2. Here primary stimulation occurred in vivo, whereas the secondary stimulation was performed in vitro. CTL activity was assessed as described above using effector:target ratios of 10:1 and 5:1. Precursor CTL frequencies were determined as previously described (37). Briefly, splenocytes were harvested 7 days after LCMV immunization and serially diluted in 96-well flat bottom plates containing LCMV-infected irradiated (2,000 rad) macrophages and irradiated syngeneic feeder splenocytes. After 8 days, each well was tested with ^{51}Cr-labeled LCMV-infected BALB C17 target cells in a standard 5 h ^{51}Cr release assay. CTL precursor frequencies were calculated as follows: pCTL(f) = (4.6-ln[percentage of negative wells])/number of splenocytes per well). The pCTL frequencies were defined as the slope of the linear regression along at least three separate data points. Positive cultures were defined by a specific ^{51}Cr release more than three standard errors above background lysis.

RESULTS

c-Maf inhibits disease onset of transgene-mediated spontaneous diabetes. To examine the influence of c-Maf on diabetes, we took advantage of previously established c-Maf transgenic mice (34). Expression of c-Maf in these mice is controlled by a modified CD4 promoter that allows expression in both CD4 and CD8 T-cells (34). Increases in the type 2 cytokines IL-4, IL-5, and IL-10 occur in both CD4 and CD8 T-cells from c-Maf transgenic mice after stimulation with anti-CD3 plus anti-CD28 (Fig. 1). Increased type 2 cytokine levels are evident at early time points after both primary and secondary stimulation. Notably, c-Maf transgenic T-cells retain the ability to produce IFN-γ, consistent with previous studies (34).

The influence of c-Maf on diabetes onset was initially evaluated using the TCR-SFE/Ins-HA transgenic model of spontaneous diabetes in which disease is mediated by antigen-specific CD4 cells (8). Here CD4 T-cells bearing the major histocompatibility complex (MHC) class II restricted T-cell receptor TCR-SFE are specific for a hemagglutinin peptide that is expressed by islet β-cells (Ins-HA transgenic mice). TCR-SFE/Ins-HA mice develop aggressive disease, and 100% are diabetic within 6 weeks of life (Fig. 2*A*). Strikingly, the onset of disease is significantly delayed and the overall incidence decreased in mice that carry the c-Maf transgene (Fig. 2*A*). Thus, constitutive T-cell expression of c-Maf leads

