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Low-Dose Methotrexate Prevents Primary and Secondary Humoral Immune Responses and Induces Immune Tolerance to a Recombinant Immunotoxin

Emily M. King,*[,1] Ronit Mazor,*[,1] Nicolas Çuburu,[†] and Ira Pastan*

Recombinant immunotoxins (RITs) are chimeric proteins being developed for cancer treatment. They are composed of an Ab fragment that targets a cancer Ag and a cytotoxic portion of *Pseudomonas* exotoxin A. They are effective for patients with hematologic malignancies with defective immunity, but their efficacy against solid tumors is limited by anti-drug Ab (ADA) responses in immune-competent patients. Pre-existing Abs or immune memory owing to previous toxin exposure represent additional hurdles because they induce rapid and strong ADA responses. Here, we evaluated the efficacy of methotrexate (MTX) to prevent ADA formation against the mesothelin-targeting RIT LMB-100 in naive mice and in mice with pre-existing Abs. We found that low-dose MTX combined with LMB-100 completely suppressed the formation of ADAs in a dose- and frequency-dependent manner. Suppression of the immune response restored blood levels of LMB-100 and prevented its neutralization. Furthermore, combination of MTX with LMB-100 did not compromise the immune response against a second Ag given after stopping MTX, indicating specific immune tolerance. Adoptive transfer of splenocytes suppressed Ab responses to LMB-100 in recipient mice, indicating a durable immune tolerance. We conclude that combination of MTX and LMB-100 is effective at preventing immune responses in a durable, Ag-specific manner. We propose combining low-dose MTX in immune-competent cancer patients receiving RIT therapy to prevent immunogenicity. This approach could be applied to other immunogenic therapeutic agents and to proteins for which there is pre-existing immunity. ***The Journal of Immunology*****, 2018, 200: 000–000.**

Recombinant immunotoxins (RITs) are therapeutic proteins composed of a tumor Ag-targeting Ab fragment linked to the cytotoxic domain of *Pseudomonas* exotoxin A. RITs have been effective in clinical trials for patients with hematologic malignancies. The CD22-targeting RIT moxetumomab pasudotox achieved an overall response rate of 86% and a complete remission rate of 46% in patients with relapsed-refractory hairy cell leukemia (1). However, the therapeutic efficacy of RITs against solid tumors is limited by their immunogenicity in immune-competent patients (reviewed in Ref. 2). In clinical trials in mesothelioma patients with SS1P, a RIT targeting mesothelin, 90% of patients developed neutralizing anti-drug Abs (ADAs) against SS1P after one cycle of treatment. When lymphodepleting chemotherapy was combined with SS1P, neutralizing Ab formation was delayed and the overall response rate increased with several major and sustained remissions (2, 3). This finding indicates a high therapeutic potential for RITs against solid tumors, once immunogenicity is surmounted. The current immune-suppressive regimen is toxic to patients and in most cases only allows one cycle of therapy before ADAs develop (4).

To decrease the immunogenicity of the protein, we used protein engineering to create an improved RIT in which the major human B cell epitopes were removed or suppressed and the murine anti-human mesothelin Ab fragment was humanized (5). This second-generation RIT, called LMB-100, shows antitumor activity in animal models and is currently being evaluated in clinical trials for the treatment of mesothelioma and pancreatic cancer (https://clinicaltrials.gov numbers NCT02798536, NCT02810418) (6, 7). However, it is anticipated that immunogenicity will be reduced but not abolished.

Methotrexate (MTX) is a folate antagonist that interferes with purine biosynthesis, and has been used to treat breast and lung cancers, leukemias, lymphomas, and osteosarcomas (8). Due to its anti-inflammatory properties, low-dose MTX is a standard-of-care treatment for autoimmune diseases including rheumatoid arthritis (reviewed in Ref. 9). Combination of MTX with anti-TNF Abs including adalimumab reduces the incidence of ADAs in patients with rheumatoid arthritis, psoriasis, and inflammatory bowel disease (reviewed in Ref. 10, 11). In recent studies MTX was combined with rituximab and bortezomib to successfully prevent the primary ADA response against enzyme replacement therapy for infantile Pompe disease (12). Several mechanisms are thought to contribute to the anti-inflammatory effects of MTX, including release of the anti-inflammatory molecules (13), depletion of activated T cells (14), changes in Ag processing (15, 16), and so on. Recently, Joly et al. (16) demonstrated that low-dose MTX prevented the primary ADA response against human alglucosidase alfa in mice in an Ag-specific manner which was dependent on IL-10. They also demonstrated that low-dose MTX induced immune

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Abbreviations used in this article: ADA, anti-drug Ab; AUC, area under the curve; MTX, methotrexate; RIT, recombinant immunotoxin.

tolerance to murine anti-thymocyte globulin, and reduced alloantibodies, in a murine allograft model (17).

