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Transient Low-Dose Methotrexate Induces Tolerance to Murine Anti-Thymocyte Globulin and Together They Promote Long-Term Allograft Survival

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Rabbit anti-thymocyte globulin (Thymoglobulin) effectively treats transplant rejection but induces anti-rabbit Ab responses, which limits routine readministration. Aiming to tolerate anti-rabbit responses, we coadministered a brief methotrexate regimen with a murine version of Thymoglobulin (mATG) for effects on anti-mATG Abs and cardiac allograft in mice. Although both single and three courses of methotrexate could significantly inhibit anti-drug Ab titers to repeated mATG administration, surprisingly, the single course given at the first mATG administration was most effective (>99% reduction). The transient methotrexate treatment also significantly improved pharmacokinetics and pharmacodynamics of repeated mATG administration. In the cardiac allograft model, the combination of transient mATG and methotrexate given only at the time of transplant dramatically improved allograft survival (>100 d) over either agent alone (<30 d). Anti-drug Ab titers were reduced and mATG exposure was increased which resulted in prolonged rather than enhanced mATG-mediated effects when combined with methotrexate. Moreover, methotrexate administration significantly reduced alloantibodies, suggesting that methotrexate not only decreases anti-drug Ab responses but also reduces Ab responses to multiple tissue-derived alloantigens simultaneously. These data suggest that mATG and methotrexate together can provide long-term allograft survival potentially through the induction of immune tolerance. The Journal of Immunology, 2012, 189: 732–743.

Methotrexate is currently used as a continuous, low-dose treatment to suppress immune responses in inflammatory diseases such as rheumatoid arthritis and psoriasis (1–3). Some patients can be treated with infliximab or other anti-TNF agents alongside continuous methotrexate treatment to further reduce disease activity (4–8). In such cases, inflammatory responses against diseased tissue as well as the anti-TNF therapies used are reduced (4–10). The mechanism by which methotrexate exerts these effects is presumed to be through killing proliferating cells by inhibiting dihydrofolate reductase and inducing immunosuppression (9, 10). Our laboratory has also demonstrated that methotrexate can significantly reduce inflammatory responses against protein therapeutics (11, 12). In particular, we have shown that methotrexate significantly decreases the development of anti-drug Abs, however, not through immunosuppression but rather through the induction of immune tolerance because only a few short courses of methotrexate can significantly reduce Abs that develop in mice against the enzyme replacement therapies, Fabrazyme and Myozyme, for extended periods of time (11, 12). We have also shown that in the context of Fabrazyme, methotrexate-induced control of anti-drug Abs is Ag specific (12).

We sought to extend our observations of methotrexate-induced immune tolerance to an immunomodulating protein therapy, Thymoglobulin, and to evaluate functional effects in a transplantation setting. Thymoglobulin is a rabbit anti-human thymocyte polyclonal Ab (anti-thymocyte globulin [ATG]) that is currently prescribed for the prevention and treatment of acute solid organ transplantation, acute graft-versus-host disease, and severe aplastic anemia (13–16). ATG, however, is not generally administered repeatedly over extended periods of time, because Thymoglobulin-treated transplant recipients have sporadically been reported to experience serum sickness, acute renal failure, or cardiovascular reactions. These reactions have been linked to anti-drug Abs (17–21). We explored whether low-dose methotrexate can reduce anti-ATG Ab responses in nonclinical studies using a murine version of Thymoglobulin, mATG. mATG is purified IgG from rabbits immunized with mouse thymocytes and behaves similarly in mice to Thymoglobulin in humans (22). mATG depletes CD4+ and CD8+ T cells, expands the percentage of functional T regulatory cells in mice (22), and through these mechanisms is effective at delaying rejection of allogeneic heart and skin transplants in mice (Ref. 23; J. Williams, unpublished observations). We explored whether the same regimen that is effective in reducing anti-drug Abs to the enzyme replacement therapy, Myozyme, or even fewer cycles of methotrexate, could reduce Ab responses that develop against mATG in the context of repeated administration. In addition, although T cell-directed therapies such as Thymoglobulin are efficacious, B cell and alloantibody responses also can contribute to chronic rejection as patients still can develop alloantibodies to their grafts (24–26). As a result, we investigated whether alloantibody responses in a fully mismatched allogeneic
murmur cardiac transplant model could also be controlled by methotrexate. Importantly, methotrexate is not currently used in transplantation, and we assessed whether the combined effects of mATG and our transient tolerizing methotrexate treatment could extend transplant survival.

Materials and Methods

Animals, mATG, and methotrexate treatments

C57BL/6 and BALB/c mice, between 8 and 13 wk of age, were obtained from Taconic Laboratories (Germantown, NY) or Charles River Lab-
ratories (Kingston, NY, and Raleigh, NC, respectively). Mice were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals and under American Association for Accreditation of Laboratory Animal Care accreditation, and all animal procedures were approved by the Institutional Animal Care and Use Committee. A murine version of Thynglobulin (mATG) was generated by immunizing rabbits with a mixture of mouse thymocytes as described previously (22). mATG was administered as an i.p. injection of 5 mg/kg every 4 wk or as two 20 mg/kg doses given 4 d apart when in the transplant setting with the first dose given on the day of transplant (day 0). Methotrexate (Calbiochem, Merck, Darmstadt, Germany) was administered in different regimens in conjunction with the mATG dosing schedule. In studies that involved monthly mATG treatment, methotrexate was administered i.p. at 5 mg/kg at 0, 24, and 48 h following either the initial mATG treatment or the first three mATG treatments. In transplant studies where mATG was dosed at days 0 and 4, methotrexate was given daily at 2 mg/kg from days 0 to 6, daily at 0.5 mg/kg from days 0 to 6, or daily at 0.5 mg/kg from days 0 to 11.

