Murine Antithymocyte Globulin Therapy Alters Disease Progression in NOD Mice by a Time-Dependent Induction of Immunoregulation

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OBJECTIVE—Antilymphocyte serum can reverse overt type 1 diabetes in NOD mice; yet, the therapeutic parameters and immunological mechanisms underlying the ability for this agent to modulate autoimmune responses against β-cells are unclear, forming the rationale for this investigation.

RESEARCH DESIGN AND METHODS—A form of antilymphocyte serum, rabbit anti-mouse thymocyte globulin (mATG), was utilized in a variety of in vivo and in vitro settings, each for the purpose of defining the physiological, immunological, and metabolic activities of this agent, with particular focus on actions influencing development of type 1 diabetes.

RESULTS—We observed that mATG attenuates type 1 diabetes development in an age-dependent fashion, only proving efficacious at disease onset or in the late pre-diabetic phase (12 weeks of age). When provided at 12 weeks of age, mATG reversed pancreatic insulitis, improved metabolic responses to glucose challenge, and rapidly increased frequency of antigen-presenting cells in spleen and pancreatic lymph nodes. Surprisingly, mATG therapy dramatically increased, in an age-dependent fashion, the frequency and the functional activity of $CD4^+CD25^+$ regulatory T-cells. Adoptive transfer/cotransfer studies of type 1 diabetes also support the concept that mATG treatment induces a stable and transferable immunomodulatory repertoire in vivo.

CONCLUSIONS—These findings indicate that an induction of immunoregulation, rather than simple lymphocyte depletion, contributes to the therapeutic efficacy of antithymocyte globulin and suggest that time-dependent windows for the ability to delay or reverse type 1 diabetes exist based on the capacity to enhance the functional activity of regulatory T-cells. ***Diabetes*** **57:405–414, 2008**

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Received for publication 29 September 2006 and accepted in revised form 16 November 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 26 November 2007. DOI: 10.2337/db06-1384.

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Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db06-1384.

ALS, antilymphocyte serum; ATG, antithymocyte globulin; APC, antigen-presenting cell; G-CSF, granulocyte colony–stimulating factor; IL, interleukin; ILN, inguinal lymph node; PLN, pancreatic lymph node; Teff, effector T-cell; Treg, regulatory T-cell.

Type 1 diabetes is a disorder of insulin deficiency resulting from the autoimmune destruction of β-cells (1). Monoclonal antibodies directed against a variety of lymphocyte subsets or their products (e.g., CD20, CD154, γ-interferon, etc.) have been investigated for therapeutic efficacy in type 1 diabetes, other autoimmune disorders, and transplantation-based settings (2–5). Of these, studies of anti-CD3 monoclonal antibodies have recently provided strong support for the promise of such approaches to modulate autoimmune disorders, proving efficacious for disease reversal in both NOD mice (6–8) and humans (9,10) with type 1 diabetes. However, in the late 1970s, studies reported the capacity for another antibody-based reagent, antilymphocyte serum (ALS), to reverse type 1 diabetes in BB rats (11). More recently, ALS has been demonstrated as an effective means to reverse type 1 diabetes in NOD mice (12,13). Those latter studies were, however, limited in terms of their mechanistic descriptions and lacked definition of therapeutic benefits corresponding to the natural history of type 1 diabetes (i.e., at which stages of disease aside from hyperglycemic onset the agent would prove effective). One clinical equivalent of ALS, antithymocyte globulin (ATG), has long been known to deplete lymphocytes in vivo and can effectively be used in a variety of therapeutic settings including renal transplantation, graft versus host disease, and aplastic anemia (14–17). Studies evaluating the mechanistic actions of ATG have not, however, been subject to significant address in a time frame contemporaneous with improvements in our understanding of and appreciation for the role of so-called regulatory T-cells (Treg), largely denoted for their coexpression of CD4 and CD25, and of forkhead transcription factor Foxp3 (18,19). This report addresses this void in understanding and evaluates the effectiveness for an antimurine form of ATG (anti-mouse thymocyte globulin [mATG]) to delay or reverse type 1 diabetes through its application at various stages in the natural history of this disease.

RESEARCH DESIGN AND METHODS

Female NOD, NOD.rag$^{-/-}$, and Balb/c mice were purchased from Jackson Labs, housed in specific pathogen-free facilities at the University of Florida, and provided autoclaved water and food ad libitum. All studies were approved

by the institution animal care and use committee at the University of Florida. NOD mice were monitored 2–3 times per week for blood glucose values indicating hyperglycemia, with type 1 diabetes defined as two consecutive nonfasting blood glucose levels >250 mg/dl separated by 24 h.

mATG administration. mATG was prepared by immunizing rabbits with pooled thymus cells prepared from NOD, C3H/He, DBA/2, and C57BL/6 mice (Genzyme). Tests for quality control and quality assurance for functional activities were performed in accordance with standard procedures by the manufacturer. For studies of type 1 diabetes prevention, at 4, 8, or 12 weeks in age, 12–18 female NOD mice (per group) were provided intraperitoneal injections of 500 μg mATG or 500 μg rabbit immunoglobulin (rIgG) (Jackson Immunologicals) diluted in 200 μl saline. After 72 h, a second dose of 500 μg of either mATG or rIgG was once again administered, bringing a total dose to 1.0 mg per animal. To provide for mechanistic analysis, mice were randomly selected and killed from each group for investigations 7, 14, or 28 days following mATG or rIgG administration. Using an identical dosing schedule, a separate set of studies was performed utilizing NOD mice newly diagnosed with type 1 diabetes. In these efforts, following two consecutive blood glucose readings above 250 mg/dl over 24 h, mice were provided mATG or rIgG. Animals were monitored 2–3 times per week for up to 12 weeks, with no exogenous insulin treatment. In accordance with veterinary guidance, animals demonstrating physiological effects of continuing hyperglycemia were killed. Insulitis scoring was performed on hematoxylin and eosin–stained pancreatic sections, as previously described (20).

Lymphocyte enumeration and serum cytokine determination. Blood was obtained via the tail vein from NOD and Balb/c mice treated with mATG or rIgG at selected times (0, 1, 3, 7, 14, and 30 days) postinjection for determination of lymphocyte counts. Samples were subjected to automated counting using a MASCOT Hemavet 850 CBC Analyzer (Drew Scientific). An additional 20 μl blood was collected from mATG- or rIgG-treated NOD mice at 0, 1, 3, 5, and 12 h and at previously indicated times and resulting serum subjected for cytokine analysis of interleukin-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IFN-γ, tumor necrosis factor (TNF)-α, granulocyte macrophage colony–stimulating factor, macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, KC, RANTES (regulated on activation, normal T-cell expressed and secreted), inducible protein-10, and granulocyte colony–stimulating factor (G-CSF) using Lincoplex kits (LincoResearch), as previously described (20).

Glucose tolerance testing. In 4-, 8-, and 12-week-old NOD mice subjected to mATG or rIgG treatment, 30 days following the first injection, animals underwent intraperitoneal glucose tolerance testing. Following 5 h of fasting, glucose (1 g/kg body weight) was provided by intraperitoneal injection in 200 μl saline. Blood glucose values were obtained at 0, 5, 15, 30, 60, and 120 min using a OneTouch Ultra (LifeScan) meter.

