Transient Low-Dose Methotrexate Generates B Regulatory Cells That Mediate Antigen-Specific Tolerance to Alglucosidase Alfa


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Transient Low-Dose Methotrexate Generates B Regulatory Cells That Mediate Antigen-Specific Tolerance to Alglucosidase Alfa


Biologic drugs, including enzyme-replacement therapies, can elicit anti-drug Abs (ADA) that may interfere with drug efficacy and impact patient safety. In an effort to control ADA, we focused on identifying regimens of immune tolerance induction that may be readily available for clinical use. Data generated in both wild-type mice and a Pompe disease mouse model demonstrate that single-cycle, low-dose methotrexate can be as effective as three cycles of methotrexate in providing a long-lived reduction in alglucosidase alfa-specific ADA. In addition, we show that methotrexate induces Ag-specific tolerance as mice generate similar Ab responses to an irrelevant Ag regardless of prior methotrexate treatment. Methotrexate-induced immune tolerance does not seem to involve cell depletion, but rather a specific expansion of IL-10– and TGF-β–secreting B cells that express Fopx3, suggesting an induction of regulatory B cells. The mechanism of immune tolerance induction appears to be IL-10 dependent, as methotrexate does not induce immune tolerance in IL-10 knockout mice. Splenic B cells from animals that have been tolerized to alglucosidase alfa with methotrexate can transfer tolerance to naive hosts. We hypothesize that methotrexate induction treatment concomitant with initial exposure to the biotherapeutic can induce Ag-specific immune tolerance in mice through a mechanism that appears to involve the induction of regulatory B cells. The Journal of Immunology, 2014, 193: 3947–3958.

Methotrexate was first described in 1948 by Farber and Diamond (1) as a folate antagonist capable of treating acute leukemia in children. Since then, methotrexate has been a widely used therapy for a number of diseases such as neoplasms and autoimmunity (2). In oncology, high-dose courses of methotrexate are used to kill proliferating, neoplastic cells (3). In autoimmune diseases, such as rheumatoid arthritis and psoriasis, continuous, low-dose methotrexate can successfully treat disease (4–8). The mechanism by which continuous low-dose methotrexate can control autoimmune disease most likely involves the killing of proliferating cells through folate antagonism (6, 7, 9), although additional mechanisms may contribute. For instance, methotrexate has been associated with neutrophil chemotaxis, apoptosis, and the inhibition of IL-1, IL-6, TNF-α, IL-4, IL-13, IFN-γ, and GM-CSF (10–15), which may play a therapeutic role (15). Also, methotrexate has been shown to increase adenosine release (15, 16), a molecule suggested to mediate the effects of regulatory T cells and hamper immune responses (17, 18).

A continuous, immunosuppressive regimen of methotrexate has been associated with reduced titers of Abs that can develop against therapeutic proteins (9). One example has been described with coadministration of weekly methotrexate treatment and antitumor necrosis factor (TNF) agents, such as infliximab, to provide additive treatment effects (19–23). In such cases, not only are inflammatory responses against diseased tissue reduced, but also the development of Abs against the anti-TNF agent (9, 19–23). Anti-drug Abs (ADA) that develop against anti-TNF agents have been shown to reduce treatment effects in rheumatoid arthritis by decreasing the exposure of the anti-TNF agent (9, 24). Therefore, reduced ADA by methotrexate can provide significant clinical benefit (25, 26).

Our laboratory previously demonstrated that a low-dose induction regimen of methotrexate can reduce ADA responses against different protein therapies in mice and induce immune tolerance in normal and disease settings (27–29). Low-dose methotrexate induction treatment was subsequently included in a clinical immune tolerance protocol that coadministered the B cell–depleting agent, Rituximab, with optional IVIG (30–32). The dose of methotrexate used in mice and in patients is considered to be low, as the human equivalent dose is 0.4 mg/kg s.c. in infants. For a 5 kg infant, this would equate to 6 mg methotrexate per week. In a 70 kg adult patient, the human equivalent oral dose of methotrexate is 7 mg, which for three doses would equate to 21 mg methotrexate in 1 wk. These doses are within the dosing range of rheumatoid arthritis treatment (5–25 mg/week) and well below the doses used for oncology treatment (6). This combination treatment successfully reduced ADA and induced immune tolerance to recombinant human acid–α-glucosidase (rhGAA, alglucosidase alfa) in a subset of infantile-onset Pompe patients who experience poor clinical outcomes associated with ADA (30–32). Importantly, this approach is associated with improved clinical outcomes within this patient population (30–32). Rituximab, however, induces a general, long-term B cell depletion and is not globally available. Therefore, the use of methotrexate alone to induce immune tolerance could provide a more tolerable and readily available means of controlling ADA in patients with a lower risk of immunogenicity effects.
The mechanism by which a low-dose induction regimen of methotrexate could induce immune tolerance remains to be discovered. Murine studies were initiated to investigate the mechanism of methotrexate-induced immune tolerance. The studies reported in this work demonstrate that low-dose induction treatment of methotrexate can increase B cell subsets that express IL-10, TGF-β, and Foxp3. Moreover, adoptive transfer of purified B cells from methotrexate-tolerized mice appears to transfer tolerance to naïve hosts. These results suggest a novel function of methotrexate in inducing regulatory B cells to help mediate immune tolerance.