Serum anti-rabbit mATG Ab titers

For studies in normal animals, anti-mATG Ab titers were analyzed weekly through the course of each study. For transplant studies, anti-drug Ab titers were analyzed weekly for up to 4 wk posttransplantation. The levels of anti-drug IgG in mouse serum were analyzed by ELISA. Briefly, 96-well plates (Corning, Corning, NY) were coated overnight with rabbit IgG and blocked with Super Block Blocking Buffer (Thermo Scientific, Rockford, IL), and serial dilutions of serum were added in duplicate. Following incubation, the plates were washed, and the signal was detected by first adding HRP-conjugated goat anti-rabbit IgG secondary Ab (Southern Biotechnology Associates, Birmingham, AL) and, following another wash, developed with 3,3′,5,5′-tetramethylbenzidine substrate (BioFx, Owings Mills, MD). The reaction was stopped by the addition of 1 N HCl, and absorbance values were read at 450/650 nm on an ELISA plate reader (Molecular Devices, Sunnyvale, CA). End-point Ab titers were defined as the lowest dilution above an absorbance of 0.1 by using Softmax software (Molecular Devices).

Serum mATG levels

For pharmacokinetic measures after mATG administration to mice, mATG levels in mouse serum were determined by ELISA. Briefly, 96-well plates (Corning, Corning, NY) were coated with goat anti-rabbit IgG-Fc fragment-specific Ab (Bethyl Laboratories, Montgomery, TX) and blocked with 0.5% BSA (high purity) standard controls, and serum samples were diluted in duplicate. Following incubation and washing, the mATG was detected using HRP-conjugated goat anti-mouse IgG secondary Ab (Bethyl Laboratories) and 3,3′,5,5′-tetramethylbenzidine substrate (BioFx). The reaction was stopped by adding 1 N HCl, and absorbance values were read at 450/650 nm on an ELISA plate reader (Molecular Devices). Final concentrations were interpolated off the standard curve and are reported as nanograms per milliliter. The presence of anti-drug Abs within the test serum was determined not to interfere with the detection of mATG up to Ab titers of 218,000 and even at those and higher titers the interference was modest (data not shown).

Cell preparations from various tissues

For splenocyte and lymph node cell preparations, single-cell suspensions were generated from harvested mouse spleens or pooled inguinal and mesenteric lymph nodes by homogenization between frosted glass slides. For splenocyte preparations, RBCs were lysed by 1–2 min of incubation with RBC lysis solution (BD Biosciences) for 20–30 min. For all tissue preparations, live cells were enumerated using the ViCell automated counter (Beckman Coulter, Fullerton, CA) prior to use in the assays described below.

Flow cytometry

Cell populations within different tissues were prepared as single-cell suspensions as described above and were incubated with fluorochrome-conjugated Abs that included anti-mouse CD4, CD8, CD19, and CD25 (BD Biosciences). Intracellular Foxp3 expression analysis was performed according to the anti-Foxp3 manufacturer’s protocol (eBioscience, San Diego, CA). Following incubation with the Abs, cells were washed and analyzed by flow cytometry (FACSCanto; BD Biosciences and FCS Express Software, De Novo Software, Los Angeles, CA). Cell populations evaluated were defined as follows: total CD4+ T cells, CD4+CD8−; total CD8 T cells, CD8+CD4−; total B cells, CD19+; and T regulatory cells, CD4+CD25+Foxp33. In vitro blocking studies determined that up to 100 μg/ml mATG did not prevent detection of these populations.

Cardiac allograft model

Donor mice (either an allogeneic C57BL/6 or syngeneic BALB/c) were first anesthetized with an i.p. injection of ketamine (Fort Dodge Animal Health/ Pfizer, Fort Dodge, IA) and xylazine (Lloyd, Shenandoah, IA), and a median sternotomy was performed. The donor heart was slowly perfused in situ with 1 ml cold heparinized Ringer’s lactate solution (Baxter Healthcare, Deerfield, IL) through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were then transected, the graft was removed from the donor, and the heart was stored in ice-cold saline until engraftment. A recipient mouse (BALB/c) was similarly anesthetized and prepared as described above for donor mice, except the abdominal cavity was opened. Using a surgical microscope to view the opened abdominal cavity, the abdominal aorta and the inferior vena cava were isolated. The donor heart was placed into the recipient abdomen (upside down), and the grafts were revascularized with end-to-side anastomoses between the donor pulmonary artery and the recipient inferior vena cava as well as the donor’s aorta and the recipient abdominal aorta. After hemostasis was confirmed, the abdominal muscle was closed with a running 5-0 Vicryl suture (Ethicon/Johnson and Johnson, Somerville, NJ), and the skin was closed with a running 5-0 Ethilon suture (Ethicon/Johnson and Johnson). Standard postoperative pain assessment and management were performed. Grafts were assessed by palpation five to seven times per week for the first 30 d and then three to four times per week until the end of the study.