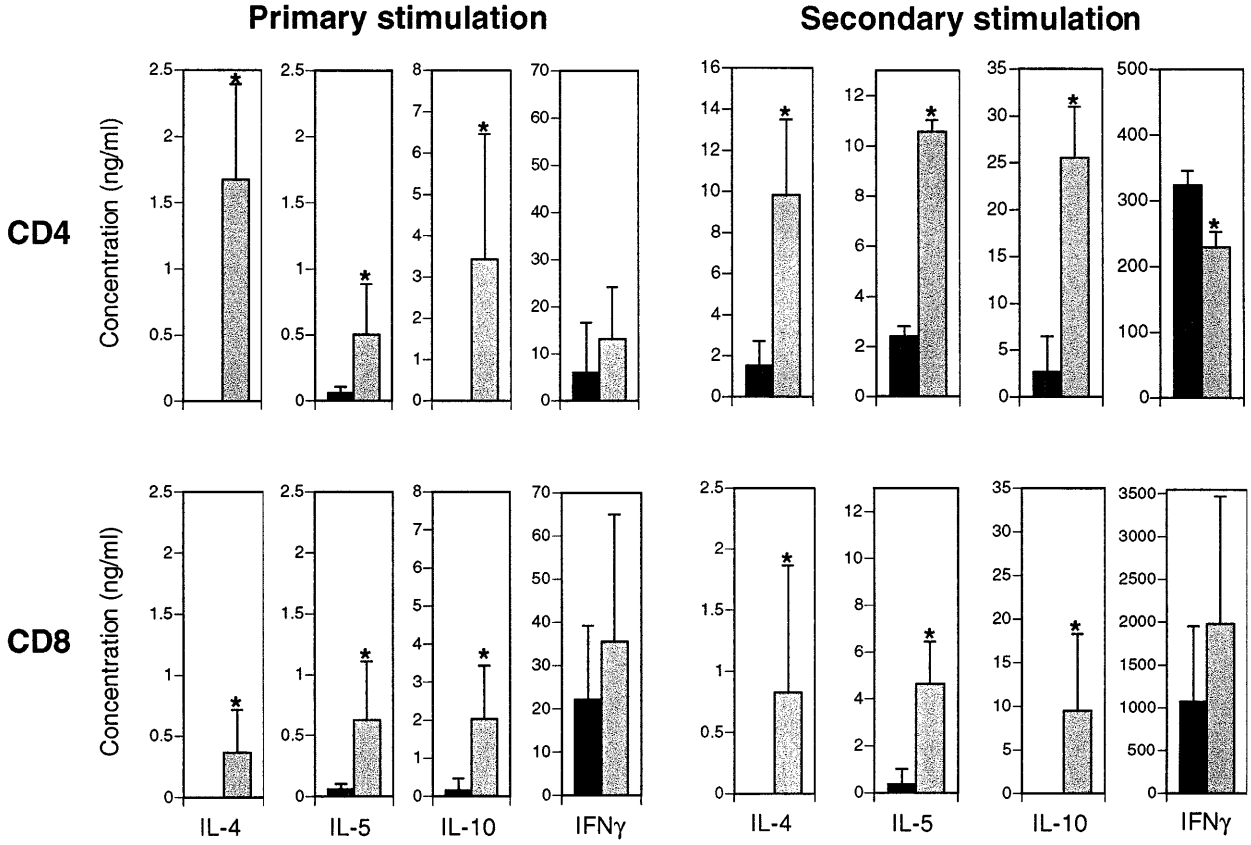

FIG. 1. Type 2 cytokine production is increased in CD4 and CD8 cells from c-Maf transgenic mice. Purified lymph node CD4 (top) or CD8 (bottom) cells from c-Maf single transgenic (□) or nontransgenic littermates (■) were stimulated with plate-bound anti-CD3 and anti-CD28. Cytokine levels were determined by ELISA from culture supernatants harvested 2 days after primary stimulation or 1 day after secondary stimulation. Data are plotted as mean ± SD obtained from >3 mice per group assayed in 2–3 independent experiments. *Statistically significant differences between transgenic and nontransgenic values, in which $P < 0.05$ using one-tailed Student's t test.

to inhibition of diabetes in this CD4-dependent model of spontaneous disease.

c-Maf does not inhibit T-cell proliferation, insulitis, or adoptive transfer of disease. As with CD4 cells from c-Maf single transgenic mice, type 2 cytokine production is increased among CD4 cells from c-Maf/TCR-SFE double transgenic mice indicating that the TCR-SFE transgene does not adversely influence the effect of c-Maf (Fig. 2*B*). Reduced disease incidence among c-Maf/TCR-SFE/Ins-HA mice is probably not the result of direct c-Maf–mediated inhibition of T-cell proliferation because CD4 T-cells from c-Maf transgenic mice do not differ from nontransgenic littermates in proliferative responses to anti-CD3 plus anti-CD28 (Fig. 2*C*). Furthermore, the accumulation of lymphocytes in the pancreatic islets of Langerhans appears unchanged by c-Maf as judged by the incidence and severity of peri-insulitis (Fig. 3).

Diabetes also occurs after passive transfer of TCR-SFE transgenic CD4 T-cells into Ins-HA mice (8,12,28). Disease onset in this transfer model is rapid (within 5 weeks of transfer) but requires lymphocyte-depleted recipients, so the mechanisms leading to disease here may differ from the spontaneous disease seen in TCR-SFE/Ins-HA transgenic mice (12,28). In contrast to the protection afforded by c-Maf in the spontaneous transgenic model, c-Maf does not have a significant effect on disease onset mediated by adoptively transferred SFE-specific CD4 cells (Fig. 2*D*). Furthermore, CD4 cells from nondiabetic c-Maf/TCR-SFE/Ins-HA mice also cause disease when transferred into RAG-$1^{-/-}$/Ins-HA recipients (Fig. 2*E*). While c-Maf changes the kinetics of type 2 cytokine expression and inhibits disease in the TCR-SFE/Ins-HA transgenic model of spontaneous diabetes, it cannot prevent disease mediated by adoptively transferred T-cells.

c-Maf also inhibits virus-induced diabetes in RIP-LCMV-NP mice. To test the effect of c-Maf in an inducible model of diabetes, we used the well-characterized RIP-LCMV-NP disease model (6,7,27,37–39). RIP-LCMV-NP mice express the nucleoprotein of LCMV in the thymus and islet β-cells and only become diabetic after infection with LCMV (typically within 8 weeks postinfection). c-Maf transgenic RIP-LCMV-NP mice are significantly more resistant to disease than the control littermate mice (Fig. 4*A*).