Materials and Methods

Animals, rhGAA, murine antithymocyte globulin, anti–TGF-β, and methotrexate treatment

C57BL/6j (Jackson ImmunoResearch Laboratories), E4-8GAA knockout (KO) (Supplemental Fig. 1; Genzyme, a Sanofi company), or B6.129P2–C57BL/6J (Jackson ImmunoResearch Laboratories) mice, between 8 and 12 wk, were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals, under the American Association for Accreditation of Laboratory Animal Care accreditation. All animal procedures were approved by the Institutional Animal Care and Use Committee. rhGAA (Genzyme, a Sanofi Company), 20 mg/kg, was given by tail vein injection once or weekly for up to 24 doses. All rhGAA-treated mice were treated prophylactically with 5–30 mg/kg diphencypramine (Westward Pharmaceutical, Eatontown, NJ) i.p. prior to rhGAA treatment. Methotrexate (Calbiochem catalog 454125), 5 mg/kg, was delivered by i.p. injection as either a single cycle or three cycles. A single cycle of methotrexate represents three consecutive daily doses (0, 24, and 48 h) of 5 mg/kg initiated within 15 min of the first rhGAA treatment at time 0. Animals that received three cycles of methotrexate were administered each cycle with the first three weekly rhGAA treatments. Anti–TGF-β, a mouse IgG1 mAb (1D11; Genzyme, a Sanofi Company), or murine isotype control Ab (13C4; Genzyme, a Sanofi Company) was administered to mice at 5 mg/kg i.p., weekly, every other day, through the end of study.

Serum anti-rhGAA–specific Ab titers

rhGAA-specific IgG was measured by ELISA. Briefly, 96-well plates (Corning, Corning, NY) were coated overnight with 5 mg/ml rhGAA in sodium phosphate buffered saline (pH 5.0). After blocking with 0.1% BSA in PBS, serial dilutions of serum were added in duplicate to rhGAA-coated plates and incubated at 37°C for 1 h. The plates were washed, and HRP-conjugated goat anti-mouse IgG secondary Ab (Southern Biotechnology Associates, Birmingham, AL) was added and allowed to incubate for 1 h at 37°C. Following a final wash, 3,3',5,5'-tetramethylbenzidine substrate (KPL, Gaithersburg, MD) was added and allowed to develop for 15 min at room temperature. 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). Endpoint titers were defined as the reciprocal of the sample dilution resulting in an absorbance value of 0.2 using Softmax software (Molecular Devices).

Serum anti–murine antithymocyte globulin Ab titers

The levels of anti-drug IgG in mouse serum were analyzed by ELISA. Murine antithymocyte globulin (mATG) is raised in rabbits; thus, Ab responses are assessed using a specific rabbit IgG ELISA. Briefly, 96-well plates (Corning) 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 washed and signal was detected by first adding HRP-conjugated goat anti-mouse 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). Endpoint Ab titers were defined as the lowest dilution above an absorbance of 0.1 using Softmax software (Molecular Devices).

Cell preparation, B cell purification, and adoptive transfer

Animals were sacrificed at various times after initiation of treatment. Spleen, mesenteric, and inguinal lymph node were collected for flow cytometric analysis of T and B cell subsets, and sera were collected for ELISA. Spleens were processed between glass slide, and RBC were lysed with lysing buffer purchased from BD Biosciences (catalog 555899), according to manufacturer’s instructions, and washed in PBS containing 0.5% FCS. Lymph nodes were processed similarly. Cells were resuspended in 200 μL PBS containing 4% FBS and 25 μg/ml total mouse IgG and blocked for 30 min at 4°C. Approximately 3 million spleen cells and 1 million lymph node cells were stained with different Ab cocktails, as described, and analyzed with a high throughput sampler on a BD Biosciences CANTOII flow cytometer. A total of 75,000–100,000 live cell events (based on 7-aminoactinomycin D [7AAD] positivity) was acquired within the lymphocyte gate. For studies requiring B cell purification, spleens were prepared in single-cell suspension, as described above, and loaded onto the RoboSep (StemCell Technologies) instrument, according to the manufacturer’s instructions, and subjected to B cell–negative selection. Purified B cells (≥90%; verified by flow cytometry) were seeded at 500,000 cells/well in 96-well round-bottom plates (Costar catalog 3799), incubated in culture medium RPMI 1640, 10% FCS, penicillin/streptomycin) for 2 d, and stained for various B cell subsets and cytokine expression. For adoptive transfer, purified B cells were injected at various cell concentrations by i.v. administration.

Flow cytometry

Anti-mouse Ab cocktails consist of PE-CD21/35 (catalog 552975), FITC-CD23 (catalog 553138), PE-CD13 (catalog 553714), PE-CD127 (catalog 552543), allophycocyanin-Cy7-CD19 (catalog 557655), FITC-CD43 (catalog 553270), PE-Cy7-CD4 (catalog 552775), FITC-CD3 (catalog 553602), allophycocyanin-CD11b (catalog 553124), PE-Cy7-CD4 (catalog 552867), PE-Cy7-CD11b (catalog 552850), PE-CD93 (catalog 558039), allophycocyanin-CD69 (catalog 560689), PE-Cy7-CD24 (catalog 560536), FITC-CD1d (catalog 553845), allophycocyanin-CD5 (catalog 550035), and PerCP-Cy5-7-1AD (catalog 559925), all purchased from BD Pharmingen. FITC-Foxp3 intracellular staining kit (catalog 11-7775) was purchased from eBioscience. Pacific Blue (PB)-CD25 (catalog 100220), PB-CD23 (catalog 101616), and PB-CD86 (catalog 105022) were purchased from BioLegend. Fluorochrome-matched and isotype-matched controls were used to help define the gating strategy (Supplemental Fig. 3). Samples were run on a BD FACS Canto II and acquired using BD FACSDiva software. The analysis of lymphocyte subset and phenotypes was performed with FCS express version 3 software provided by Novo Software. Percentages of each cell population within the live, lymphocyte gates were generated with the batch-processing option, and absolute numbers were calculated according to the cell counts obtained. Spleen and lymph node cell counts were obtained with a Beckman Coulter Vi-cell XR cell viability analyzer, according to the manufacturer’s instructions. The cell types assessed include total and activated CD4+ T cells (CD4+CD69+), CD8+ T cells (CD8+CD69+), regulatory T cells (CD4+CD25+Fasp3+, CD69−), follicular B cells (CD19+IgMlow/highCD23−/+, total and activated marginal zone B cells (CD19+CD23+CD69−), total CD19+CD1d−/−CD5−/−CD86−/− B cells, total and activated transitional 1 (CD19+CD23+城际hiCD5+CD86+l), transitional 2 (CD19+CD1d−/−CD5−/−CD86−), and transitional 3 (CD19+IgM−CD23+CD86+) B cells (33, 34).