Histopathology and immunohistochemistry

Cardiac grafts were fixed in 10% neutral buffered formalin, bisected along the longitudinal axis to expose the right and left ventricles and the outflow tract, and routinely processed for paraffin embedding. Sections were cut at 5 μm and were stained with hematoxylin and eosin (H&E) or Masson’s trichrome. Serial sections were also immunostained as described below. Each H&E-stained section was evaluated qualitatively for various features of allograft rejection pathology (e.g., vasculitis, myocardial degeneration and necrosis, and myocardiitis) using a histologic grading scheme modified from Ref. 27.

Immunohistochemistry was performed using Bond-Max automated immunostaining system (Leica Microsystems, Buffalo Grove, IL). To detect CD3, CD4, and Foxp3 dual immunostaining, graft tissue sections were subjected to double immunostaining with anti-CD3 and anti-Foxp3 Abs using Bond Polymer Refine Detection kit and Bond Polymer AP Red kit (Leica Microsystems) following the manufacturer’s guidelines. Briefly, deparaffinized sections of paraffin-embedded grafts were subjected to heat-induced epitope retrieval (25 min at 99˚C) and incubated with serum-free protein block (DakoCytomation, Carpinteria, CA), rabbit monoclonal anti-CD3 Ab (Lab Vision/Neo Marker), peroxidase-conjugated polymer, peroxidase block, and diamobenzidine detection reagent, followed by rat anti-mouse Foxp3 Ab (eBioscience) and then a rabbit anti-rat Ab (Vector Laboratories, Burlingame, CA). Slides were then incubated with Bond Polymer AP and mixed red detection reagent and finally counterstained with hematoxylin. In negative control slides, primary Abs were replaced with phosphate-buffered saline (DakoCytomation). Immunohistochemical analysis was performed with an a priori scoring system designed to assess the extent of immune cell infiltration. Sections were assessed by three independent observers and scored for the percentage of each cell type present within the perivascular region and the myocardium (25).

Cell populations were calculated as mean ± SD and are shown as dot plots. Data were analyzed by Student’s t test for two groups or one-way ANOVA with Tukey’s post hoc test for multiple groups. Confidence intervals for the area under the curve were calculated by the trapezoidal rule and assigned as *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001. All data were analyzed using GraphPad software (GraphPad Software, La Jolla, CA).
fluorescent intensities of serum-stained fibroblasts were divided by isotype control-stained fibroblasts to normalize alloantibody levels between experiments. We did not observe any binding of serum Abs to SV40-transformed BALB/c (syngeneic) fibroblasts indicating that they are only reactive to alloantigens (data not shown).

**Results**

**mATG generates significant anti-drug Abs, which can be controlled by a single course of methotrexate**

The immunogenicity of mATG is unknown in mice though mATG is expected to generate anti-drug Abs because it is a nonmurine protein. To investigate mATG immunogenicity in normal animals, C57BL/6 mice were administered five monthly treatments of 5 mg/kg mATG. Anti-drug Ab responses were then quantified weekly through 20 wk of monthly treatments. During this period, despite CD4⁺ T cell depletion by mATG, Ab titers reached as high as 5 million (Fig. 1A). Interestingly, animals that received nonspecific rabbit IgG at the same dose level and schedule as mATG showed low anti-rabbit IgG responses (Fig. 1A). One possibility for the enhanced immunogenicity of mATG may be the specific binding of mATG to APCs such as follicular dendritic cells, which when in the presence of complement may significantly enhance B cell responses (28).

Having established that mATG is highly immunogenic in mice, the ability of methotrexate to control anti-drug Ab responses was next evaluated. For these studies, methotrexate was tested as an immune tolerance inducing agent to mATG and was administered in a similar regimen to that used to tolerize Myozyme and Fabrazyme (11, 12). Administration of 5 mg/kg methotrexate within 15 min of as well as 24 and 48 h following the initial three mATG treatments, significantly reduced anti-drug Ab titers by 69% through 6 mo of monthly mATG administration (Fig. 1B). Additional studies were conducted to assess whether the methotrexate immune tolerance induction regimen can be further optimized. Earlier work in our laboratory with another protein therapy suggested that the immune tolerizing effects of methotrexate decreased as more methotrexate was administered (data not shown). These data implied that the methotrexate regimen could possibly be shortened further. To pursue this idea, the effect of a single cycle of 5 mg/kg methotrexate on anti-drug Ab responses through five monthly mATG treatments was assessed. In this setting, mATG anti-drug Abs were significantly reduced by 99% when comparing the area under the effect curves of Ab titers generated in methotrexate-treated animals with those generated in mATG alone-treated mice (Fig. 1C). The tolerance-inducing effect of methotrexate was further investigated by resting animals from mATG dosing for 2 mo and then rechallenging them with a single treatment of 5 mg/kg mATG. Ab titers were assessed through 4 wk following the final mATG treatment. Although mATG anti-drug Abs increased in mATG-only treated mice, they remained lowest in mice that received the single-cycle regimen compared with those that received the three-cycle regimen (Fig. 1D).