$CD4^+CD25^+$ T-lymphocyte suppression assay. $CD4^+CD25^+$ cells were purified using a MACS (Miltenyi Biotec) magnetic bead purification system and mixed in 96-well tissue culture plates at varying ratios with $CD4^+CD25^-$ effector T-cell (Teff) lymphocytes. In six replicates, 1.0×10^5 accessory cells (irradiated with 3,300 rads) were used in combination with each of the following Treg-to-Teff ratios: 2:1, 1:1, and 0.5:1 (where $1 = 1.0 \times 10^4$ cells). To each well, 5.0 μg/ml anti-CD3 antibody and 2.5 μg/ml anti-CD28 antibody were added. In other wells, accessory cells were plated alone both with and without 5.0 μg/ml anti-CD3 and 2.5 μg/ml anti-CD28 antibody. The cells were then incubated at 37°C, 5% CO_2, and 95% humidity for 5 days. On day 4, 1.0 μCi ^3H thymidine was added to each well. Following 18 h of incubation, the cells were harvested and the ^3H thymidine incorporation determined using a 1450 Microbeta Trilux β-scintillation counter (Wallac).

Flow cytometry. Spleen, dendritic cells, bone marrow, inguinal lymph node (ILN), and pancreatic lymph node (PLN) were collected (as noted) from mice and subjected to flow cytometric analysis using either a FACScan or FACScalibur flow cytometer (Becton Dickinson). Flow cytometric analysis of prepared cells was performed with 5.0×10^4 to 1.0×10^5 cells from each sample. Data were analyzed using the FCS Express analysis program (De Novo Software). For each mouse, cells were labeled using antibodies (as well as relevant isotype controls) purchased, with two exceptions, from a single commercial vendor (BD Pharmingen), including: anti-CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD28, CD86, CD154 and anti-major histocompatibility complex class II. Anti-Foxp3 and F4/80 antibodies were purchased from eBiosciences. In all situations, antibodies were utilized according to the manufacturer's recommendations.

Adoptive transfer. Splenocytes were obtained from NOD mice of various treatment groups at 30 weeks of age (i.e., at the end of study) and adoptively transferred or cotransferred via intravenous injection into $NOD.rag^{-/-}$ mice. A group of mice receiving 2.0×10^7 splenocytes from four untreated mice with recent-onset type 1 diabetes served as a methodological control. For transfer studies, $NOD.rag^{-/-}$ mice received 2.0×10^7 splenocytes from 12-week-old rIgG-treated mice or 2.0×10^7 splenocytes from 12-week-old mATG-treated mice. In studies of adoptive cotransfer, $NOD.rag^{-/-}$ mice were provided 1.0×10^7 splenocytes from untreated recent-onset type 1 diabetic mice mixed with 1.0×10^7 splenocytes from 12-week-old rIgG- or mATG-treated mice. All mice were followed for onset of type 1 diabetes, as previously described.

Statistical analysis. Statistical analysis was performed using Kaplan-Meier life table analysis, one-way ANOVA, or Fisher's Exact (two-tailed) testing. All data are presented as means ± SD. P values <0.05 were deemed significant.

RESULTS

mATG administration transiently depletes peripheral blood lymphocytes in the NOD model. To evaluate whether the in vivo activities of mATG demonstrate strain-specific differences in terms of its capacity for lymphocyte depletion, whole blood samples were collected at various times from 4-, 8-, or 12-week-old NOD and Balb/c mice both before and up to 30 days following intraperitoneal administration of mATG. Treatment of both strains of mice with mATG induced a significant degree of lymphopenia within 1 day (Fig. 1*A*); however, peripheral blood lymphocyte counts returned to preadministration levels by 30 days postadministration. No significant differences were observed in lymphocyte counts between mATG-treated NOD and Balb/c mice at any treatment age (all P = NS), represented by similar patterns of depletion and subsequent restoration over a 30-day period (12-week data; Fig. 1*A*). Hence, mATG treatment imparts a period of transient lymphocyte depletion followed by a robust recovery of cells, with no age- or strain-dependent variations noted in terms of either lymphocyte depletion or recovery.

Depletion of $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocyte populations is transient following mATG treatment. Multiple cells of the immune system have been associated with the pathogenesis of type 1 diabetes, with much focus on the importance of T-lymphocytes (21). To identify the actions of mATG on a variety of these T-cell subsets and uncover any bias in terms of its actions in vivo, flow cytometry was used to evaluate the levels of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell populations 7, 14, and 30 days following treatment of NOD mice treated with mATG or rIgG. Treatment of NOD mice with mATG versus rIgG imparted a transient decline in $CD3^+$ T-cells (12.1 ± 0.8 vs. 53.7 ± 6.8%, respectively; $P < 0.01$), which by 30 days postadministration returned to preadministration levels (Fig. 1*B*). This pattern was also observed with $CD4^+$ (8.5 ± 0.2 vs. 38.4 ± 2.4%) (Fig. 1*C*) and $CD8^+$ (2.6 ± 0.2 vs. 16.1 ± 1.8%) (Fig. 1*D*) T-cell populations. Throughout this 30-day period, the CD4-to-CD8 ratio of mATG-treated mice was not significantly altered from that of rIgG-treated mice (P = not significant), and the T-lymphocyte subsets of rIgG-treated mice were not reduced either.

Transient serum cytokine increases following mATG treatment. Because ATG treatment in humans has been associated with what is commonly referred to as a "cytokine storm," we questioned whether such a phenomenon would follow mATG treatment and whether the actions of this therapy might be characterized by elevations in specific cytokines. Therefore, serum samples from mice were collected at 0, 1, 3, 6, 12, and 24 h as well as 3, 7, 14, and 30 days following treatment of NOD mice provided mATG or rIgG. Consistent with observations in clinical settings, mATG imparted a cytokine release pattern in vivo that was marked by transient but statistically significant increases in many cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, γ-interferon,