In vitro and cytokine analysis

C57BL/6 (Jackson ImmunoResearch Laboratories) or E4-8GAAKO (Genzyme, a Sanofi company) mice, 8–12 wk old, were given methotrexate (Calbiochem catalog 454125), 5 mg/kg, by i.p. injection for 1 cycle (3 consecutive daily doses) commencing with i.v. rhGAA 20 mg/kg treatment. Animals were sacrificed on day 6 or 7 post-rhGAA initiation, spleens on the mouse surface were prepared fresh (as previously described), and suspended and loaded onto the RoboSep (StemCell Technologies) instrument, according to the manufacturer’s instructions, and subjected to B cell–negative selection. Purified B cells were seeded at 500,000 cells/well in 96-well round-bottom plates (Costar catalog 3799) and incubated with no stimulation. Cells were allowed to incubate for at least 48 h at 37°C. Samples were transferred to V-bottom wells (USA Scientific catalog 551128), and spun at 500,000 rpm at 4°C for 5 min at 4°C. Cells were then resuspended in 100 μL PBS containing 4% FBS and 25 μg/ml total mouse IgG and blocked for 30 min at 4°C. Plates were spun again and resuspended in 90 μL PBS with 2% FCS with addition of 10 μM Ab mixture, as described above, and incubated for 20 min at 4°C with addition of 5 μL 7AAD for the last 10 min of the staining procedure. Addition of 100 μL buffer to the samples with subsequent spin sufficed as a wash; samples could be resuspended in buffer for surface analysis of protein and immediate acquisition or resuspended in Fix/Perm (eBioscience catalog 11-5773) for intracellular staining of IL-10 (BioLegend catalog 550080), TGF-β (latent-associated peptide [LAP]/TGF-β1; BioLegend catalog 141404),
and Foxp3 (eBioscience catalog 11-5773-82), according to the manufacturer’s instructions. Calculation of cell number was obtained with the addition of 50 μL CountBright absolute counting beads (Molecular Probes catalog C36950). All samples were acquired and analyzed, as described above.

**Results**

**Single cycle and three cycles of methotrexate similarly reduce anti-rhGAA IgG titers**

To investigate whether ADA titers against rhGAA could be reduced with a brief course of methotrexate in Pompe mice, anti-rhGAA–specific IgG titers were assessed after single or three cycles of methotrexate in mice with 12 weekly i.v. treatments of 20 mg/kg rhGAA in E4-8GAAKO mice (a model of Pompe disease; Supplemental Fig. 1). A single cycle of methotrexate represents three consecutive daily doses of 5 mg/kg (at 0, 24, and 48 h) initiated within 15 min of the first rhGAA treatment at time 0. Animals that received three cycles of methotrexate were administered each cycle along with the first three weekly rhGAA treatments. Both induction regimens similarly reduced anti-rhGAA IgG titer (Fig. 1). Subsequently, rhGAA treatment was halted for 4 wk, and the animals were then rechallenged. Anti-rhGAA IgG responses were not increased in methotrexate-treated mice as they were in rhGAA-alone–treated mice. The overall reduction of rhGAA-specific IgG by single- and three-cycle methotrexate compared with rhGAA alone was 78% (p = 0.014) and 71% (p = 0.025), respectively, as measured by area under the curve (AUC) (Fig. 1). No statistical difference was observed between the two different regimens of methotrexate.

**Methotrexate-induced immune tolerance appears to be Ag specific**

The ability of a single cycle of methotrexate to induce Ag-specific tolerance to rhGAA was investigated. Wild-type (WT), C57BL/6 mice were treated weekly with 20 mg/kg i.v. rhGAA for 26 wk. A subset of animals was dosed with a single cycle of 5 mg/kg methotrexate. Starting at week 12, each group of animals was also treated monthly with mATG (5 mg/kg, i.p.). Monthly treatments of mATG in C57BL/6 mice generate robust Ab responses (29), and thus enabled our investigation of the Ag-specific nature of methotrexate-induced immune tolerance. Methotrexate reduced anti-rhGAA–specific titers by 78% as measured by AUC (Fig. 2A). Irrespective of methotrexate treatment, both groups of animals generated similar anti-mATG IgG responses (Fig. 2B). These data suggest that animals tolerated to one Ag with a single cycle of methotrexate remain fully responsive to subsequent challenge with other Ags.