**FIGURE 1.** A single course of methotrexate (MTX) induces long-lived reduction in anti-mATG Abs through repeated mATG administration. C57BL/6 mice were administered 5 mg/kg mATG (or a nonspecific rabbit IgG control Ab; rbIgG) every 4 wk for five treatments with coadministration of 5 mg/kg MTX given for three consecutive days starting on day 0 relative to the first mATG treatment (single-cycle MTX, 1XMTX) or under the same conditions relative to the first, second, and third mATG treatment (three-cycle MTX, 3XMTX). Anti-mATG titers were monitored by ELISA weekly over the course of the study. (A) mATG alone compared with rbIgG control. (B) Three-cycle MTX compared with mATG alone. (C) Single-cycle MTX compared with mATG alone. (D) Following the fifth treatment of mATG, mice were rested for 2 mo and rechallenged a sixth time with mATG in the absence of MTX. Anti-mATG titers were monitored weekly through 4 wk after the additional challenge. Data are expressed as the mean ± SE and represent data of at least three experiments (n = 10). Area under the effect curves were calculated and compared, highlighting the overall reduction in Ab responses by MTX.
Single-cycle methotrexate restores mATG-mediated T cell depletion in the context of repeat administration

Because anti-drug Abs can directly impact the pharmacodynamic effects of protein therapies, mATG-mediated T cell depletion was evaluated following repeated monthly administration. CD4+ and CD8+ T cell depletion was assessed in blood, spleen, and lymph nodes following the fifth monthly dose of mATG. Tissues were harvested 24 h prior to and 72 h after the fifth mATG treatment. The CD4+ and CD8+ T cell subsets were evaluated by flow cytometry, and the absolute cell numbers were compared between the two time points to calculate the percentage of cells depleted for each population after the fifth monthly dose of mATG (Fig. 2A). Animals that were given only mATG exhibited little depletion of both cell subsets in blood, spleen, and lymph nodes (Fig. 3B; data not shown). Methotrexate-treated mice, in contrast, showed significantly more depletion in all tissues when compared with mATG-alone treated mice (Fig. 3B; data not shown). As observed in previously reported studies, mATG has a greater effect on CD8+ T cell depletion than CD4+ T cell depletion (22), and this bias is maintained with methotrexate administration (Fig. 2B). Finally, consistent with the effects observed on anti-drug Abs, a single cycle of methotrexate can be more effective at restoring the depleting effects of mATG than three methotrexate cycles (Fig. 2B). Although single-cycle treatment is more consistently effective than three cycles of methotrexate in reducing anti-drug Ab responses, we have observed instances where three cycles of methotrexate treatment can be as effective at restoring mATG-mediated depletion as a single cycle. Although a significant correlation was observed between anti-drug Ab titer and the depletion of CD4+ and CD8+ T cells ($R^2 = 0.702$ and $R^2 = 0.836$, respectively; Fig. 3C), it appeared that rather than a direct correlation, there was a threshold whereby mATG-mediated cell depletion was affected when anti-drug Ab titers were greater than 100,000 (Fig. 2C).

![FIGURE 2. Pharmacodynamic activity (% T cell depletion) of repeated mATG administration is increased by a single cycle of methotrexate (MTX). C57BL/6 mice were administered 5 mg/kg mATG every 4 wk for five treatments in the absence of MTX or in combination with either one or three cycles of MTX as described in Fig. 1. (A) One day prior to and 3 d after the fifth mATG treatment, the number of CD4 and CD8 T cells in the spleen and blood were determined by flow cytometry. (B) The percent depletion was determined, based on the absolute cell number of these populations 1 d prior to and 3 d following the fifth mATG treatment. (C) Anti-mATG titers were plotted relative to the CD4 and CD8 T cell depletion observed following the fifth dose of mATG. Dots on the graphs represent individual mice and the dotted line indicates the apparent threshold of anti-mATG titers where titers above this line will significantly impact mATG pharmacodynamics. Data are expressed as the mean ± SEM and are representative of two separate experiments ($n = 6$). \(*p < 0.05, **p < 0.001, ***p < 0.0001.\)
Single-cycle methotrexate restores mATG exposure in the context of repeat administration

Because mATG-specific anti-drug Abs appeared to interfere with the ability of mATG to deplete T cells, it was of interest to determine whether mATG exposure was also decreased. The circulating levels of mATG were therefore assessed following the first, third, and fifth monthly mATG treatment in animals that received either both methotrexate and mATG or mATG alone (Fig. 3A). The serum levels of mATG were overlapping among the two treatment groups after the first mATG dose (Fig. 3B). However, following both the third and fifth monthly dose of mATG, little to no circulating mATG was detected in animals that were administered mATG alone (Fig. 3B). In contrast, animals that were given the combination treatment exhibited similar circulating mATG levels following both the third and fifth doses as those observed after the initial dose (Fig. 3B). Because anti-drug Ab titers are significant at the third and fifth month of treatment, a direct comparison between anti-drug Ab titer and circulating levels of mATG was performed. This comparison revealed that when end-point anti-drug Ab titers are >10,000, the level of circulating mATG is significantly reduced (Fig. 3C). This level of anti-drug Ab titer is lower than the titer associated with interference of pharmacodynamic T cell-depletive effects. However, because only a very small percentage of total mATG binds specifically to T cells (29), the anti-drug Ab titer required to inhibit the binding and depletion of lymphocytes is measurably greater than the anti-drug Ab titer level that can interfere with the circulating levels of total mATG.