To investigate whether disease attenuation might be attributable primarily to the c-Maf–mediated change in CD4 helper function (Fig. 1) or might involve additional changes in CD8 effector functions, we examined the CTL activity of c-Maf transgenic T-cells. c-Maf transgenic or nontransgenic littermates were primed in vivo with LCMV (10^5 PFU) 7 days before the harvest of spleens for analysis of primary antigen-specific CTL activity. For analysis of memory CTL activity, spleens were harvested 30–60 days postinfection and then restimulated in vitro before analysis. Both the primary and memory virus-specific CTL responses of c-Maf transgenic mice were reduced compared with nontransgenic littermates (Fig. 4*B*). We also examined lytic precursor frequencies after

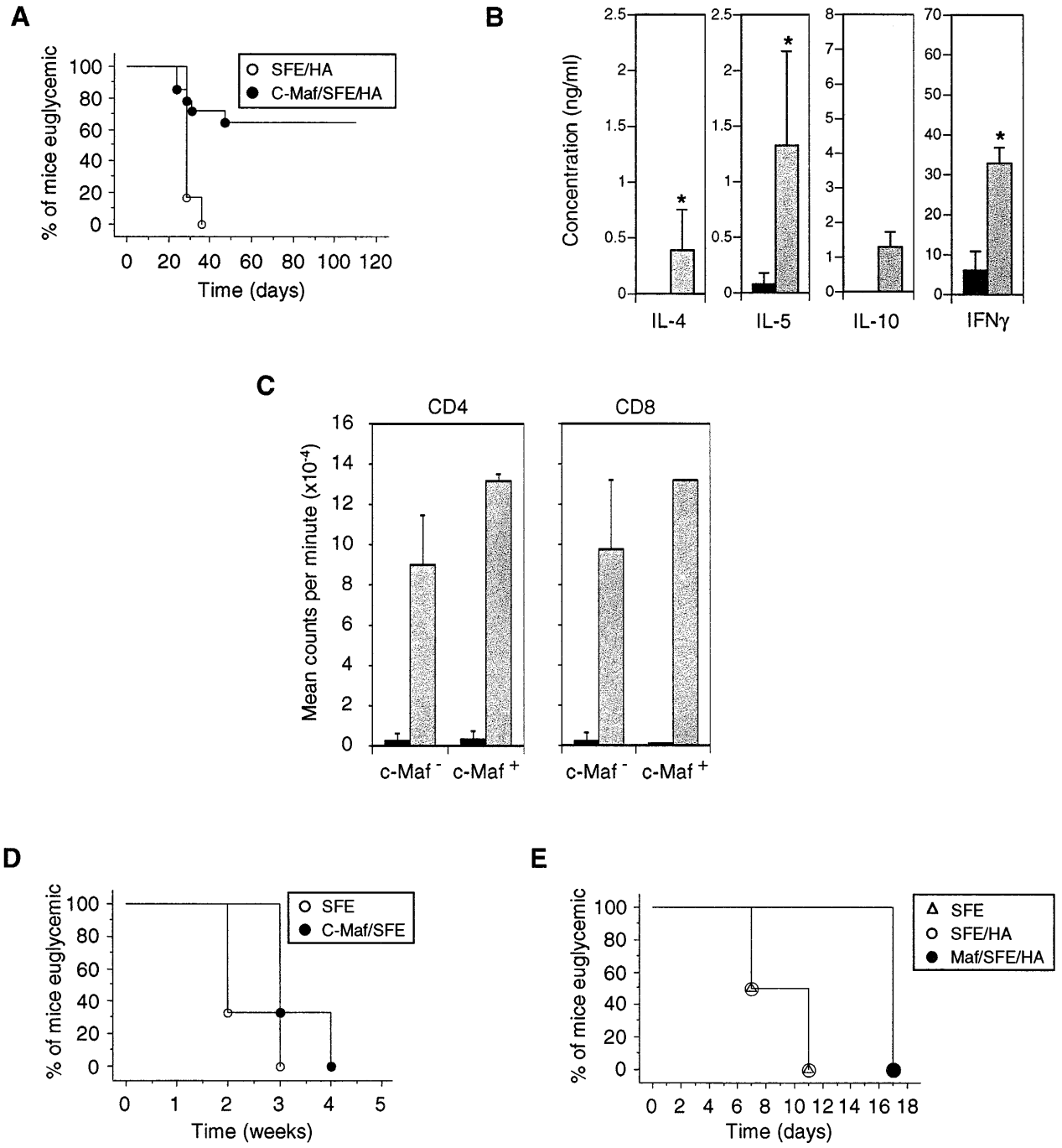

FIG. 2. Diabetes onset is significantly attenuated in c-Maf/TCR-SFE/Ins-HA mice but not in adoptive transfer models of disease. *A*: Blood glucose levels were monitored weekly, beginning at ~3 weeks of age, in a cohort of randomly chosen c-Maf/TCR-SFE/Ins-HA mice (n = 14) or TCR-SFE/Ins-HA littermates (n = 6). Data are represented in a Kaplan-Meier plot in which the x-axis indicates time from birth. Statistical significance is indicated as determined by the log-rank (Mantel-Cox) test (P = 0.0084). *B*: Purified lymph node CD4 cells from c-Maf/TCR-SFE double transgenic (□) or TCR-SFE single transgenic littermates (■) were stimulated with plate-bound anti-CD3 and anti-CD28. Cytokine levels were determined by ELISA from culture supernatants harvested 2 days after primary stimulation. Data are plotted as mean ± SD obtained