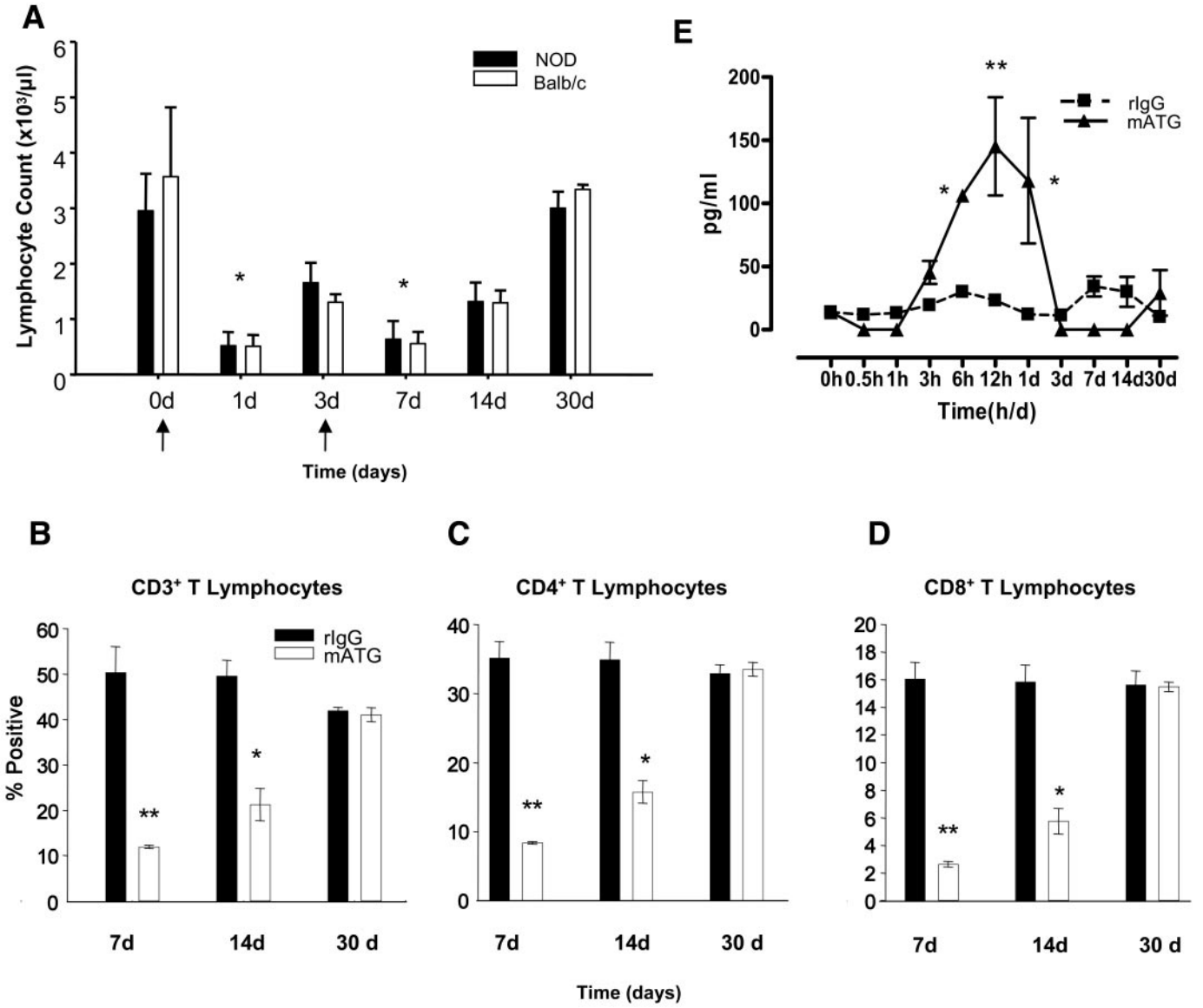

FIG. 1. mATG administration diminishes the frequency of peripheral blood lymphocytes in vivo and does so with equivalent efficacy in strains both prone and nonprone to type 1 diabetes. To determine the physiological consequences of mATG administration and to select a dose for in vivo analyses seeking to modulate the development of type 1 diabetes, a series of experiments were performed wherein Balb/c mice (8–12 weeks of age) were provided intraperitoneal doses of mATG, ranging from 0.1 to 25.0 mg/animal over a 72-h period. As a result of these initial studies, a 1.0 mg/animal dose was considered optimal because it represented the minimal dose of mATG providing an equivalent degree of peripheral blood lymphocyte depletion (i.e., ~0.5 × 10^3/µl) targeted in human therapeutic settings utilizing ATG (42). As such, this dose was selected as the standard for testing in all studies noted hereafter. *A*: 12-week-old NOD or Balb/c mice (n = 5 per group) were treated on days 0 and 3 with 1.0 mg/animal of mATG (i.e., two 500 µg/animal injections at times 0 and 72 h [noted with arrows]). Whole blood was collected from tail veins and subjected to automated determination of lymphocyte counts. Shown are the lymphocyte counts ± SEM. *P < 0.02 in comparison with preadministration lymphocyte counts by ANOVA. *B–D*: mATG treatment transiently depletes $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocyte populations in vivo. Following the administration of mATG or rIgG into 12-week-old NOD mice (1.0 mg/animal; two 500-µg doses 72 h apart; n = 3 per group), the frequency of specific cell populations in peripheral blood at various points in time was determined by flow cytometry, including assessment of markers for $CD3^+$ (*B*), $CD4^+$ (*C*), and $CD8^+$ (*D*) cells. *P < 0.01 and ** P < 0.001 for comparison of the frequency of this cell population in mATG- vs. rIgG-treated animals. *E*: mATG (1.0 mg/animal; two 500 µg/animal injections at times 0 and 72 h) induces transient increases in serum IL-2 in vivo. Following administration of 1.0 mg/animal of rIgG or mATG (two 500 µg doses 72 h apart) into 12-week-old NOD mice, serum samples were collected (n = 3 per group) at 0, 1, 3, 6, and 12 h and at 1, 3, 7, 14, and 30 days. Samples were subjected to multiplex analysis for 21 cytokines including, as shown here, IL-2. *P < 0.05 and **P < 0.02 for comparison of the serum concentration of IL-2 in mATG- vs. rIgG-treated animals.

TNF-α, granulocyte macrophage colony–stimulating factor, macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, KC, RANTES, inducible protein-10, and G-CSF). Indeed, of these, only G-CSF and IL-12 p70 were not significantly elevated in mATG- versus rIgG-treated mice. For example, serum IL-2 levels increased in mATG-treated mice from an average baseline of <20 ± 1.9 to 145 ± 46.1 pg/ml within 12 h but declined to baseline levels by day 3 (Fig. 1*E*). Despite these transient elevations in serum cytokine levels, no overt clinical symptoms of distress were observed, and there was not any acute mortality noted with mATG administration. As expected, NOD mice treated with control rIgG did not exhibit a significant increase in any serum cytokine concentration, including IL-2 (Fig. 1*E*), throughout the measured time points (P = NS). These observations provide another example for rapid and transient immunological consequences of mATG treatment on cytokine production in