**B cell subpopulations are enriched in methotrexate-treated mice**

To investigate how the induction regimen of methotrexate generates immune tolerance, the absolute numbers of various lymphocyte subsets from C57BL/6 WT and E4-8GAAKO mouse spleen and lymph nodes were assessed by flow cytometry. Included in the subset analysis were total and activated CD4+ T cells (CD4+CD69++), total and activated CD8+ T cells (CD8+CD69++), total and activated natural T regulatory cells (CD4+CD25+Foxp3+CD69+++), and total and activated B cells (CD19+CD86++). No significant, reproducible changes were observed in the numbers of total or activated CD4+, CD8+, or T regulatory cells from the lymph node and spleen days 4–8 following rhGAA treatment on day 1 between animals treated with and without methotrexate (data not shown; Supplemental Fig. 2). On days 7 and 8, significant increases were observed in the absolute numbers of activated, splenic B cells isolated from WT animals treated with rhGAA plus methotrexate when compared with B cells isolated from animals treated with rhGAA alone (Fig. 3A). Similarly, the numbers of activated B cells in rhGAA plus methotrexate-treated E4-8GAAKO mice were significantly increased by ~60% when compared with mice treated with rhGAA alone (Fig. 3B). In both WT and E4-8GAAKO mice, activated B cells were not expanded in the lymph node (data not shown). In contrast, no significant differences were observed between rhGAA-treated and saline-treated mice or between saline- and methotrexate-alone–treated mice (Fig. 3B).
Additional B cell subsets were then investigated that included follicular, marginal zone, transitional 1, transitional 2, transitional 3, and B10-like (CD1d-high CD5+) B cells (Table I). The activation status of most of these subsets was assessed by CD86 expression. The B10-like B cells, activated transitional 2 B cells, and activated follicular B cell subpopulations isolated from the spleens of animals co-treated with rhGAA plus methotrexate were consistently and significantly increased in cell number by an average of 50, 70,
and 70%, respectively, when compared with animals treated with rhGAA alone (Fig. 3D). No increases were observed between saline-treated and rhGAA alone–treated mice or between saline-treated and methotrexate only–treated mice in any of the populations examined (Fig. 3D).

Methotrexate treatment increases the numbers of CD1d<sup>high</sup>CDS<sup>+</sup>B cells that are IL-10, TGF-β, and Foxp3 positive

IL-10 expression was assessed in CD1d<sup>high</sup>CDS<sup>+</sup>B cells isolated from mice treated with rhGAA plus methotrexate or rhGAA alone in three independent experiments to determine whether methotrexate induces the expansion of B10 cells. B10 B cells are regulatory IL-10–secreting CD1d<sup>high</sup>CDS<sup>+</sup>B cells (35, 36). Specifically, splenic B cells were purified from WT animals 7 d after treatment, cultured without any stimulation for 2 d, and then analyzed by flow cytometry. A subset of CD1d<sup>high</sup>CDS<sup>+</sup>B cells from animals treated with rhGAA alone appears to express IL-10. The numbers of IL10<sup>+</sup>CD1d<sup>high</sup>CDS<sup>+</sup>B cells isolated from rhGAA plus methotrexate–treated mice were significantly increased by an average of 70% over those isolated from animals treated with rhGAA alone (Fig. 4A) across three experiments in WT mice. In addition, the total mean fluorescence intensity (MFI) of IL-10 in CD1d<sup>high</sup>CDS<sup>+</sup>B cells obtained from methotrexate–treated mice was also significantly increased by an average of 33% across three separate experiments (Fig. 4D).

TGF-β and Foxp3 are additional proteins that have been associated with immunosuppressive effects of regulatory B cells (37–39). The expression of these proteins in CD1d<sup>high</sup>CDS<sup>+</sup>B cells was investigated by flow cytometry, as described above. CD1d<sup>high</sup>CDS<sup>+</sup>B cells isolated from rhGAA–treated mice appear to show TGF-β, specifically the propeptide LAP/TGF-β1, and Foxp3 expression in a small subset of the population. In contrast, mice treated with rhGAA plus methotrexate appear to consistently exhibit an average 70% increase in numbers of both TGF-β<sup>+</sup>CD1d<sup>high</sup>CDS<sup>+</sup>B and Foxp3<sup>+</sup>CD1d<sup>high</sup>CDS<sup>+</sup>B cells (Fig. 4B, 4C). Moreover, the overall expression levels of TGF-β and Foxp3, as measured by MFI, within CD1d<sup>high</sup>CDS<sup>+</sup>B cells were significantly increased by 42 and 33%, respectively, in CD1d<sup>high</sup>CDS<sup>+</sup>B cells cultured from rhGAA plus methotrexate–treated mice as compared with rhGAA alone–treated mice within each and among all three experiments (Fig. 4E, 4F).

Methotrexate treatment increases the numbers of transitional 2 and follicular B cells that are IL-10, TGF-β, and Foxp3 positive

Because methotrexate treatment appeared to influence the expression of IL-10, TGF-β, and Foxp3 in CD1d<sup>high</sup>CDS<sup>+</sup>B cells, we investigated whether methotrexate induced similar effects in

![Table I. B cell subsets assessed by flow cytometry](http://www.jimmunol.org/)

Methotrexate treatment influences the expression of IL-10, TGF-β, and Foxp3 in CD1d<sup>high</sup>CDS<sup>+</sup>B cells, allowing us to investigate the effects on the expression of these proteins. The table below lists the B cell subsets assessed by flow cytometry.