from >3 mice per group assayed in 2 independent experiments. *Statistically significant differences between single and double transgenic values, where $P < 0.05$ using one-tailed Student's t test. *C*: Transgenic c-Maf does not inhibit T-cell proliferation directly. Proliferation of purified lymph node CD4 or CD8 cells from c-Maf single transgenic (+) or nontransgenic (–) mice was assessed using standard [^{3}H]thymidine incorporation assays as described in RESEARCH DESIGN AND METHODS. Cells were cultured in the presence (□) or absence (■) of plate-bound anti-CD3 plus anti-CD28 for 48 h before harvest. Data presented are means ± SD of triplicate samples obtained from individual animals. These data are representative of at least three experiments. *D*: Adoptive transfer of 1×10^7 CD4$^+$ T-cells from age-matched TCR-SFE or c-Maf/TCR-SFE mice were performed by intravenous injections into irradiated (700 rad) Ins-HA recipients. Blood glucose levels were followed weekly posttransfer. Data are plotted and statistical analysis performed as described for Fig. 2*A*. Data are representative of two independent experiments in which n = 3 mice per group (P = 0.1029). *E*: Adoptive transfer of 5×10^6 CD4$^+$ T-cell equivalents from age-matched TCR-SFE, diabetic TCR-SFE/Ins-HA, or euglycemic c-Maf/TCR-SFE/Ins-HA mice were performed by intravenous injections into RAG-1^{ko}/Ins-HA recipients. Blood glucose levels were determined every 3–4 days posttransfer. Data are plotted and statistical analysis performed as described for Fig. 2*A*. Data are representative of two independent experiments in which n = 2 mice per group (P = 0.0896).