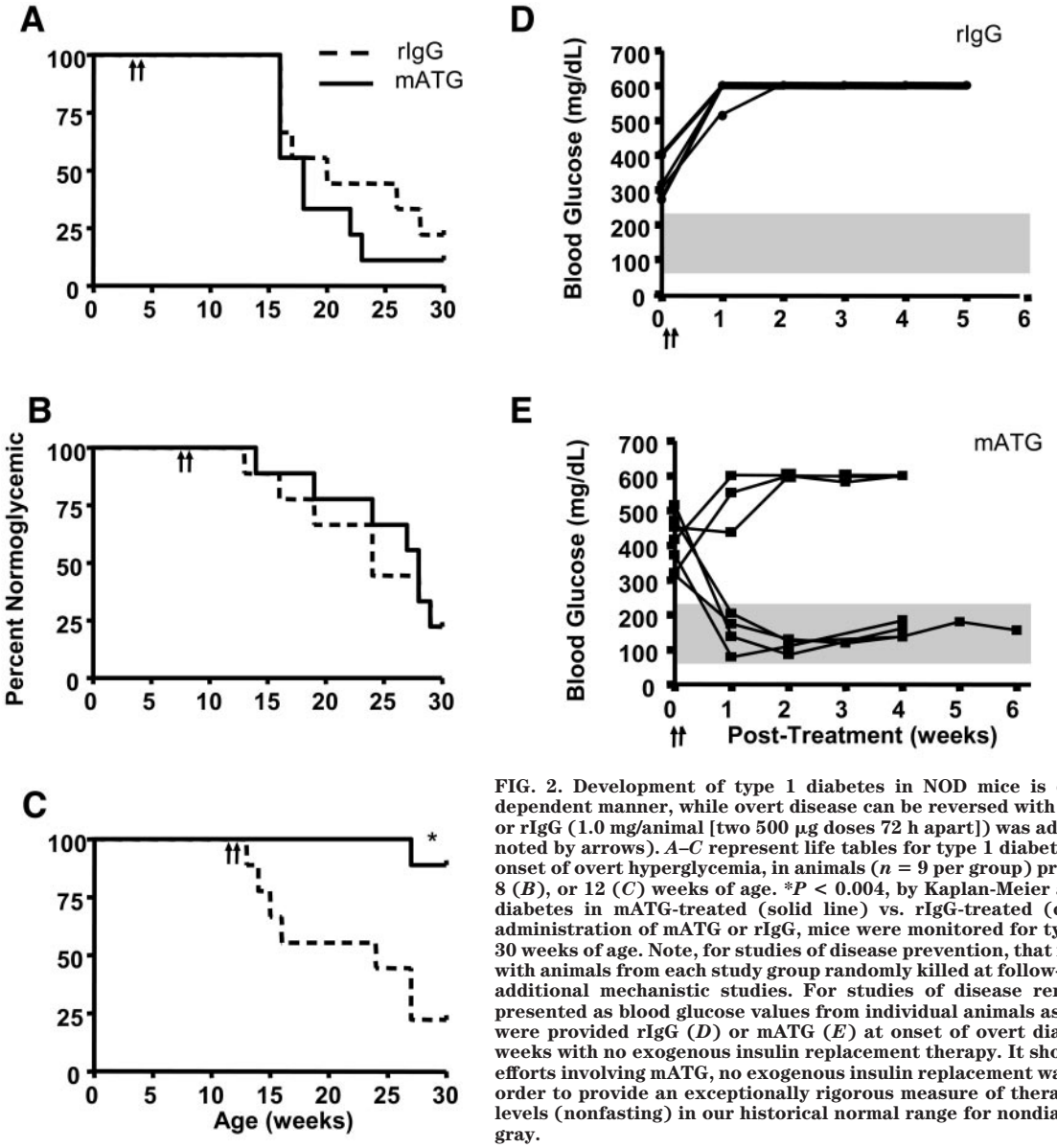

FIG. 2. Development of type 1 diabetes in NOD mice is delayed by mATG in a time-dependent manner, while overt disease can be reversed with this therapeutic agent. mATG or rIgG (1.0 mg/animal [two 500 μg doses 72 h apart]) was administered to NOD mice (time noted by arrows). *A–C* represent life tables for type 1 diabetes progression, defined by the onset of overt hyperglycemia, in animals (n = 9 per group) provided mATG or rIgG at 4 (*A*), 8 (*B*), or 12 (*C*) weeks of age. *P < 0.004, by Kaplan-Meier analysis, on the rate of type 1 diabetes in mATG-treated (solid line) vs. rIgG-treated (dashed line) mice. Following administration of mATG or rIgG, mice were monitored for type 1 diabetes development to 30 weeks of age. Note, for studies of disease prevention, that initial group sizes were larger, with animals from each study group randomly killed at follow-up time periods to provide for additional mechanistic studies. For studies of disease remission (*D* and *E*), data are presented as blood glucose values from individual animals as a function of time. NOD mice were provided rIgG (*D*) or mATG (*E*) at onset of overt diabetes and monitored for 4–6 weeks with no exogenous insulin replacement therapy. It should be emphasized that in our efforts involving mATG, no exogenous insulin replacement was provided to these animals in order to provide an exceptionally rigorous measure of therapeutic efficacy. Blood glucose levels (nonfasting) in our historical normal range for nondiabetic NOD mice are shaded in gray.

vivo, the biological consequences of which remain unclear.

A time-dependent delay of type 1 diabetes is imparted by mATG treatment. To study the ability of mATG to delay type 1 diabetes, NOD mice (n = 12–18 per group) were provided mATG or rIgG at 4, 8, or 12 weeks of age. No differences in either overall frequency or rate in progression of type 1 diabetes (i.e., life-table analysis) were seen between NOD mice administered with rIgG or mATG at either 4 or 8 weeks of age (Fig. 2*A* and *B*) (P = not significant). In contrast, treatment of NOD mice with mATG at 12 weeks of age resulted in a significant decrease in development of type 1 diabetes in comparison with that in rIgG-treated littermates (Fig. 2*C*). Indeed, at 30 weeks of age, 89% (8 of 9) of mATG-treated mice remained euglycemic, while only 22% (2 of 9) of rIgG-treated mice were without type 1 diabetes (P = 0.015).

A reversal of overt hyperglycemia in NOD mice can be afforded by mATG treatment. As previous studies utilizing ALS and anti-CD3 have sought to assess the ability of these agents to reverse disease (6,12,13), we also addressed the issue of type 1 diabetes reversal in NOD mice at the overt onset of hyperglycemia. Utilizing the same treatment schedule used for studies of disease prevention (i.e., 1.0 mg/animal, two 500-μg doses 72 h apart), mATG was observed to provide a significant degree of disease reversal in comparison with that provided by rIgG administration (4 of 7 [57%] vs. 0 of 6 [0%], respectively; P = 0.05). Considered collectively with the studies of disease prevention, the therapeutic benefits afforded by mATG treatment demonstrated a clear age dependence, suggesting that additional factors related to stage in the natural history of type 1 diabetes development were associated with the ability to delay disease. Among the potential mechanisms that could underlie this observation are those related to the local influences (both qualitative and quantitative) of mATG on the insulitis lesion or the pancreatic lymph node and systemic influences activating components promoting immunoregulatory mechanisms affording islet cell protection.

mATG treatment attenuates insulitis and improves response to glucose levels in response to metabolic challenge. To determine the nature of protection from type 1 diabetes onset afforded by mATG treatment at 12