<table>
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<tr>
<th>B Cell Subset Name</th>
<th>Surface Phenotype Assessed</th>
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<tr>
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<td>CD19&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;+&lt;/sup&gt;/IgD&lt;sup&gt;+&lt;/sup&gt;/CD23&lt;sup&gt;+&lt;/sup&gt;</td>
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<sup>a</sup>Activation status of these cell types was assessed by CD86 expression.

<sup>b</sup>These cells are termed B10-like because initially we did not assess IL-10 expression.
transitional 2 and follicular B cells. The number of IL-10+ transitional 2 B cells increased significantly and consistently by an average of 45% in mice treated with rhGAA plus methotrexate when compared with animals treated with rhGAA alone in three individual experiments (Fig. 5A). The total MFI of IL-10 expression in transitional 2 B cells appears to be enhanced in methotrexate-treated mice (by 43%), although the level of increase is variable between experiments (Fig. 5D). Analogous increases in cell numbers were observed with TGF-β+ and Foxp3+ transitional 2 B cells (40 and 50%, respectively), and these increases were significant across three independent experiments (Fig. 5B, 5C). The average MFI of TGF-β+ and Foxp3+ also was increased (by 46 and 52%, respectively; Fig. 5E, 5F). These increases were generally significant in each individual experiment, but the level of increase varied among the three experiments. Moreover, the increase in total TGF-β expression in transitional 2 B cells was less consistent, as it was significant in only two of the three studies.

In three separate experiments, IL-10, TGF-β, and Foxp3 were observed in ~1% of follicular B cells obtained from rhGAA-treated mice. The numbers of IL-10+ follicular B cells, TGF-β+ follicular B cells, and Foxp3+ follicular B cells were significantly expanded with methotrexate treatment by an average of 55, 57, and 72%, respectively, across three experiments (Fig. 6A–C). The most variability across experiments was observed with increases in the numbers of Foxp3+ follicular B cells (Fig. 6C). A significant shift in MFI for these proteins was not observed within the total follicular B cell population. However, when activated CD86+ follicular B cells were analyzed, we observed slight but significant increases in the MFI of IL-10, TGF-β, and Foxp3 in cells obtained from mice treated with rhGAA plus methotrexate compared to animals treated with rhGAA alone. Specifically, the MFI of IL-10, TGF-β, and Foxp3 was increased by 32, 34, and 40% respectively (Fig. 6D–F).

**IL-10, but not active, secreted TGF-β, appears to be necessary for methotrexate-induced immune tolerance**

Because IL-10 has consistently been described as a critical mediator of B regulatory cell function (35), we assessed whether IL-10 was required for methotrexate-induced immune tolerance to rhGAA. Both WT C57BL/6 mice and IL-10KO mice of the same genetic background were treated weekly with 20 mg/kg rhGAA, i.v., with and without single cycle methotrexate. Anti-rhGAA–specific IgG titers were assessed biweekly by ELISA on weeks 4, 6, and 8. The C57BL/6 WT mice responded as expected with an 80% reduction in AUC of titers over time in rhGAA plus methotrexate–treated mice compared with rhGAA alone–treated mice (Fig. 7A). In contrast, no reduction in anti-rhGAA–specific titers was observed in IL-10KO mice treated with rhGAA plus methotrexate when compared with those treated with rhGAA alone (Fig. 7B). Expectedly, the anti-rhGAA titer values were lower in the IL-10KO mice than in the WT mice. IL-10 is also known to promote plasma cell differentiation and Ab secretion (40, 41). Therefore, these studies were initiated with the expectation that

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**FIGURE 5.** Methotrexate treatment increases the numbers of transitional 2 B cells that are IL-10, TGF-β, and Foxp3 positive. B cells were purified from splenocytes isolated and processed from C57BL/6 animals treated with either rhGAA alone or in combination with single-cycle methotrexate, as described above. B cells isolated from each treatment group were pooled and cultured in replicates of six without stimulation for 2 d. Flow cytometry was then performed on each replicate to assess the presence of IL-10, TGF-β, and Foxp3 in transitional 2 B cells, as described in Materials and Methods. Representative dot plots are shown for each population assessed from rhGAA and rhGAA plus methotrexate–treated animals. The average total number of IL-10+ transitional 2, TGF-β+ transitional 2, and Foxp3+ transitional 2 B cells obtained in three independent experiments is graphed, with each character representing the average number for each experiment (A–C). Representative histograms IL-10, TGF-β, and Foxp3 positive transitional 2 B cells are depicted, and the average MFI observed in three independent experiments are graphed (D–F). In addition to the p values noted on the figure, significance is represented as *p≤0.05, **p≤0.01.
anti-rhGAA–specific IgG titers would be lower in the IL-10KO mice, but not too low to evaluate the effect of methotrexate. As demonstrated in Fig. 7D, single-cycle methotrexate can successfully reduce titers when maximal titer values in a study are in the range of $2\times10^3$ to $5\times10^4$, which is within the range observed in the IL-10KO mice treated with rhGAA.

After observing that methotrexate could not control titers in IL-10KO mice, we evaluated the numbers of splenic IL-10+ B10 B cells, TGF-β+ B10 B cells, and Foxp3+ B10 B cells in C57BL/6 WT mice and IL-10KO mice that were treated with rhGAA alone or rhGAA plus methotrexate. Not surprisingly, the numbers of IL-10+ B10 B cells in the IL-10KO mice were not increased with methotrexate treatment as compared with WT mice (Fig. 7C). However, the numbers of TGF-β+ B10 B cells and Foxp3+ B10 B cells were similarly increased in IL-10KO mice and C57BL/6 WT mice (Fig. 7C).