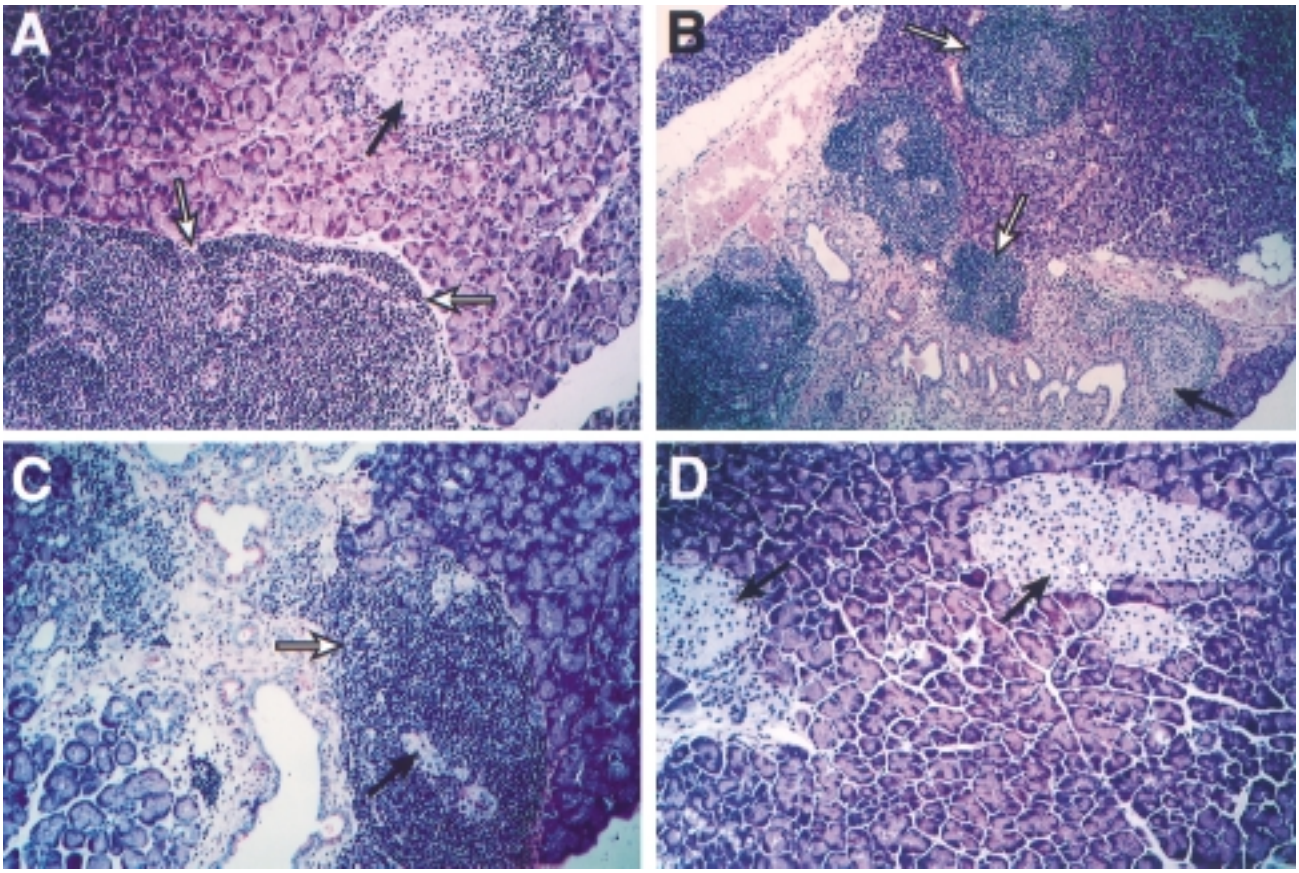

FIG. 3. Profound insulitis occurs in c-Maf/TCR-SFE/Ins-HA mice. Sections from paraffin-embedded pancreas tissue obtained from c-Maf/TCR-SFE/Ins-HA (*A*, 84 and *B*, 93 days old) or TCR-SFE/Ins-HA (*C*, 29 days old) mice were stained with PAS and viewed by light microscopy at 200× (*A*, *C*, and *D*) or 100× (*B*). Both triple transgenic mice were euglycemic at the time of analysis, but the TCR-SFE/Ins-HA mouse was diabetic. Large leukocyte infiltrates (⇨) surround islet tissue (→) in *A*, *B*, and *C*. The low magnification in *B* shows the extensive nature of the insulitis occurring in nondiabetic triple transgenic mice. As a comparison, *D* shows normal islet tissue obtained from an Ins-HA mouse.

in vivo infection with LCMV in c-Maf^{+or-}/RIP-LCMV-NP mice. LCMV-specific precursor CTL frequencies were significantly reduced in c-Maf/RIP-LCMV-NP mice compared with nontransgenic littermate controls, 1/18,000 (± 5,000) and 1/3,200 (± 1,500), respectively. Although disease attenuation in the RIP-LCMV-NP model parallels the c-Maf–mediated increase in type 2 cytokine production (Fig. 1), it also reflects a reduction in CTL development and a reduced precursor frequency.

Despite its ability to increase IL-4 cytokine production, c-Maf does not delay disease onset in NOD mice. Spontaneous autoimmune diabetes is typically a multigenic disorder (40–42). Both TCR-SFE/Ins-HA and RIP-LCMV-NP models are on the B10.D2 background. Studies have shown that this genetic background, closely related to C57BL/6 and C57BL/10, is permissive for autoimmune disease in transgenic models (13,42). In comparison, the NOD mouse strain is permissive for spontaneous autoimmune diabetes even in the absence of transgenes (41,42). Because diabetes in NOD mice is also thought to be driven by Th1 effectors (18), we tested whether the c-Maf transgene could protect NOD mice from diabetes. After backcrossing the c-Maf transgene five generations to NOD, we found that nontransgenic littermates developed diabetes at a high frequency (Fig. 5). Surprisingly, the presence of the c-Maf transgene had no protective effect; indeed, there appears to be a slight acceleration of disease that is not quite statistically significant ($P = 0.0515$; Fig. 5). Although the c-Maf transgene caused some increased IL-4 expression in CD4 T-cells after stimulation with anti-CD3 plus anti-CD28 (Fig. 6), the effect was not as strong as that seen in CD4 cells from B10.D2 mice (compare Figs. 1 and 6). Type 2 cytokine upregulation was also not significantly increased among NOD/c-Maf transgenic CD8 cells.