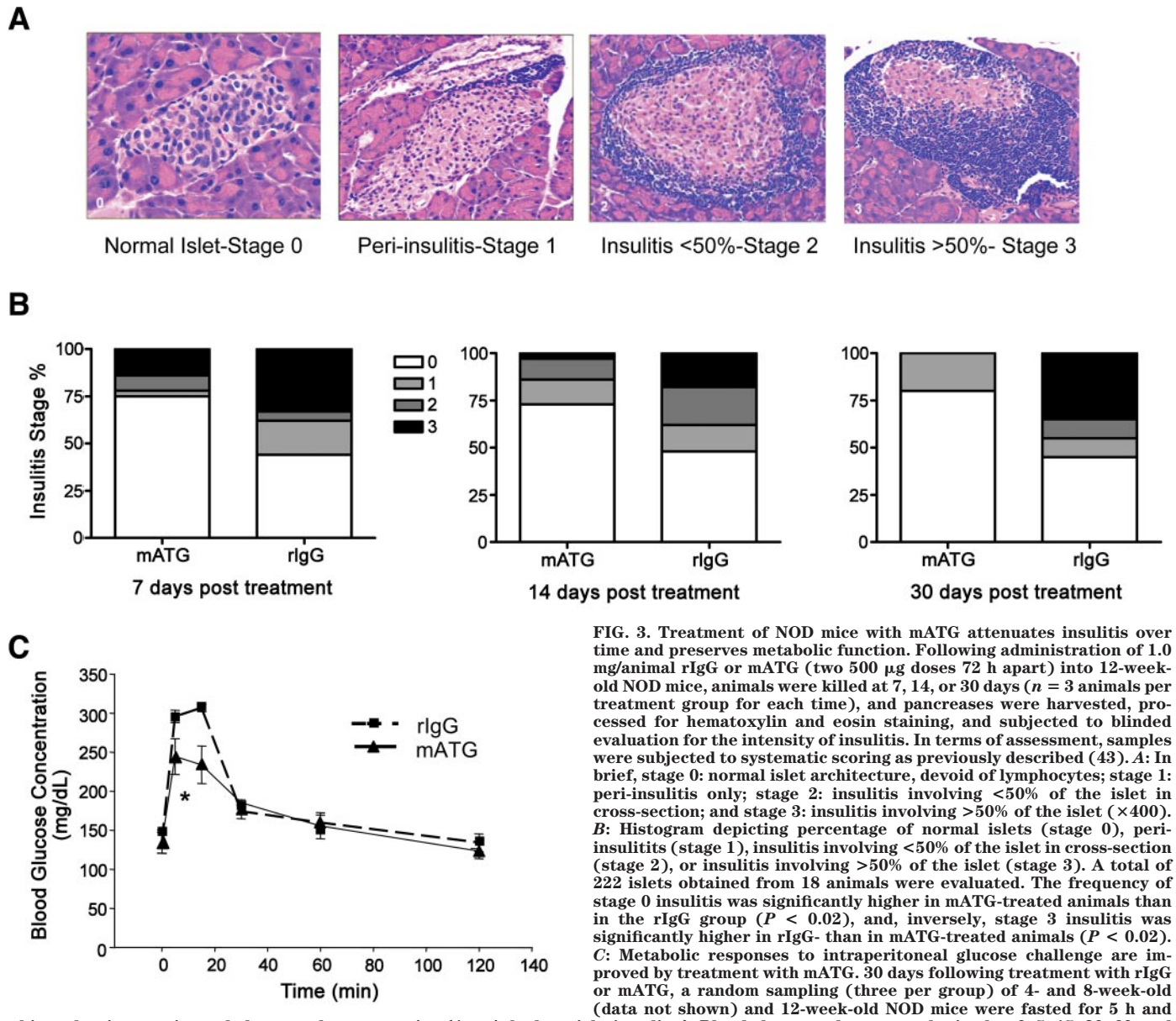

FIG. 3. Treatment of NOD mice with mATG attenuates insulitis over time and preserves metabolic function. Following administration of 1.0 mg/animal rIgG or mATG (two 500 μg doses 72 h apart) into 12-week-old NOD mice, animals were killed at 7, 14, or 30 days (n = 3 animals per treatment group for each time), and pancreases were harvested, processed for hematoxylin and eosin staining, and subjected to blinded evaluation for the intensity of insulitis. In terms of assessment, samples were subjected to systematic scoring as previously described (43). *A*: In brief, stage 0: normal islet architecture, devoid of lymphocytes; stage 1: peri-insulitis only; stage 2: insulitis involving <50% of the islet in cross-section; and stage 3: insulitis involving >50% of the islet (×400). *B*: Histogram depicting percentage of normal islets (stage 0), peri-insulitits (stage 1), insulitis involving <50% of the islet in cross-section (stage 2), or insulitis involving >50% of the islet (stage 3). A total of 222 islets obtained from 18 animals were evaluated. The frequency of stage 0 insulitis was significantly higher in mATG-treated animals than in the rIgG group (P < 0.02), and, inversely, stage 3 insulitis was significantly higher in rIgG- than in mATG-treated animals (P < 0.02). *C*: Metabolic responses to intraperitoneal glucose challenge are improved by treatment with mATG. 30 days following treatment with rIgG or mATG, a random sampling (three per group) of 4- and 8-week-old (data not shown) and 12-week-old NOD mice were fasted for 5 h and subjected to intraperitoneal glucose tolerance testing (1 mg/g body weight in saline). Blood glucose values were obtained at 0, 5, 15, 30, 60, and 120 min postinjection. Area under the curve analysis (P < 0.05), as well as determination of peak glucose levels (noted as *P < 0.02), revealed an improved metabolic response to glucose stimulation in mATG- vs. rIgG-treated animals. (A high-quality digital representation of this figure can be found at http://dx.doi.org/10.2337/db06-1384.)

weeks of age, the degree of pancreatic insulitis following treatment was assessed. Mice treated with mATG at 12 weeks of age exhibited significantly lower levels of infiltration in comparison with rIgG-treated animals (Fig. 3 *A* and *B*). Interestingly, this pattern demonstrating a less severe form of insulitis increased over time, suggesting that, above and beyond the initial depletion afforded by mATG, the agent may induce protective mechanism(s) attenuating migration of cells to pancreatic islets. Further confirmation of attenuated autoimmunity in the group treated with mATG at 12 weeks of age was observed in i.p. glucose tolerance tests. At thirty days after the first injection, 4-, 8-, and 12-week-old mATG- or rIgG-treated mice underwent intraperitoneal glucose tolerance testing. Glucose levels were not significantly different in mice treated with mATG at 4 and 8 weeks of age compared with those of rIgG-treated mice (data not shown). In contrast, mice treated with mATG at 12 weeks of age demonstrated a significant divergence between the mATG- and control rIgG-treated mice (P < 0.05), by area under the curve analysis, following glucose administration (Fig. 3*C*). In addition, while mice from both treatment groups had similar fasting glucose levels, on glucose administration, average glucose levels of rIgG-treated mice rose to a peak of 307 ± 5.0 mg/dl, whereas those of mATG-treated mice only elevated to 244.7 ± 22.8 mg/dl, demonstrating a more severe impairment in glucose response in rIgG mice (P < 0.02). These metabolic findings, noting improved function in mATG treated mice, combined with their reduced levels of insulitis, suggest an age-specific attenuation of β-cell autoimmunity and preservation of the capacity for insulin secretion.

Flow cytometric analysis of antigen-presenting cell and regulatory T-cell populations in vivo following mATG treatment. Due to the broad spectrum of cellular targets that have been described for ATG, beyond T-lymphocytes, we thought it was essential to evaluate the levels of various antigen-presenting cells (APCs) including