An induction regimen combining mATG and methotrexate results in long-term cardiac allograft survival along with reductions in anti-drug Ab titers and increased mATG exposure

Because methotrexate can improve mATG exposure and pharmacodynamics in normal mice following repeated mATG administration, studies were conducted to evaluate whether these effects could provide a functional benefit under conditions of a fully mismatched allogeneic heart transplant. In our cardiac transplant model, 20 mg/kg mATG is typically administered on the day of transplantation and 4 d after transplantation and a reproducible extension of graft survival is observed. Specifically, untreated mice reject around 7 d after transplant, whereas mATG-treated animals reject between days 15 and 25. To determine whether there is any benefit to coadministering methotrexate with mATG, we treated animals with a single course of both mATG and methotrexate around the time of cardiac allotransplantation. To maintain the 0, 24, and 48 h dosing of methotrexate at each mATG treatment and to keep the total dose of methotrexate consistent with previous studies, we administered 2 mg/kg methotrexate daily from the day of transplant through 6 d later (14 mg/kg total methotrexate dose). In addition, we investigated 4-fold lower doses of methotrexate (0.5 mg/kg) given under the same regimen or with an extended regimen of 12 consecutive days. Groups of mice either received no treatment, mATG alone, methotrexate alone, or a combination of the mATG and methotrexate regimens. Although both mATG or methotrexate treatment alone provided a modest benefit of an average extended survival to 15 and 20 d, respectively, the coadministration of mATG and any of the methotrexate regimens...
evaluated demonstrated a dramatic benefit in cardiac graft survival with the majority of mice retaining their grafts for up to over 100 d (Fig. 4A). Because cardiac graft survival continues long after the early, brief induction treatments of mATG and methotrexate, this regimen appears to be tolerogenic rather than immunosuppressive.

Similar to studies in normal mice, methotrexate coadministration with mATG reduced anti-drug Ab titers to mATG regardless of regimen used (Fig. 4B, Table I). Moreover, coincidental to the reduction in Ab titers was an observed increase in mATG exposure in this transplant setting (Fig. 4C). Given the likely adjuvant effect under the conditions of a potent, coincident immune response against the transplanted tissue, the anti-drug Ab titers increased even faster than in a normal mouse setting and resulted in mATG levels being near undetectable within 7 d of the first mATG administration (Fig. 4C). This emphasizes that under conditions of an ongoing inflammatory response, anti-drug Ab responses can be accelerated and perhaps have an even greater impact on pharmacodynamics and efficacy. Importantly even under these conditions, methotrexate had a profound inhibitory effect on mATG anti-drug Abs and enhanced mATG exposure. However, because circulating mATG levels were still low to undetectable by 21 d with combination mATG and methotrexate treatment, additional tolerance mechanisms are likely at play given the >100 d graft survival. These results demonstrate a profound synergy between mATG and methotrexate treatment on the survival of the allogeneic grafts and show a similar level of reduction in mATG anti-drug Abs and enhancement of mATG exposure as observed in normal mice.

mATG and methotrexate cotreatment reduces allograft rejection pathology without inducing further increases in T regulatory cells

To both assess the histological changes in long-surviving grafts as well as begin to understand the mechanism of mATG and methotrexate combination on graft survival, cardiac grafts were collected and evaluated for pathology as well as cellular composition. In particular, as T regulatory cells have been associated with long-term graft survival in transplantation (23, 24, 30, 31), are induced by Thymoglobulin and mATG (22, 25, 26, 32, 33), and have previously been demonstrated to be responsible for delayed graft rejection following mATG (16), CD3+Foxp3+ cells, which bear a phenotype consistent with regulatory T cells, were evaluated. To assess histologic changes in long-surviving grafts, cardiac grafts were collected from the mATG and methotrexate combination-treated group and the untreated syngeneic group at least 100 d after transplantation. Grafts from untreated, mATG alone, or methotrexate alone-treated mice were taken after graft rejection for comparison. Tissue sections stained with H&E or Masson’s trichrome or immunostained anti-CD3 and anti-Foxp3 Abs were microscopically evaluated for histologic changes indicative of transplant rejection, namely mild to moderate myocarditis, myocardial degeneration and necrosis, cardiac allograft vasculopathy (CAV), and T cell infiltration. At day 100, allografts from animals cotreated with mATG and methotrexate revealed minimal to mild CAV lesions and none to minimal myocardial degeneration and myocarditis. Histologic changes suggestive of graft rejection were not apparent in syngeneic grafts at this late time point (Fig. 5A). Allografts from the combination-treated group exhibited mild T cell infiltration in the myocardium with a few cells infiltrating the myocardial blood vessels. Syngeneic grafts contained rare T cells within the myocardium. Clusters of T cells with occasional dual CD3 and Foxp3 immunopositive cells were present in the epicardium of both syngeneic grafts and combination-treated allografts. Thus, long-surviving grafts show minimal signs of graft rejection, which correlates with reduced inflammation.