DISCUSSION

The transcription factor c-Maf is critical for the development of Th2 cells (33–35), which may serve to regulate the onset of autoimmune diabetes (21–27). In this study, we evaluate whether transgene-mediated expression of c-Maf in T-cells inhibits diabetes. Constitutive expression of c-Maf in T-cells does not cause constitutive cytokine expression, but it does alter the baseline regulation of gene expression such that T-cells only overexpress IL-4 after antigen activation. IL-5 and IL-10 levels are secondarily increased, presumably through indirect mechanisms. The current study shows that the combined upregulation of multiple type 2 cytokines by c-Maf transgenic T-cells correlates with disease attenuation in some, but not all, models of diabetes.

In the TCR-SFE/Ins-HA transgenic model of spontaneous diabetes, c-Maf significantly inhibits disease. Because diabetes in this model is dependent on MHC class II restricted

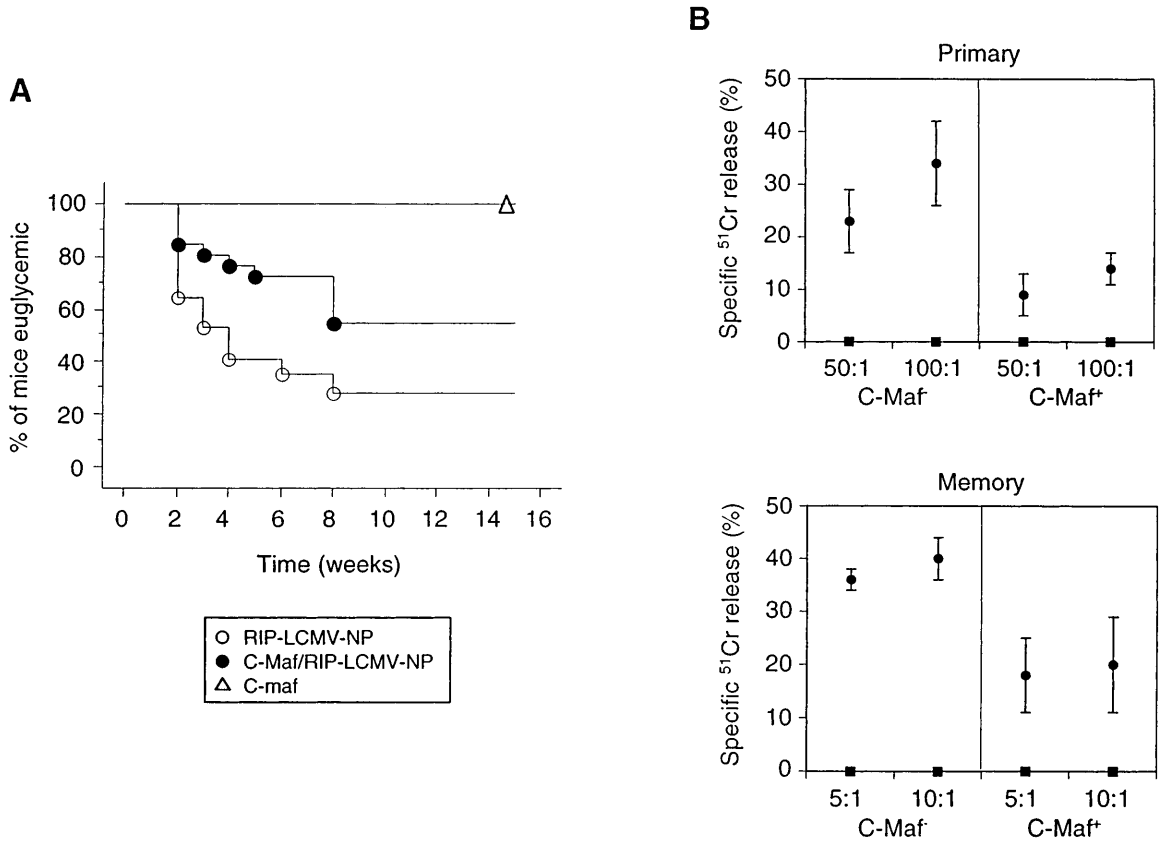

FIG. 4. c-Maf–mediated attenuation of LCMV-induced diabetes is at least partially due to a reduction in antigen-specific CTL activity. *A*: Blood glucose levels were monitored weekly after LCMV infection of RIP-LCMV-NP (n = 17), c-Maf/RIP-LCMV-NP (n = 26), or control c-Maf (n = 14) mice. Diabetes was significantly inhibited in c-Maf/RIP-LCMV-NP compared with nontransgenic mice as determined by log-rank (Mantel-Cox) analysis (P < 0.0291). c-Maf alone does not impart susceptibility to hyperglycemia because single transgenic mice remained euglycemic after infection with LCMV. *B*: Splenic CTL activity was assessed after in vivo LCMV infection of c-Maf transgenic or nontransgenic (B10.D2) mice in ^{51}Cr release assays using noninfected (■) or LCMV-infected (●) H-2^{d} BALB C17 fibroblast target T-cells. Primary responses (top graph) were assayed directly ex vivo 7 days postinfection. For secondary responses (bottom graph), splenocytes were harvested 30–60 days postinfection and then reinfected in vitro for 1 week before CTL analysis. Splenocytes from c-Maf transgenic mice exhibited significantly reduced primary and secondary antigen-specific CTL activity as determined by the Student's t test (P < 0.05). Effector:target ratios are as indicated. Data are expressed as the mean ± SD of triplicate samples from a single experiment and are representative of two independent experiments.