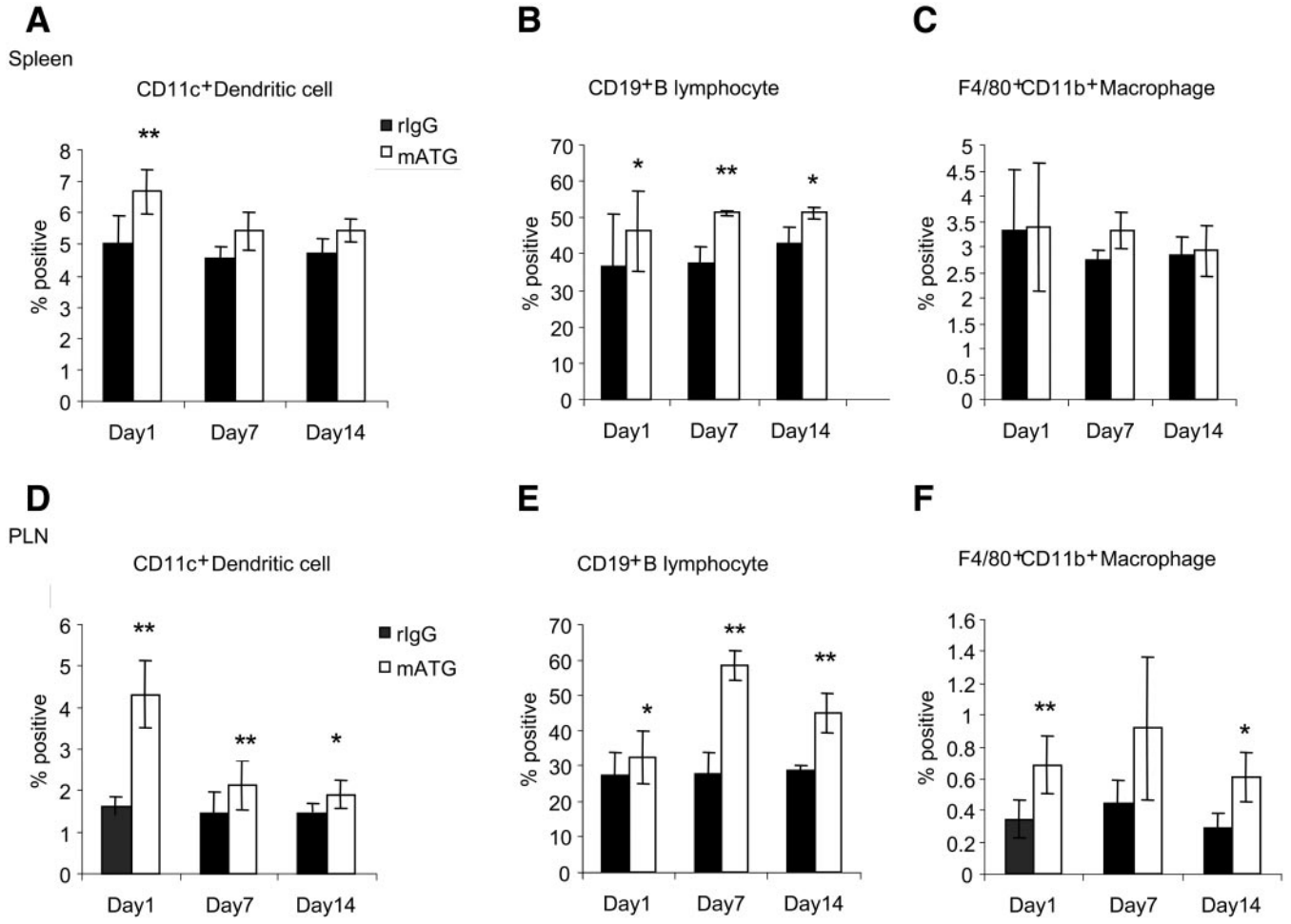

FIG. 4. The distribution of APCs is modulated in vivo following mATG treatment. Twelve-week-old NOD mice were administered rIgG or mATG at days 1, 7, and 14, with a variety of organs including spleen and PLN harvested for subsequent flow cytometric analysis of resident APC populations. Antibodies specific for dendritic cell, B-lymphocyte, and macrophage cell surface makers were utilized, including: $CD11c^+$ dendritic cells, $CD19^+$ B-lymphocytes, and $F4/80^+$ $CD11b^+$ macrophages. $^{**}P < 0.01$ and $^{*}P < 0.05$ in mATG- vs. rIgG-treated animals. $n = 6$ per group for animals at day 1; $n = 3$ per group for animals analyzed at days 7 and 14.

dendritic cells, B-lymphocytes, and macrophages following mATG or rIgG treatment. To perform such assessments, NOD mice at 12 weeks of age were injected intraperitoneally with mATG or polyclonal rabbit IgG as control and killed 1, 7, or 14 days later. Spleen, PLN, ILN, and bone marrow cells were harvested for flow cytometric analysis of markers associated with the aforementioned cell populations (Fig. 4). In vivo, mATG treatment rapidly (1 day) increased the frequency of dendritic cells (number of dendritic cells/total cells) in a number of lymphoid tissues, including spleen (rIgG vs. mATG: 5.0 ± 0.88 vs. 6.7 ± 0.71%, respectively; $P < 0.01$), PLN (1.6 ± 0.22 vs. 4.3 ± 0.81%; $P < 0.01$), and ILN (1.7 ± 0.27 vs. 4.5 ± 0.85%; $P < 0.01$) but not in thymus or bone marrow (P = not significant). A significant increase persisted at day 7 and 14 in PLN but not spleen or ILN. Although much of this increase likely reflects the relative reduction in T-cell number associated with mATG, absolute dendritic cell counts (total cell number from one organ × dendritic cell frequency) confirmed this significant increase in PLN (cells/node × 10^3 at day 1; rIgG vs. mATG, 59.18 ± 16.28 vs. 114.26 ± 36.17, respectively; $P < 0.001$) but not ILN or spleen.

As dendritic cells have been associated with the generation of Treg cells, the aforementioned population of cells thought intimately associated with immune regulation, we addressed the question of whether the altered dendritic cell profile was associated with similar alterations in Treg. To this end, we observed that the frequency of $CD4^+CD25^+$ $Foxp3^+$ T-cells was increased at day 1 in PLN (7.8 ± 1.6 vs. 15.8 ± 1.7%; $P < 0.01$) and ILN (7.8 ± 0.79 vs. 15.8 ± 2.3%; $P < 0.01$) following mATG treatment. By day 7, there was still a nonsignificant trend toward an increase in both of these lymphoid compartments (PLN 6.6 ± 1.3 vs. 14.43 ± 7.4% [$P = 0.2$] and ILN 8.1 ± 1.1 vs. 25.11 ± 10.7% [$P = 0.052$]). Luo et al. (22) recently demonstrated that β-cell peptide-pulsed NOD dendritic cells can generate islet-specific $CD4^+CD25^+$ $Foxp3^+$ T-cells ex vivo that, upon adoptive transfer, can prevent development of type 1 diabetes. Thus, the increase in PLN dendritic cells observed following mATG treatment might be responsible for maintaining $CD4^+CD25^+$ $Foxp3^+$ T-cells in the face of $CD4^+CD25^-$ depletion, which has the potential to prevent type 1 diabetes. As with the dendritic cell populations, there were also significant changes in the frequency of other APCs following mATG treatment, specifically, a significant increase in the percentage of B-cells at day 1, 7, and 14 in the spleen (Fig. 4*A*) and PLN (Fig. 4*B*).