**FIGURE 4.** Synergistic effects of mATG and methotrexate (MTX) on allograft heart survival correspond with MTX-mediated decreases in anti-mATG titers and increased circulating mATG levels. BALB/c mice were transplanted with allogeneic C57BL/6 hearts as described in Materials and Methods. Following transplantation, groups of mice were left untreated, treated with 20 mg/kg mATG alone on the day of transplant (day 0) and 4 d afterward, treated with 2 mg/kg methotrexate (MTX) alone on days 0–6, or treated with a combination of the mATG and MTX. For the combination treatment, MTX was administered as 2 mg/kg days 0–6, 0.5 mg/kg days 0–6, or 0.5 mg/kg days 0–11. (A) Graft survival was monitored. Data are presented as the percentage of mice maintaining surviving grafts over time and are pooled from at least three experiments. (B) Serum from the transplanted mice treated with mATG, MTX, or the combination regimen using 2 mg/kg MTX days 0–6 was also assessed for anti-mATG titers and mATG levels over time. (C) Data are expressed as the mean ± SE from at least three experiments.
Because the effects of mATG and methotrexate combination treatment were likely to more actively occur closer to the time of transplantation, we also evaluated pathology and characterized the cellular infiltrate of transplanted heart allografts at 7 d (for untreated mice) or 14 d (for all treatment groups) after transplantation. Untreated allografts displayed graft rejection pathology including myocarditis, myofibril degeneration, and necrosis in both epicardial and intramyocardial branches of coronary arteries (Fig. 5B). In contrast, allografts isolated from animals treated with both mATG and methotrexate revealed less severe CAV (Fig. 5B). This was in comparison with allografts from untreated mice as well as from animals treated with either mATG or methotrexate (Fig. 5A). As expected, syngeneic grafts from untreated mice revealed little or no pathology at these time points. CD3+ T cell infiltration was observed in the myocardium and epicardium in allografts from untreated mice and those treated with mATG or methotrexate alone (Fig. 5B). A few CD3+ T cells were also present in the inflammatory cell infiltrate associated with the CAV lesions in these grafts. In contrast, allografts from animals treated with the combination of mATG and methotrexate exhibited substantially lower CD3+ T cell infiltration in the myocardium and only minimal CD3+ T cell infiltration in the epicardium. Syngeneic cardiac grafts showed minimal CD3+ T cell infiltration only in the epicardium. A small proportion of T cells within the inflammatory cell infiltrates in the epicardium appeared to have a T regulatory cell phenotype as indicated by dual CD3 and Foxp3 immunoreactivity, but this frequency appeared no greater within inflammatory infiltrates than in other groups. Therefore, reduced pathology is also observed early after treatment with mATG and methotrexate and is associated with both reduced and epicardium-restricted T cell infiltration.

Because the coadministration of mATG and methotrexate appeared to reduce inflammation and yield a different distribution of Foxp3+ T cells within allograft tissue, we were interested in whether similar T cell effects would be evident in the spleens of the transplanted animals at an early time point. Methotrexate treatment alone did not affect the absolute numbers of CD4+ and CD8+ splenic T cells 14 d after treatment, whereas as expected, mATG reduced the numbers of these T cell populations. Animals that received both methotrexate and mATG exhibited a further reduction in the numbers of splenic CD4+ and CD8+ T cells 14 d after transplant. This additional decrease in T cells is presumably due to enhanced mATG effects resulting from increased exposure at this time point (Figs. 5C, 4B, 4C). In contrast, the increases in T regulatory cell frequencies seen with mATG treatment in the spleen were not further enhanced by methotrexate treatment, and methotrexate treatment alone did not exhibit an effect on this population (Fig. 5C). Overall, the combined treatment of mATG and methotrexate attenuated the severity of graft rejection pathology and was associated with decreased T cell infiltrate in the graft but not with increases in cells with a regulatory T cell phenotype. Finally, because methotrexate treatment inhibits Ab responses against mATG in this setting, we evaluated whether total B cell numbers were decreased by methotrexate. Methotrexate treatment did not reduce total B cell numbers in the spleens of transplanted animals because no significant differences were observed between the absolute B cell numbers among the differently treated allogeneic transplant groups (Fig. 5C). These data suggest that methotrexate effects on anti-drug Abs may not be mediated by anti-folate-induced depletion of activated B cells. As expected, mATG treatment also did not impact total B cell numbers in this heterotopic cardiac allograft model model.

Delayed repopulation of T cells, prolonged maintenance of T regulatory cells, and no obvious effects on total B cell numbers by methotrexate cotreatment with mATG in normal mice

Because the absolute cell numbers of splenic CD4+ and CD8+ T cell populations appeared lowest in animals that were coadministered mATG and methotrexate 14 d after transplant, we investigated whether this observation was due to delayed repopulation or a direct enhancement of cell depletion. A time-course experiment was performed in normal mice to evaluate circulating and splenic CD4+ and CD8+ T cells over time following mATG, methotrexate, or the combination treatment. Methotrexate coadministered with mATG did not appear to increase the magnitude of T cell depletion at the peak depletion time point (5 d); however, the recovery of both CD4+ and CD8+ T cells was delayed by 1–2 wk in the combination treatment group (Fig. 6A, 6B). Similarly, although mATG increased the frequencies of T regulatory cells as expected, methotrexate cotreatment did not amplify the magnitude of the T regulatory cell increase but did extend the elevations prior to them declining to baseline (Fig. 6C). Notably, treatment with methotrexate and control rabbit IgG did not alter the absolute numbers of splenic CD4+, CD8+, or CD4+CD25Foxp3+ cells, indicating that methotrexate itself was not responsible for T cell depletion or the induction of T regulatory cells. Thus, these data indicate that methotrexate merely prolonged the effects of mATG by reducing mATG anti-drug Abs rather than directly enhanced those effects. In addition, this effect is not long-lived because these T cell populations do return to baseline levels within a couple weeks of mATG treatment alone, indicating that there are likely to be additional mechanisms to account for the significant prolongation of graft survival. Finally, as previously suggested in the transplant model, B cells did not appear to be affected by methotrexate, mATG, or the combined treatment with methotrexate and mATG (Fig. 6C).