The impact of TGF-β inhibition on anti-rhGAA–specific IgG titer by methotrexate-induced immune tolerance was assessed by administering a TGF-β–neutralizing murine IgG1 mAb, 1D11. The 1D11 neutralizes all active, secreted forms of TGF-β. The 1D11 or its isotype control Ab (13C4) was administered three times per week throughout the course of the study. The 1D11 treatment had no impact on the ability of methotrexate to induce immune tolerance to rhGAA (Fig. 7D).

**FIGURE 6.** Methotrexate treatment increases the numbers of follicular B cells that are IL-10, TGF-β, and Foxp3 positive. B cells were purified from splenocytes isolated and processed from C57BL/6 mice treated with either rhGAA alone or in combination with single-cycle methotrexate, as described above. Cells isolated from each treatment group were pooled and cultured in replicates of six without stimulation for 2 d. Flow cytometry was then performed on each replicate to assess the presence of IL-10, TGF-β, and Foxp3 in follicular B cells, as described in Materials and Methods. Representative dot plots are shown for each population assessed from rhGAA and rhGAA plus methotrexate–treated animals. The average total number of IL-10+ follicular, TGF-β+ follicular, and Foxp3+ follicular B cells obtained in three independent experiments is graphed, with each character representing the average number for each experiment (A–C). Representative histograms IL-10, TGF-β, and Foxp3 in activated (CD86+) follicular B cells are depicted, and the average MFI observed in three independent experiments are graphed (D–F). In addition to the $p$ values noted on the figure, significance is represented as *$p<0.05$.

Splenoc B cells isolated from mice tolerized to rhGAA by methotrexate transferred tolerance to naïve animals

Adoptive transfer studies were performed to assess whether total splenic B cells, which include B10, activated transitional 2, and activated follicular B cells, could transfer tolerance to naïve hosts (Fig. 8). Total splenic B cells were therefore purified from donor mice, 7 d after treatment with either rhGAA alone or rhGAA plus methotrexate. Following negative B cell separation, donor B cells were 94–95% pure (Fig. 8B). Five million B cells from rhGAA alone–treated mice were transferred to naive recipient mice. Recipients of B cells purified from rhGAA plus methotrexate–treated mice were administered between 500,000 and 5 million B cells. Following transfer, the recipient mice were treated weekly with 20 mg/kg rhGAA i.v. Anti-rhGAA IgG titers were then assessed biweekly through 16 wk. The highest average titer was observed in animals that received B cells from rhGAA alone–treated donor mice. These titers were similar in range to those reported through week 16 for naive C57BL/6 mice treated with weekly rhGAA (Fig. 8A). Recipient mice of B cells from rhGAA plus methotrexate–treated donor mice exhibited the lowest anti-rhGAA–specific IgG titers with a percent reduction in AUC ranging from 88 to 99% (Fig. 8C). Recipients of B cells from rhGAA alone–treated donor mice also exhibited little variability in titer among the animals within each of the four recipient groups (Fig. 8C). Of note, two of the eight animals that received splenic B cells from rhGAA alone–treated mice did not mount an Ab response to rhGAA, and one animal exhibited a very low anti-rhGAA IgG titer. The reason for this lack of response is unclear. These animals were not included in the data presented in Fig. 8C. Statistical significance was conservatively assessed by removing the two animals with no response and comparing the AUC difference between the recipients of 5 million nontolerized cells and...
recipients of 5 million tolerized cells ($p = 0.02$), recipients of 2 million tolerized cells ($p = 0.008$), recipients of 1 million tolerized cells ($p = 0.01$), and recipients of 500,000 tolerized cells ($p = 0.01$). When all eight recipient mice of rhGAA alone–treated B cells were included in the statistical comparison, the $p$-value range was 0.052–0.1, in which the comparison between the recipient mice of 5 million nontolerized and 2 million tolerized mice was the closest to reaching statistical significance.

A schematic representation of the proposed effects of low-dose methotrexate induction treatment on antibody responses is shown in Fig. 9.

**Discussion**

These studies expand our understanding of the function of methotrexate. Methotrexate has classically been described as a dihydrofolate reductase antagonist (2). Folate inhibition by methotrexate interferes with purine metabolism and DNA synthesis, and thus induces death in proliferating cells (2). Through this mechanism, methotrexate has served as a successful oncology therapy for many years. Continuous, low-dose, weekly administration of methotrexate has been associated with a similar proposed mechanism of action in the treatment of rheumatoid arthritis (7, 9), although other mechanisms of methotrexate could also contribute in this setting (14, 15). Cytokine inhibition, apoptosis, adenosine release, and chemotaxis may be additional means by which methotrexate may modulate inflammation (10–13, 15, 16).

Recently, low-dose, methotrexate induction treatment has been shown to induce immune tolerance to protein therapies in several different mouse models (27–29). In the context of murine-specific antithymocyte globulin (mATG), a single cycle of three daily doses of methotrexate at the initiation of monthly mA TG treatment more effectively reduced ADA and enhanced mA TG pharmacodynamic activity than three consecutive cycles of methotrexate (29). Our present investigation of single- versus three-cycle methotrexate in rhGAA tolerance demonstrated equivalent ADA reduction by both regimens. Importantly, reduced Ab titers were similarly maintained through rhGAA rest and rechallenge, suggesting durable immune tolerance with methotrexate induction treatment. In addition, regardless of whether rhGAA-treated mice received methotrexate or not, both sets of animals were able to
produce comparable responses against a different Ag, mA TG. Therefore, methotrexate can induce Ag-specific tolerance when administered as a single cycle.