LMB-100 contains a highly immunogenic bacterial protein. Although LMB-100 has been engineered for diminished immunogenicity in humans, it is still highly immunogenic in mice. Previous studies with MTX have employed proteins from mammals, but pathogenic bacterial proteins that can activate both the innate and adaptive immune system (18, 19) have not been evaluated.

Pre-existing immunity against protein therapeutics poses an additional barrier to immunogenicity mitigation, because the secondary response can be very strong and rapid. Many patients have pre-existing immunity to *Pseudomonas* exotoxin A used to make LMB-100 because of environmental exposure (20). The effect of MTX on the secondary immune response has not been experimentally evaluated. Our goal in this study was to evaluate the safety and efficacy of MTX to prevent Ab responses to LMB-100 in naive mice and mice with pre-existing immunity.

Materials and Methods

Compounds

LMB-100 was manufactured by Roche Diagnostics (Mannheim, Germany) as previously described (21) and provided through a Collaborative Research and Development Agreement. MTX injection, USP at 25 mg/ml (clinical grade) (Hospira, Lake Forest, IL) was diluted in PBS to 0.2 mg/ml for injection. OVA was purchased from Sigma (Saint Louis, MO); DNase and collagenase were purchased from Roche Diagnostics.

Mice

Female, wild-type BALB/c mice 8–10 wk of age were acquired from Charles River (Frederick, MD). All mice experiments followed National Institutes of Health guidelines approved by the Animal Care and Use Committee of the National Cancer Institute. Mice were injected with LMB-100 or OVA by i.v. injection unless otherwise noted, and MTX was injected i.p. at 0, 24, and 48 h after each LMB-100 injection for a total of five MTX injections per week. For ADA assays, blood was collected into heparinized tubes. Samples were centrifuged at 3000 rpm for 5 min and plasma was collected and stored at −20°C. Mouse weight was measured twice a week and treatment was withheld if mice experienced weight loss >10% of their initial body weight.

Complete blood counts

Mouse blood was collected via mandibular vein into EDTA-coated tubes. Samples were diluted with 200 μl PBS and blood counts were determined by Siemens ADVIA 120 hematology analyzer in the Department of Laboratory Medicine, National Institutes of Health.

Preparation of splenocytes

Mice were sacrificed 24 h after the final injection for flow cytometry, or 72 h after the final injection for adoptive transfer experiments and B cell isolation. Spleens were harvested into ice-cold HBSS, injected with digestion media (RPMI 1640, 10% heat-inactivated FBS, 1 mM penicillin/streptomycin, 0.1 mg/ml DNase, 0.5 mg/ml collagenase), and incubated at 37°C for 10 min. Spleens were then mechanically dissociated through 70 μm mesh. RBCs were lysed with ammonium-chloride-potassium lysis buffer, and splenocytes were resuspended to desired concentration in RPMI 1640 (for adoptive transfer), media (RPMI 1640, 10% heat-inactivated FBS, 1 mM penicillin/streptomycin) for B cell isolation, or FACS buffer (for flow cytometry).

B cell isolation and IL-10 ELISPOT

Following splenocyte preparation, B cells were isolated using a magnetic bead negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions. ELISPOT plates (Mabtech, Cincinnati, OH) were prewashed and coated overnight with 2 μg/ml mouse IL-10 capture Ab and blocked with media. Purified B cells from each mouse were plated at 300,000 cells per well in quadruplicate and treated with 1 μg/ml LPS. Plates were incubated for 20 h at 37°C. Spots were detected using 1 μg/ml biotinylated secondary Ab, streptavidin–alkaline phosphatase and BCIP/NBT substrate and counted by ImmunoSpot 5.0 image analysis software (Cellular Technology, Shaker Heights, OH).

Flow cytometry

Splenocytes were fixed, washed and stained as previously described (22) using the following Abs from BioLegend (San Diego, CA): CD3 (clone 17A2), CD4 (clone GK1.5), CD8 (clone 53-5.8), CD19 (clone 6D5), B220 (clone RA3, 6B2), CD11c (clone N418), IAIE (clone M5/114.15.2), CD11b (clone M1/70), Ly6G (clone 1A85), and Ly6C (clone HK1.4). Data were collected on a FACS CANTO II flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo version X (Tree Star).