Alloantibodies decreased by transient methotrexate treatment

Although methotrexate coadministration with mATG resulted in decreased anti-drug Abs and prolonged mATG exposure and T cell depletion, these effects were not long-lived and thus do not fully explain the profound synergy between the brief treatments with
FIGURE 5. Pathology within the cardiac allografts is reduced with combination mATG and methotrexate (MTX) treatment in 14-d heart allografts but is not associated with increases in T regulatory cells. BALB/c mice were transplanted with either BALB/c hearts (syngeneic control) or C57BL/6 (allogeneic) hearts as described in Materials and Methods. The mice given allografts were untreated or treated with mATG and MTX as described in Fig. 4. For both (A) and (B) representative photomicrographs at original magnification $\times 400$ are shown. The upper panels are stained with H&E. The lower panels are stained by immunohistochemistry using 3,3'-diaminobenzidine and mixed red immunohistochemistry with hematoxylin counterstain. Asterisks indicate blood vessel lumen, arrows indicate T cells, and arrowheads mark the epicardial surface. (A) Grafts were collected after graft rejection or 100 d, whichever came first, and were evaluated for pathology and for CD3$^+$ and CD3$^+$Foxp3$^+$ cells. Syngeneic graft at 100 d posttransplantation is histologically unremarkable. Untreated allograft shows extensive myocardial necrosis and heavy infiltration in the epicardium. mATG- or MTX-treated allograft shows mild to moderate myocardial degeneration and inflammatory cell infiltration, whereas mATG and MTX combination-treated allograft shows (Figure legend continues)
mATG and methotrexate in this transplant setting. Given the effect of methotrexate on Ab responses in this context and because graft rejection can also be influenced by alloantibodies that develop against the graft, we evaluated whether methotrexate could also affect alloantibody levels. We examined alloantibody levels in the serum of allograft recipient mice 21 d following transplantation that were untreated or treated with mATG, methotrexate, or a combination. An allogeneic fibroblast line was used to assess alloantibody responses to minor and major histocompatibility Ags because MHC mismatch is primarily responsible for graft rejection in this model. Alloantibody binding to the allofibroblast line was increased in serum from allogeneic heart-transplanted mice compared with serum from normal mice (Fig. 7A). Treatment of allotransplanted mice with mATG alone did not alter the alloantibody levels compared with untreated allotransplanted mice (Fig. 7A, 7B), indicating that even though mATG depletes CD4+ T cells significantly, this level of CD4 depletion is not sufficient to impact the generation of alloantibodies. In contrast, methotrexate treatment alone and in combination with mATG showed significantly reduced alloantibody levels. Because we have no evidence that methotrexate by itself under these conditions depletes T cells, depletes B cells, or increases regulatory T cells, this effect of methotrexate appears to be independent of these mechanisms of Ab suppression. These results suggest that methotrexate can control Ab responses not only to mATG but also to coincident alloantigens of a transplanted graft.

Discussion

These studies demonstrate that a single, brief course of low-dose methotrexate can control Ab responses in mice to the polyclonal Ab, mATG. By significantly reducing anti-drug Abs, methotrexate increased mATG exposure in both normal mice treated repeatedly with mATG and transplanted animals after a single mATG treatment. Although the brief combination treatment of methotrexate and mATG showed dramatic prolongation of graft survival, enhancements in mATG exposure and pharmacodynamic effects could not fully explain this effect. It was found that methotrexate not only reduces mATG anti-drug Abs but also reduces alloantibodies to a transplanted graft. In addition, the requirement for only transient administration of both agents suggests an induction of tolerogenic mechanisms rather than long-lived immunosuppression. Taken together, these data suggest that although mATG-mediated T cell depletion and regulatory T cell enhancement induces T cell tolerance (22) and reduces T cell-mediated rejection, short-term methotrexate treatment reduces anti-allograft Ab responses. Thus, the combination appears to result in a two-pronged inhibition of both arms of the adaptive immune response that results in long-term graft survival.
Alloantibodies in cardiac transplanted mice are significantly reduced by methotrexate (MTX). Mice receiving allogeneic heart transplants that were treated with mATG, MTX, or a combination as described in Fig. 4 were assessed for serum alloantibodies as described in Materials and Methods. (A) Representative flow cytometry plots show the relative amounts of alloantibodies in the different mice. (B) The ratio of alloantibodies compared with the unstained control is shown. The data are expressed as the mean ± SEM from at least three experiments. Statistically significant decreases compared with no treatment are indicated with the p value, ***p < 0.0001.

Methotrexate has previously been reported to control Ab responses to protein therapies. As an approved therapy for rheumatoid arthritis, weekly methotrexate treatments between 7.5 and 25 mg/wk can be coadministered with infliximab to further control disease (4, 34–36). In this setting, methotrexate has been shown to control the development of anti-infliximab Abs. The presumed mechanism of methotrexate control of infliximab-specific anti-drug Abs in the context of weekly dosing is through the inhibition of proliferating T and B lymphocytes by interfering with thymidine uptake through dihydrofolate reductase antagonist (10). An additional contributing feature of repeated methotrexate treatment has recently been suggested in rats that involves the expansion of T regulatory cells during low-dose administration of methotrexate every other day (37).

In contrast to regimens of continual methotrexate dosing, our laboratory has demonstrated previously that a brief course of methotrexate can induce long-term, Ag-specific control of Ab responses throughout repeat administration of protein therapeutics in mice (11, 12). This effect is being evaluated in the clinical setting where patients treated with a combination of rituximab and an immune tolerizing regimen of methotrexate appear to develop immune tolerance to the enzyme replacement therapy, Myozyme (38, 39). These observations were extended further in the studies reported in this paper, which demonstrated that a single cycle of methotrexate treatment can effectively induce immune tolerance to anti-drug Abs and control Ab responses to multiple protein Ags simultaneously in a transplantation setting. Importantly, methotrexate treatment alone has not demonstrated any effects on CD4+ T cells, CD8+ T cells, total B cells, and T regulatory cells in these and other studies exploring short-term regimens of methotrexate, yet this single agent can significantly reduce anti-drug Abs and alloantibody responses.