Although these results support the utility of methotrexate in immune tolerance induction, they do not explain the mechanism by which methotrexate exerts such effects. Because methotrexate treatment has been associated with the death of activated, proliferating cells, as well as with apoptosis induction (15), we investigated whether cell numbers were altered following methotrexate treatment. Specifically, T and B cell subsets within spleen and lymph nodes were assessed as these populations would be activated upon rhGAA treatment to enable B cell differentiation into Ab-secreting plasma cells. In addition, T regulatory cell numbers were investigated because methotrexate has been described to induce adenosine, and adenosine has been associated with T regulatory cell function (16–18). No differences were observed in the total numbers of Th, T cytotoxic, T regulatory, or B cells between mice treated with rhGAA alone or with rhGAA plus methotrexate in either tissue. These results were expected because only a small percentage of total T and B cells should be responsive to rhGAA, and minor shifts in cell numbers could be easily masked within assessments of the total populations. Because methotrexate specifically targets activated cells rather than resting cells (15), the numbers of activated Th, T cytotoxic, T regulatory, as measured by CD69 expression, and activated B cells, as measured by CD86 expression, were investigated. No changes in the cell numbers of these populations were observed in the lymph nodes. Interestingly, rhGAA plus methotrexate induced significant increases in the absolute numbers of activated splenic B cells, whereas no changes were observed in any activated T cell population. The increases observed in the numbers of activated B cells were consistent among both WT and E4-8GAKO mice, and the timing of the increase was a bit faster in the E4-8GAKO mouse. There were no significant differences between saline-treated mice and rhGAA alone–treated mice, nor between the control groups, saline-treated mice, and methotrexate only–treated mice.

To further understand the effect of methotrexate on B cells, different splenic B cell subsets were examined. These included follicular, marginal zone, transitional 1, transitional 2, transitional 3, and B10-like B cells. These peripheral subsets represent fully mature B cells, B cells transitioning from immaturity to maturity, as well as regulatory B cells (35, 42). Of these subsets, significant increases in the cell numbers of activated follicular, activated transitional 2, and B10-like B cells were reproducibly observed in animals treated with rhGAA plus methotrexate when compared with animals treated with rhGAA alone. CD1d<sup>hi</sup>CD5<sup>-</sup> B cells that secrete IL-10 have been described in both mouse and humans as B10 regulatory B cells (35, 36). B10 B cells have been implicated in controlling immune responses in murine disease settings that include, but are not limited to experimental autoimmune encephalomyelitis (43), scleroderma-tous graft-versus-host disease (44), and chronic intestinal inflammation (45). In addition, B10 B cells have been shown to reduce IgG responses in mice (46). IL-10 secretion has been identified as a critical mediator of B10 B cell regulatory function (35). Therefore, we investigated whether the CD1d<sup>hi</sup>CD5<sup>-</sup> B10-like B cell subset that is increased upon methotrexate treatment expresses IL-10. We observed IL-10<sup>+</sup> CD1d<sup>hi</sup>CD5<sup>-</sup> Cells consistently found significant increases in the numbers of IL-10<sup>+</sup> CD1d<sup>hi</sup>CD5<sup>-</sup> cells in rhGAA plus methotrexate–treated mice when compared with those isolated from rhGAA alone–treated animals. Moreover, the overall expression level of IL-10, as measured by MFI, within the CD1d<sup>hi</sup>CD5<sup>-</sup> population was increased in methotrexate-tolerized mice. Cytokine expression by regulatory B cells is typically measured following in vitro culture with stimulation by LPS or PMA and ionomycin along with monensin treatment (47). Although the increases in IL-10 MFI are ~2-fold, our assessments of IL-10 are conducted in primary B cells cultured for 2 d in the absence of any stimulation, suggesting that rhGAA plus methotrexate is a potent inducer of this response. These data suggest that methotrexate when administered with rhGAA can induce the expansion of B10 B cells, and that B10 B cells may be involved in mediating the immune-tolerizing effects of methotrexate.

In addition to B10 B cells, several other regulatory B cell subsets have been described in mice (48, 49). Some of these subsets appear to express TGF-β, which is linked with their immunosuppressive properties (48, 50–53). Although TGF-β expression has not yet been described for B10 B cells, we evaluated whether TGF-β expression is influenced by methotrexate treatment in B10 B cells. There are three isoforms of TGF-β, as follows: TGF-β1, TGF-β2, and TGF-β3 (54). The predominant form of TGF-β in immune
B cells isolated from rhGAA plus methotrexate–treated mice were rhGAA plus methotrexate treatment. Transitional 2 and follicular Foxp3 were only observed in activated CD86+ follicular B cells. Interestingly, mice treated with rhGAA plus methotrexate exhibited increases in not only the numbers of TGF-β CD1dhighCD5+ cells, but in the MFI of TGF-β within these cells when compared with animals treated with rhGAA alone.

Finally, although Foxp3 expression has been widely demonstrated in T regulatory cells, there is an example in which Foxp3 has been observed in human CD5+CD19+ B cells (55). Moreover, in T cells, TGF-β can induce Foxp3 expression and promote the development of T regulatory cells (56). In addition, T regulatory cells can express Foxp3, IL-10, and TGF-β, and these proteins are associated with immunosuppression (57). We investigated whether we could observe Foxp3 expression in methotrexate-induced CD1dhighCD5+ cells. Interestingly, mice treated with rhGAA plus methotrexate exhibited significant increases in the numbers of Foxp3+CD1dhighCD5+ B cells. In addition, the expression level of Foxp3 in this cell subset was increased in methotrexate-treated mice.