Cell culture and neutralization assay

KLM-1 cells (provided by U. Rudloff, National Cancer Institute) were grown in RPMI 1640 containing 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin. Cell cultures were incubated at 37°C, 5% CO_2. Cells were plated at 5000 cells per well in 96-well flat-bottom plates and incubated for 24 h for neutralization assays. Mouse plasma was diluted 1:50 in assay media and incubated with different concentrations of LMB-100 for 30 min on ice prior to treating cells. Cells were treated in quadruplicate for 72 h. Cell viability was determined by WST-8 cell counting kit (Dojindo Molecular Technologies) as per the manufacturer's instructions. Absorbance readouts at 450 nm were normalized to 0% viability (staurosporine or cycloheximide [Sigma-Aldrich] treatment) and 100% viability (assay media treatment).

ELISA assays

Total anti–LMB-100 and anti-OVA Abs were measured as previously described (23). For Ig isotype, ELISA plates (Thermo Fisher) were coated with 2 μg/ml LMB-100. Plates were blocked and serial dilutions of plasma were incubated for 1 h. Plasma Abs were bound by goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgM isotyping kits at dilutions of 1:3,000, 1:4,000, 1:4,000, 1:3,000, and 1:16,000, respectfully (Sigma) and anti-goat IgG (H+L) HRP (1:15,000) (Jackson ImmunoResearch Laboratories) was used for detection. To quantify plasma LMB-100, ELISA plates were coated with 1 μg/ml human Fc mesothelin (laboratory stock) for 18 h. Plates were blocked with blocking buffer (PBS, 25% DMEM, 25 mM HEPES, 0.5% BSA, 0.1% azide, 5% FBS) for 15 min. Plasma samples or LMB-100 standard (100 μg/ml) were diluted 1:100 in blocking buffer and serially diluted down the ELISA plate. Plates were washed and plasma LMB-100 was bound by 1 μg/ml IP12 mAb (laboratory stock) that binds to LMB-100. Goat anti-mouse IgG (H+L) HRP (1:3000) was used for detection.

Statistical analysis

Graphing and statistical analyses were performed using GraphPad Prism. Mann–Whitney *U* tests were used for comparisons of two nonparametric variables. Titer was interpolated by fitting each sample to a four-parameter curve fit and interpolation of the dilution factor at 50% of max absorbance of IP12 standard.

Results

MTX prevents the formation of ADAs against LMB-100 in a dose-dependent manner

To evaluate the number of cycles of MTX required for sustained tolerance to LMB-100, mice were treated with LMB-100 twice a week for 6 wk and 1 mg/kg MTX for 1, 2, 3, or 6 wk (Fig. 1A). A single cycle of MTX (given 0, 24, and 48 h after each LMB-100 dose during week 1 for a total of five doses) was sufficient to significantly reduce ADA titer by 4-fold (area under curve, Mann–Whitney *U* test, $p = 0.05$) and three cycles suppressed ADA formation as effectively as six cycles of MTX (>20-fold) (area under curve, Mann–Whitney *U* test, $p = 0.28$). Moreover, mice that received three cycles of MTX were challenged with 12 doses of LMB-100 over 17 wk and ADA titers were suppressed by 73% (area under the curve [AUC], Mann–Whitney *U* test, $p = 0.003$) (Supplemental Fig. 1A). This indicates that MTX not only delayed the ADA response but induced long-term suppression of ADAs. We therefore used a three-cycle schedule for tolerance induction in subsequent experiments.

To evaluate the toxicity and efficacy of different doses of MTX, mice were injected with twice-weekly doses of LMB-100 and various doses of MTX at 0, 24, and 48 h after LMB-100 injection