The mechanism by which methotrexate induces immune tolerance is unclear. As mentioned above, methotrexate is classically known as a dihydrofolate reductase antagonist that is thought to kill proliferating cells by inhibiting purine metabolism and interfering with de novo DNA synthesis (40). It seems unlikely that methotrexate uses this mechanism to induce immune tolerance because methotrexate has a short half-life and is unlikely to be in cells or circulation long enough to actively kill cells 3 and 4 mo following treatment (41, 42). Thus, immune tolerance induction appears to arise from a distinct mechanism, which may only be activated upon brief, low-dose exposure to methotrexate. In fact, some of the effects of methotrexate are not influenced by the addition of folic acid, suggesting that they may arise from alternative mechanisms (reviewed in Ref. 36).

Interestingly, the typical mechanisms of tolerance, such as the induction of regulatory T cells or prolonged effector T cell depletion, which have been associated with prolonged graft survival, do not seem to be enhanced by coadministering mATG with methotrexate, suggesting that each agent may be acting independently. In fact, although T regulatory cells associated with transplantation tolerance (24, 30, 31, 43) are induced by Thymoglobulin and mATG (23, 25, 26, 32, 33) and are responsible for mATG-mediated prolonged graft survival (16), we did not see increases in T regulatory cells with the mATG and methotrexate combination over that of mATG alone. In addition, there was no evidence of enhanced depletion of T cells, and only a modest extension of mATG effects was observed as a result of blockade of anti-drug Ab responses by methotrexate. These data argue against two mechanisms that would be expected to exert such prolonged survival effects in the context of transient treatments and suggest that alternative mechanisms may exist.

Recently, the role of B cell responses and alloantibodies in transplantation has come to the forefront as more evidence demonstrates that these responses contribute to graft rejection, particularly in the chronic setting (44–47). Clinically, there have been reports of effectiveness of B cell-depleting agents, complement blocking agents, or proteasome inhibitors, which block plasma cell Ab production, in transplantation (48–50). It has been suggested that memory B cells and alloantibodies can actually enhance alloreactive T cell priming and prevent tolerance toward the graft in a CD154-independent manner (51, 52). This is also consistent with studies showing that alloantibodies can activate endothelial cells and monocytes to produce proinflammatory cytokines that enhance graft rejection (53). In addition, although B cells and Ab were not found to be necessary for CAV to develop, alloantibodies did contribute to the development of these lesions (54). Although we do not yet know how methotrexate mediates its effects on B cell responses, given the significant contributions of B cells and alloantibodies in transplant rejection, the ability of a short course of methotrexate to block these responses is an important finding.

When considering that methotrexate can potentially affect multiple B cell responses, including the development of anti-drug Abs, the profound synergy with mATG in transplant might not be unexpected given the role of T cells, B cells, and alloantibody responses in transplantation. However, mATG has been combined with transient treatment of several other immunomodulatory drugs, some of which have been shown to interfere with B cell
responses, including cyclophosphamide, mycophenylate mofetil, dexamethasone, CTLA4Ig, and rapamycin. Of this list, rapamycin and CTLA4Ig were the only agents that prolonged graft survival when combined with mATG (Ref. 16; J. Williams, unpublished observations). Rapamycin showed only a modest delay of graft survival when administered with mATG (Ref. 19; J. Williams, unpublished observations), whereas the combination of mATG with CTLA4Ig displayed a significant benefit, with skin grafts surviving to day 50 (16). This survival benefit of the mATG and CTLA4Ig combination was attributed to an enhancement of T regulatory cells through blocking the CD28/B7 costimulatory pathways, which we did not observe in our studies with mATG and methotrexate. Interestingly, CTLA4Ig has also been associated with transient decreases in Ab responses (55, 56), but Ab responses were not evaluated in the transplantation studies evaluating mATG and CTLA4Ig (16). Notably, in our experience with anti-drug Abs against enzyme replacement therapies, Abs return after cessation of CTLA4Ig treatment, which additionally suggests that CTLA4Ig does not provide a long-lived reduction in Ab responses (Refs. 55 and 56; A. Joseph, unpublished observations). Taken together, these data suggest that methotrexate is unique in its ability to control anti-allograft Ab responses as a transient treatment.

Although early studies had suggested that methotrexate can be beneficial in preclinical and clinical transplantation settings (57, 58), methotrexate in transplantation was largely discontinued with the advent of newer drugs. In addition, methotrexate-associated toxicities arising from continued treatment and high-dose regimens have also hindered use. The immune tolerizing protocol of methotrexate described in this paper likely avoids risk of toxicity because of the transient nature of the regimen and the lower doses used. The fact that the immune modulating effect of mATG does not control either Abs responses to itself or alloantibody responses suggests that mATG has minimal effects on B cell responses, even while concomitantly depleting T cells. Conversely, methotrexate appears to primarily affect Ab responses with minimal evidence of cellular depletion of B and T cells or generation of T regulatory cells. Thus, the synergistic activity of the combination could be explained by the tolerogenic activities of the two agents on two separate arms of the immune response that contribute to allograft rejection.