CD4 T-cell effector function (8), disease inhibition is most likely a consequence of the c-Maf–mediated early shift in type 2 cytokines produced by antigen-specific CD4 cells. c-Maf had no significant direct effects on T-cell proliferation in vitro or peri-insulitis in vivo, although current studies cannot rule out unusual skewing of T-cell populations within the islet tissue itself. Thus, regulated overexpression of multiple type 2 cytokines by antigen-specific T-cells can significantly inhibit diabetes onset. Although we have previously demonstrated that the c-Maf transgene induces global changes in cytokine expression by T-cells, the effect on diabetes in the TCR-SFE/Ins-HA model is likely caused by the local effects within islet infiltrates as TCR-SFE T-cells are triggered to express type 2 cytokines by presentation of HA in and around islets. Notably, disease attenuation is much more effective with transgenic c-Maf than previously observed with regulated T-cell overexpression of IL-10 alone (28).

Although a number of studies show that type 2 cytokines can inhibit autoimmune diabetes (21–28), it is clear that profound immune deviation does not always alleviate disease. NOD mice with a targeted disruption in the IFNγ gene are unable to generate classical Th1 cells, yet still become diabetic (43). This strong skewing is avoided in c-Maf transgenic mice in which constitutive T-cell expression of c-Maf leads to increased production of multiple type 2 cytokines but not complete elimination of Th1 cells (34). While c-Maf–mediated upregulation of type 2 cytokines is effective in attenuating disease in our TCR-SFE/Ins-HA model of spontaneous disease, it cannot inhibit disease onset after the adoptive transfer of islet-specific CD4 cells, even from previously protected mice. This suggests that the modest increases in type 2 cytokines afforded by c-Maf may be insufficient to block disease when it is mediated by antigen-specific CD4 cells once they have been activated.

Virus-induced diabetes is also significantly attenuated by transgenic c-Maf. Because diabetes after LCMV infection of RIP-LCMV-NP requires both antigen-specific CD4 and CD8 participation (7,38), disease mechanisms are more complex here than in TCR-SFE/Ins-HA mice. The comparison of cytokine responses in CD4 and CD8 cells demonstrates that c-Maf can direct increases in type 2 cytokines in both cell types. Having established that c-Maf–mediated changes in CD4 cells lead to significant attenuation of diabetes in TCR-SFE/Ins-HA mice, it became important to determine if c-Maf–mediated disease attenuation is primarily the result of changes in CD4 effector function or if additional CD8 effector functions are altered. Analysis of antigen-specific CTL activity

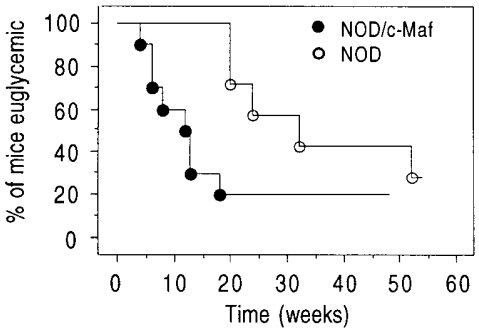

FIG. 5. Onset of diabetes is not delayed in transgenic c-Maf in NOD mice. Blood glucose levels of 5th generation female NOD/c-Maf (n = 10) or nontransgenic littermate (n = 7) mice were monitored once every 2 weeks beginning at ~4 weeks of age. Data were plotted and analyzed as in Fig. 2 (P = 0.0515). Transgenic c-Maf tended to accelerate the onset of hyperglycemia, however this was not statistically significant.

shows that c-Maf mediates an ~2-fold reduction in antigen-specific killing and a 5- to 6-fold decrease in CTL precursor frequency. Previously we have shown that IL-4 can suppress CTL activity indirectly through a STAT-6–dependent effect on antigen-presenting cells (27). Given the increased IL-4 production capacity of c-Maf transgenic T-cells, a similar mechanism may lead to the reduced CTL activity observed in c-Maf/RIP-LCMV-NP mice. It is also possible that other more direct c-Maf–mediated changes in T-cell function may occur that impact disease onset. One possibility is a change in migration ability, particularly because unique in vivo migration kinetics have been observed for Tc1 versus Tc2 cells that correspond to altered viral clearance abilities (44). However, viral clearance was not affected by transgenic c-Maf as determined by viral plaque assays (37) (data not shown). The current data indicate that changes in both CD4 and CD8 effector functions could account for attenuation of diabetes after viral infection of c-Maf/RIP-LCMV-NP mice.