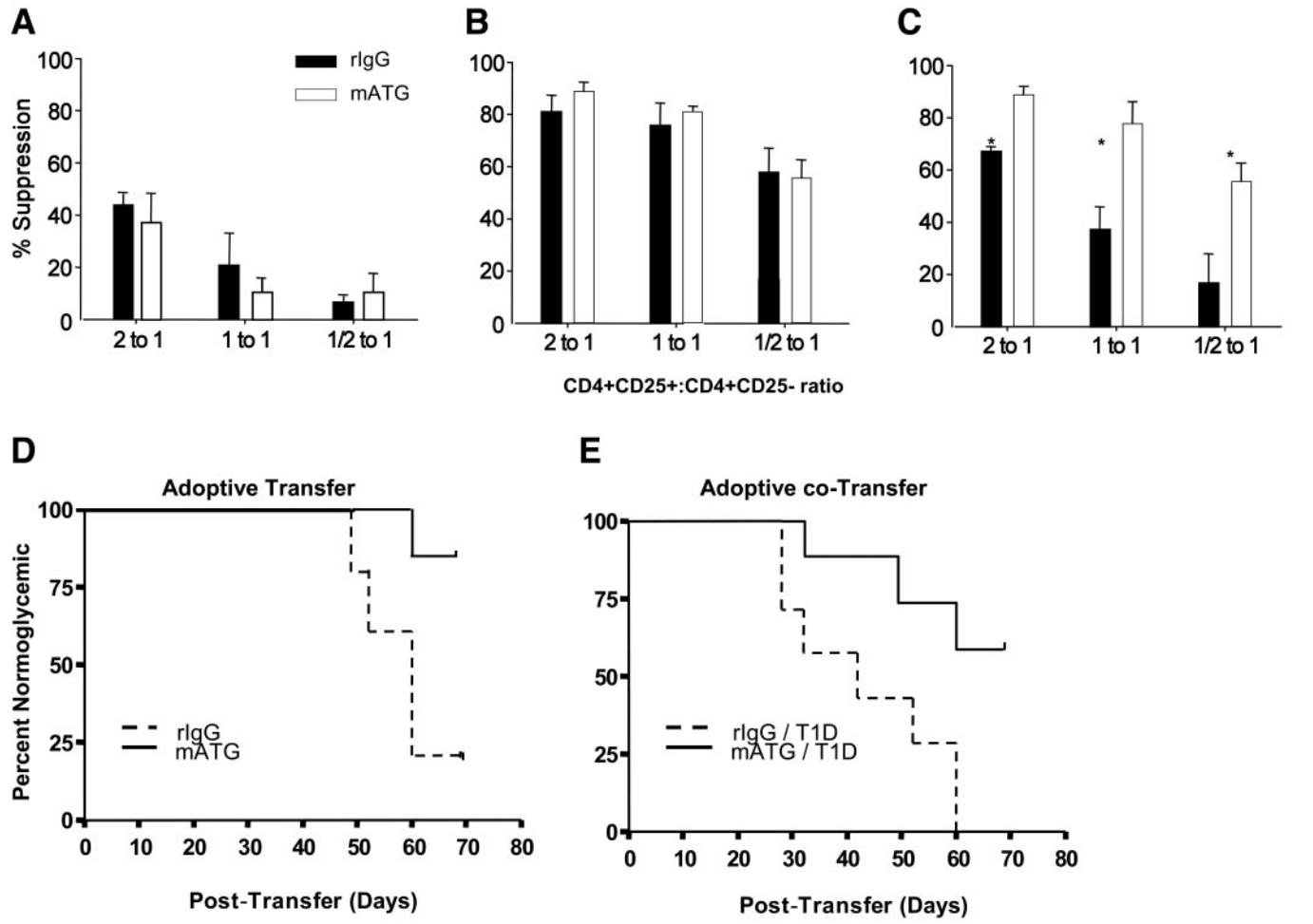

FIG. 5. In a time-dependent fashion, mATG enhances the Treg suppression of Teff in vivo. At 30 days following treatment with rIgG or mATG, a random sampling (n = 3 per group) of 4-, 8-, and 12-week-old NOD mice were killed and their splenocytes subjected to a purification scheme for $CD4^+CD25^-$ (Teff) and $CD4^+CD25^+$ (Treg) cells. Six replicate wells containing 1.0×10^5 total cells per well (in the presence of irradiated accessory cells) were used in each of the following Treg-to-Teff ratios: 2:1, 1:1, and 0.5:1. Cells were stimulated with the combination of anti-CD3 antibody and anti-CD28 antibodies, with subsequent determination of ^{3}H-thymidine incorporation. Suppression assay for treated mice aged 4 (*A*), 8 (*B*), and 12 (*C*) weeks. Mean ± cycles per minute for the groups were as follows: 4 weeks (2:1–707 ± 96, 1,616 ± 666; 1:1–1,683 ± 483, 1,984 ± 675; 0.5:1–2,191 ± 413, 2,223 ± 777 for rIgG and mATG, respectively), 8 weeks (2:1–249 ± 59, 1,889 ± 920; 1:1–514 ± 112, 3,389 ± 923; 0.5:1–1,392 ± 425, 5,718 ± 1,138), and 12 weeks (2:1–239 ± 33, 1,889 ± 920; 1:1–520 ± 112, 2,880 ± 420; 0.5:1–1,394 ± 425, 3,921 ± 637). Treatment with mATG was also shown to modulate the diabetogenic capacity of NOD mice in vivo. *D*: Adoptive transfer of 2.0×10^7 splenocytes from 30-week-old mATG-treated (n = 6) or rIgG-treated (n = 5) mice transferred into NOD.rag$^{-/-}$ mice. *E*: Adoptive cotransfer of 1.0×10^7 splenocytes from 30-week-old mATG-treated (n = 7) or rIgG-treated (n = 8) surviving mice with 1.0×10^7 splenocytes from type 1 diabetic mice transferred into NOD.rag$^{-/-}$ mice.

mATG treatment augments $CD4^+CD25^+$ cell frequencies. The $CD4^+CD25^+$ subset constitutes 5–10% of peripheral $CD4^+$ T-lymphocytes and is capable of inhibiting the responses of $CD4^+CD25^-$ and $CD8^+$ T-lymphocytes in vitro and in vivo (23). $CD4^+CD25^+$ T-cells have also been demonstrated in a number of animal models, including those for type 1 diabetes (24–28), to possess the ability to downregulate autoreactivity and are therefore considered to play a major role in the maintenance of immune tolerance to self. As a growing body of information suggests that Treg control of Teff responses are critical factors in the pathogenesis of type 1 diabetes and treatment of 12-week-old animals with mATG was observed to afford protection from disease, splenocytes from mATG- or control rIgG-treated mice analyzed at various time points for markers of cell populations previously associated with the pathogenesis of this disorder, namely, CD25, CD28, and CD154. Flow cytometric analysis (supplemental Table 1 [available in an online appendix at http://dx.doi.org/10.2337.db06-1384]) revealed increased expression of $CD4^+CD25^+$ cells at 7 (16.85 ± 2.09 vs. 8.30 ± 0.27%; $P < 0.01$) and 14 (11.06 ± 0.23 vs. 8.26 ± 0.27%; $P < 0.05$) days post–mATG treatment. In addition, increased levels of $CD4^+CD28^+$ cells (3.58 ± 0.34 vs. 1.12 ± 0.32%; $P < 0.05$) and $CD8^+CD28^+$ cells (2.78 ± 0.12 vs. 0.89 ± 0.19%; $P < 0.01$) were observed 7 days post–mATG treatment versus rIgG treatment. These frequency changes were, however, temporary, as these or other cell populations analyzed at time points 30 days posttherapy did not reveal differences in cellular frequencies. The findings do, however, suggest the capacity for mATG to induce a transient imbalance of the frequency of cells favoring regulatory T-cell activities in vivo.

mATG treatment enhances the functional activities of $CD4^+CD25^+$ T-cells. Aside from frequency, the capacity for this therapy to modulate Treg function was investigated. Toward this end, purified $CD4^+CD25^+$ T-lymphocytes from different experimental groups administered with mATG or rIgG antibodies were mixed with varying ratios to effector $CD4^+CD25^-$ T-lymphocytes and

proliferation following anti-CD3/CD28 stimulation determined (Fig. 5). Mice treated with mATG at 4 weeks of age and killed at 30 days demonstrated a reduced (albeit not statistically significant) ability to suppress effector T-cell proliferation (Fig. 5*A*). Mice administered mATG at 8 weeks of age showed an equivalent capacity to suppress stimulated effector T-cells (Fig. 5*B*) in comparison with those administered rIgG. In contrast, mice treated with mATG at 12 weeks of age demonstrated a marked decrease in average proliferation of $CD4^+$ Teff cells in the presence of Treg cells at 2:1, 1:1, and 0.5:1 ratios. Indeed, the largest difference in this capacity was seen at 1:1 ratio, in which $CD4^+CD25^+$ T-lymphocytes from mice treated with mATG suppressed lymphocyte proliferation by 78 ± 8.2% ($P < 0.01$) compared with 37.3 ± 8.2% suppression with $CD4^+CD25^+$ T-lymphocytes purified from mice treated with rIgG (Fig. 5*C*). Therefore, much like our observations involving type 1 diabetes delay with mATG suggesting age dependencies, mATG treatment also appears to augment Treg function in vivo in a limited time frame (i.e., 12 weeks of age).

mATG treatment alters diabetogenic and immunomodulatory activities in vivo. To further characterize the potential of this treatment to impart a degree of immunoregulation capable of attenuating anti–β-cell immunity and to establish whether mATG altered the inherent capacity for treated mice to develop type 1 diabetes, both adoptive transfer and adoptive cotransfer studies were performed. For studies of adoptive transfer, splenocytes were obtained at 30 weeks from nondiabetic survivors that were mATG or rIgG treated at 12 weeks of age, administered via intravenous tail vein injection into NOD.rag$^{-/-}$ mice. In animals subject to this procedure, type 1 diabetes onset was delayed ($P = 0.03$) (Fig. 5*D*) and occurred at a reduced frequency (17% [1 of 6] vs. 80% [4 of 5]; $P = 0.03$) in mice that received mATG versus rIgG, respectively.