Because rhGAA plus methotrexate treatment appeared to expand the absolute numbers of two other B cell subtypes that have also been associated with immune regulation, transitional 2 B cells and follicular B cells (58, 59), we decided to evaluate whether these populations expressed the immunosuppressive proteins IL-10, TGF-β, and Foxp3 and whether these subsets were enhanced by rhGAA plus methotrexate treatment. Transitional 2 and follicular B cells isolated from rhGAA plus methotrexate–treated mice were IL-10+, TGF-β+, and Foxp3+. The numbers of these cell subsets were significantly increased in mice treated with rhGAA plus methotrexate when compared with those isolated from animals treated with rhGAA alone. Generally, the expression of these proteins in transitional 2 B cells was increased with methotrexate treatment, although the MFI of TGF-β was only significantly increased in two of the three experiments. In contrast, such a small percentage of total follicular B cells was expanded with methotrexate treatment, shifts in the MFI of IL-10, TGF-β, and Foxp3 were only observed in activated CD86+ follicular B cells.

One open question was whether IL-10 is a critical mediator of tolerance to rhGAA in this treatment paradigm, as has been consistently reported in both human and mouse for B regulatory cell–mediated suppression (35, 36, 60). To assess this, we compared the ability of methotrexate to reduce rhGAA–specific IgG in C57BL/6 WT mice and C57BL/6-derived IL-10KO mice. An 80% reduction in titer was observed in C57BL/6 WT mice, whereas no reduction in rhGAA–specific IgG by methotrexate was observed in IL-10KO mice. These data suggest that IL-10 is a critical mediator of methotrexate-induced tolerance. Upon further evaluation, the numbers of IL-10+ B10 cells in IL-10KO mice were not increased with rhGAA plus methotrexate treatment as observed in C57BL/6 WT mice. In contrast, the numbers of TGF-β+ B10 cells and Foxp3+ B10 B cells were similarly increased in both murine strains.

These data suggest that TGF-β and Foxp3 expression in B10 B cells cannot, independently of IL-10, enable methotrexate-induced immune tolerance.

The role of TGF-β in methotrexate-induced immune tolerance to rhGAA was investigated further. A neutralizing murine IgG1 anti–TGF-β mAb (1D11) that binds to active TGF-β, TGF-β2, and TGF-β3 was used. Alongside rhGAA alone or rhGAA plus methotrexate treatment, 1D11 was given three times per week at 5 mg/kg in a regimen that has demonstrated TGF-β neutralization in mice (61). Two independent studies demonstrated that the inhibition of active TGF-β did not interfere with the ability of methotrexate to control anti-rhGAA–specific IgG, indicating that secreted, active TGF-β may not be an essential mediator of this process.

To investigate whether B cells isolated from methotrexate–tolerized mice can transfer immune tolerance to naive hosts, purified splenic B cells from animals treated with either rhGAA alone or rhGAA plus methotrexate were transferred into naive animals that began weekly rhGAA treatment the same day as cell transfer. Because small populations of B10, follicular, and transitional 2 B cells appeared to be responding to rhGAA plus methotrexate treatment, we decided to assess the effects of all of them by transferring total splenic B cells. Methotrexate–induced changes in B10, transitional 2, and follicular B cells were consistently observed 7 d following rhGAA plus methotrexate treatment in C57BL/6 WT mice; thus, this time point was chosen for harvest and adoptive transfer. In the recipient mice, anti-rhGAA–specific IgG was evaluated every other week for 16 wk. Recipients of 5 million cells isolated from nontolerized mice treated with rhGAA alone displayed the highest average titer. In contrast, recipients of either 5, 2, 1, or 0.5 million cells purified from mice tolerated to rhGAA by methotrexate exhibited lower titers than anti-rhGAA IgG and overall reductions in area under the effect curve ranging from 88 to 99%. The reduction in Ab responses remained throughout the 16-wk study. Moreover, whereas the variability in titer among the individual animals that received B cells from rhGAA alone–treated mice was large, the variability exhibited among animals within each of the four groups that received B cells...
from methotrexate-treated mice was low. The data from these studies collectively suggest that B cells represent a key mediator of methotrexate-induced Ag-specific immune tolerance.

In conclusion, the studies reported in this work identify a novel mode of methotrexate function. Particularly, low-dose induction treatment of methotrexate given together with Ag stimulation expands IL-10+, TGF-β1-, and Foxp3+ B cell populations that appear to control Ab responses and contribute to immune tolerance induction in mice (Fig. 9). Regulatory B cells with similar protein expression profiles have been observed in autoimmune patients, tolerized human transplant patients, and allergy (39, 58, 62–64). Thus, it is conceivable that methotrexate may successfully induce tolerance in humans through a similar mechanism. Moreover, methotrexate treatment in rheumatoid arthritis patients may exert similar effects that may help control disease. Currently, high-risk, infantile Pompe patients who may not respond to enzyme-replacement therapy due to ADA interference might benefit from low-dose methotrexate induction treatment along with Rituximab and optional i.v. IG (30–32). This regimen has successfully tolerized a number of these high-risk patients and has enabled effective response to therapy (30–32). The use of Rituximab can be associated with infection risk and is not available worldwide. The availability of a methotrexate-only tolerance strategy may further enable treatment responsiveness in Pompe patients, including high-risk patients unable to receive Rituximab.

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All of the authors were employed by Genzyme, a Sanofi company, and held some equity in the company while this work was being conducted.

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