While the effectiveness of transgenic c-Maf is impressive in the TCR-SFE/Ins-HA and RIP-LCMV-NP models in which disease incidence can be reduced by >60%, it has no inhibitory effect among NOD mice. It appears that the Th1-favoring genetic predisposition of NOD mice is not easily skewed by c-Maf. In fact, a trend toward accelerated disease onset occurs in the presence of c-Maf, but this is not statistically significant. This effect is not likely the result of an overwhelming allergic inflammation (21); islet infiltrates in c-Maf/NOD mice are histologically similar to NOD littermate controls (data not shown), lacking the abscess formation and profound eosinophilia that has been observed in some cases after transfer of diabetogenic Th2 cells (21). Alternatively, c-Maf could potentially influence antigen-presenting cell function indirectly through cytokine regulation, as has been suggested for IL-4 (27); however, this awaits further investigation. How such changes would affect disease onset among different mouse strains and disease models is currently unclear.

The differing results obtained from the combined evaluation of transgenic c-Maf using multiple disease models illustrates the need for caution when evaluating therapies for use among genetically diverse clinical populations. In addition, results here also suggest logical focal points for uncovering functional immune defects that might contribute to the complex multigenic susceptibility of diabetes in NOD mice. For

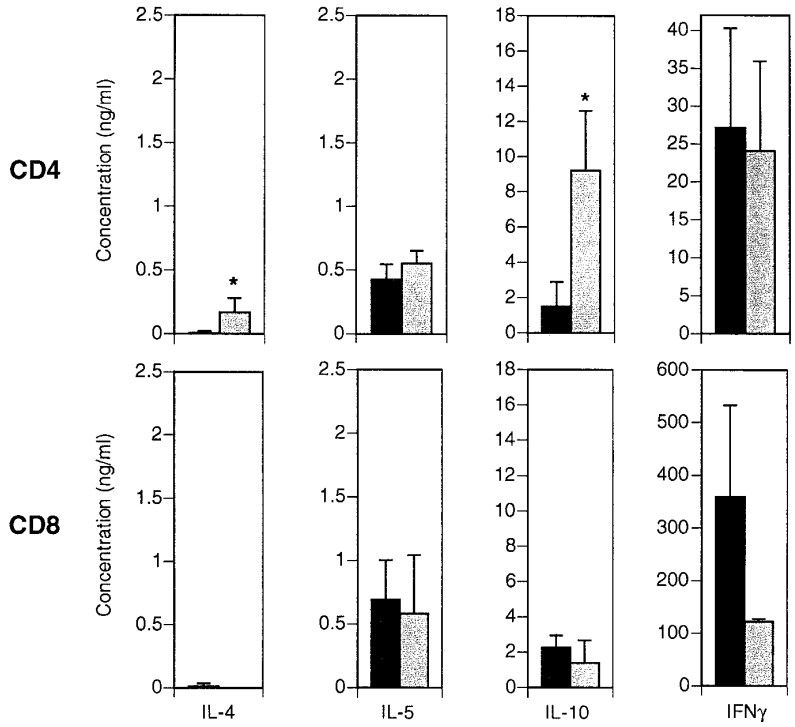

FIG. 6. Transgenic c-Maf mediates upregulation of type 2 cytokines in NOD CD4, but not CD8, cells. Cytokine responses of purified lymph node CD4 (top) or CD8 (bottom) cells from NOD/c-Maf (□) or nontransgenic (■) mice were measured by ELISA using supernatants collected 3 days after anti-CD3 plus anti-CD28 stimulation. Data are plotted as mean ± SD obtained from >3 mice per group assayed in 2–3 independent experiments. *Statistically significant differences between transgenic and nontransgenic values in which $P < 0.05$ using one-tailed Student's t test.

example, the resistance of NOD mice to changes in T-cell function mediated by transgenic c-Maf suggests that the coordinate action of factors that directly regulate the IL-4 gene may differ among susceptible and resistant mouse strains. Alternatively, differences in other factors involved in IL-4 receptor signaling might promote the Th1 predisposition of NOD mice and in this way also contribute to disease susceptibility.

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