In a parallel set of adoptive cotransfer studies, 1.0×10^7 splenocytes from 30-week-old mATG- or rIgG-treated mice were mixed at a 1:1 ratio with 1.0×10^7 splenocytes obtained from a set of untreated NOD mice with recent-onset type 1 diabetes and transferred into NOD.rag$^{-/-}$ mice. Similar to the observations involving adoptive transfer, cotransfer of 2.0×10^7 splenocytes representing the mixture from 30-week-old mATG-treated mice into NOD.rag$^{-/-}$ mice delayed ($P = 0.02$) and reduced the degree of diabetes development in comparison with cotransfers with cells from rIgG-treated animals (3 of 7 vs. 8 of 8 in mATG- and rIgG-treated mice, respectively; $P = 0.02$) (Fig. 5*E*). These in vivo data support the aforementioned in vitro data suggesting that mATG induces cells capable of attenuating autoreactive cells.

DISCUSSION

These studies demonstrate that mATG, in an age-dependent fashion, provides an intervention capable of inhibiting the development of autoimmune type 1 diabetes in NOD mice. In terms of the mechanisms underlying this protection, our results suggest that mATG protects β-cells from autoimmune destruction via two pathways: one expected, the other novel. First, a transient reduction of lymphocytes was observed in mATG-treated animals, a form of immunosuppression that would appear of obvious benefit to preventing an immune-mediated disorder such as type 1 diabetes.

We also suggest that a second mechanism involved in beneficial disease modification is active, one previously suggested but not well understood by others. Specifically, in earlier works including those by Monaco and Wood (29) using ALS to promote tolerance to skin allografts in mice, it became apparent that the long-term effects of the treatment were due to T-cell–mediated active tolerance mechanisms. Two and a half decades later, our studies describe, for the first time in the NOD mouse model involving $CD4^+CD25^+$ T-cells following mATG treatment, a refinement made accessible by improvements in the preparations of ATG in combination with the availability of new markers capable of characterizing regulatory T-cells.

Several studies have demonstrated that ATG affects a wide range of immune cell types, having antibodies reactive with an extensive number of cell surface molecules (30–33). One clear benchmark for current efforts to avert type 1 diabetes is that of anti-CD3 antibodies, and in terms of comparison with mATG, both similarities and differences exist in their activities in vivo; in addition, much remains unknown. mATG administration induced, not unexpectedly, a marked increase in serum cytokine concentrations, a finding also observed with anti-CD3 treatment (i.e., increase in serum TNF, interferon-α, IL-2, IL-3, and IL-6) (34). At the same time, it remains unclear whether in situations of mATG-based therapy, the transient but substantial increases in cytokines play a role in creating a microenvironment conducive to generating protective mechanisms. In the case of anti-CD3, much more is known. Specifically, the F(ab′)2 fragments of the antibody work as well as the whole molecule and yet, as shown in various reports using normal mice and NOD mice, they do not elicit any cytokine release (35).

With low-dose anti-CD3 antibody treatment of NOD mice, a significant fall in the proportion of grade one insulitis–containing islets was observed early but then reversed by 10 days post–antibody injection (36). Interestingly, with mATG provided at 12 weeks of age, we observed a significant reduction in insulitis that maintained and even improved 30 days post–mATG administration. Studies with anti-CD3 treatment of NOD mice indicate a marked ability to reverse overt hyperglycemia when provided at disease onset, yet the ability for this agent to impart a stable form of disease prevention when administered late in the pre-diabetic phase was far more limited (6). Such was not the case with mATG, where long-term prevention with late-phase intervention was observed. Indeed, our observation that mATG mediated inhibition of type 1 diabetes onset was dependent on stage in the natural history of type 1 diabetes implied the potential that an active protective mechanism was induced by mATG—one also susceptible to inactivation at an early age (i.e., 4 and 8 weeks).

In NOD mice (21), age-dependent defects in the ability of Treg to modulate activities of Teff have recently been described (28). Reduced levels of $CD4^+CD25^+$ T-cells in both NOD mice and humans with type 1 diabetes have been reported, although the notion in both species has been controversial (26,28,37–40). Our analysis showed significant increases in $CD4^+CD25^+$ cell frequencies shortly after mATG injection in the 12-week-old mATG-treated group, as well as an enhancement of their functional activity (i.e., Treg suppression of Teff responses in vivo). This analysis also revealed increased numbers of APC in spleen and PLN, including dendritic cells, which in

certain activation states have been shown to induce Treg. While this increase may simply reflect the relative decrease in T-cell number following mATG treatment, such an environment may promote the generation and/or maintenance of Treg. Additional data, both in vitro and in vivo, indicate that mATG administration in NOD mice results in a more mature dendritic cell population, characterized by reduced $CD8^+$ expression and increased IL-10 production (K. Womer, unpublished observations). Although $CD4^+CD25^+$ Treg percentages returned to near basal levels at 30 days post–mATG administration, inhibition assays demonstrated retention of functional competence of these cells in spleen. It is speculated that the return of Treg levels to near-normal levels at 30 days post–mATG treatment may be due to trafficking of these cells from spleen to PLN or to insulitis areas within the pancreas. Finally, adoptive transfer of spleen cells obtained from surviving 30-week-old mice from the 12-week mATG group clearly demonstrated their inability to induce type 1 diabetes in recipient NOD.rag$^{-/-}$ mice, indicating attenuation of prodiabetogenic Teff cells within this population. The adoptive cotransfer experiments directly demonstrated the functionally active nature of Treg cells induced by mATG treatment. However, it is vital that future studies involving adoptive cotransfer involve experimentation using purified T-cell subsets instead of total spleen cell preparations for a better defined dissection of the roles for Treg and Teff subsets in these processes. Furthermore, investigations regarding the influence of mATG in vivo should expand beyond those of frequency (as in our studies) to those that, in addition, evaluate absolute number to allow for further address of questions regarding the specific activities of this agent on Treg in vivo.

In contrast to treatment with some depleting anti–T-cell antibodies such as anti-CD8 where disease prevention depends on maintenance of T-cell depletion, a short course of mATG, such as that which occurs with anti-CD4 (12,41), appears sufficient to establish long-term tolerance and confer permanent protection from type 1 diabetes—as may also be the case of anti-CD3 antibody. To our knowledge, our results provide the first indications that a short course of mATG given alone can restore self-tolerance mechanisms via Treg cells, a facet that has been previously ascribed to anti-CD3. We believe additional studies would support efforts proposing ATG, either alone or in combination with self-antigens or β-cell regeneration agents, to allow for a rendering of insulin independence in type 1 diabetic patients. Given the immunoregulatory properties of this agent, this form of therapy might be applicable not only to type 1 diabetes but also to other diseases associated with dysregulated immune responses.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Juvenile Diabetes Research Foundation International, the National Institutes of Health, and the Sebastian Family and Keene Endowments for Diabetes Research.

We thank Dr. S. Brian Wilson for his review of this manuscript.

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