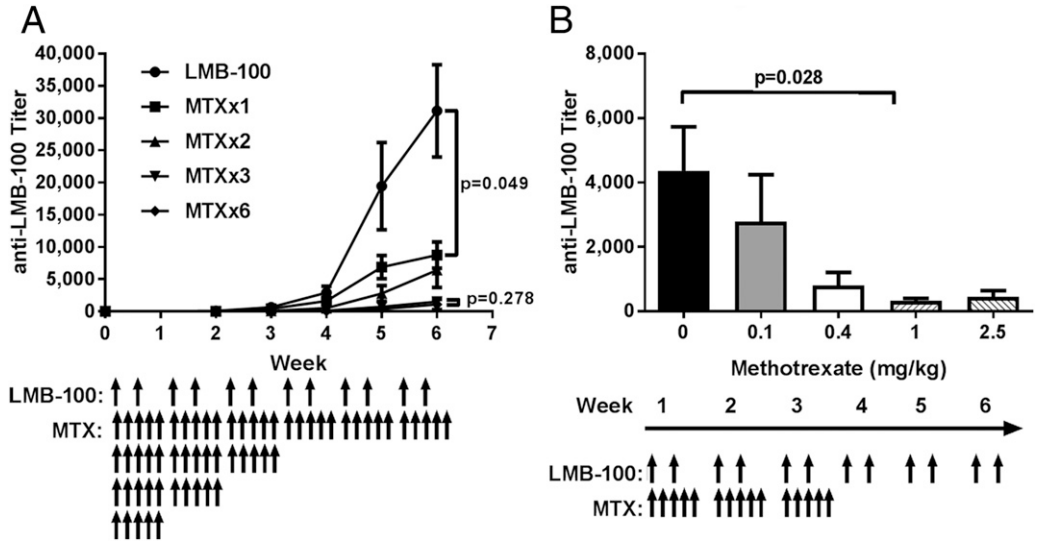

FIGURE 1. MTX prevents the formation of ADAs. (**A**) Female BALB/c mice were immunized with 40 μg LMB-100 i.p. twice per week (days 1 and 3) together with 1 mg/kg MTX i.p., given at 0, 24, and 48 h after LMB-100 for a total of 5 doses per wk. MTX was given for 1, 2, 3, or 6 wk. Plasma was collected and analyzed for anti–LMB-100 Abs by ELISA ($n = 8$). (**B**) Mice were immunized with 20 μg LMB-100 i.v. twice weekly. MTX was given by i.p. injection at the doses indicated for 5 d per wk ($n = 4$). Blood samples were collected in week 6 and analyzed for anti–LMB-100 Abs by ELISA. Titer is reported as dilution factor. Data points are mean, error bars are SEM. Each experiment was repeated at least once.

(Fig. 1B). Mice receiving MTX at 1 mg/kg had a significantly reduced anti–LMB-100 titer at week 6; the titers were reduced from 4366 for LMB-100 treated mice to 324 for mice treated with the combination (Mann–Whitney U test, $p = 0.03$) with no weight loss. In contrast, mice treated with a higher dose of 2.5 mg/kg MTX lost 10% of their starting body weight (Supplemental Fig. 1B). Therefore, a dose of 1 mg/kg was used in all subsequent experiments.

MTX treatment prevents neutralizing Abs and prevents ADA-mediated clearance

To determine whether the decrease in titers measured by ELISA was associated with a decrease in the ability of the Abs to neutralize the cytotoxic activity of the immunotoxin, cytotoxicity assays were performed using plasma from treated mice (Fig. 2A). LMB-100 was incubated with mouse plasma and then applied to KLM-1 cells, and WST-8 was used to assess cell viability. LMB-100 incubated with naive mouse plasma had an average IC_{50} of 1.3 ng/ml, whereas LMB-100 incubated with plasma from mice immunized with LMB-100 was neutralized 67-fold (Mann–Whitney U test, $p = 0.016$) resulting in an average IC_{50} of 87 ng/ml. In contrast, LMB-100 incubated with plasma from mice treated with LMB-100+MTX had an average IC_{50} of 1.2 ng/ml, which is not significantly different from naive mouse plasma (Mann–Whitney U test, $p = 0.9$) (Fig. 2B). This demonstrates that MTX prevents the formation of neutralizing Abs.

We then investigated the effect of ADAs on the concentration of LMB-100 in mouse blood (Fig. 2C, 2D). A group of mice were treated with LMB-100 i.p. in weeks 1–3 and a group of mice also received 1 mg/kg MTX given i.p. at 0, 24, and 48 h after LMB-100 to prevent Ab formation. Mice were then given a single dose of 20 μg LMB-100 i.v. in week 4, and blood samples were collected after 5, 20, 40, 240, and 480 min postinjection to measure LMB-100 concentrations by ELISA. The peak level of LMB-100 in naive mice was 20.3 μg/ml with a mean $t_{1/2}$ of 26.8 min and a mean AUC of 1666. The mean $t_{1/2}$ of LMB-100 in mice treated with LMB-100+MTX was not different at 24.6 min and AUC of 1590 (Mann–Whitney U test, $p = 0.84$ and 0.9 comparing $t_{1/2}$ and AUC, respectively) (Fig. 2C). However, mice previously treated with LMB-100 had a high ADA titer (Fig. 2D) and showed a dramatically lower peak concentration of LMB-100 5 min postinjection of 7.8 μg/ml with a mean AUC of 451 (Mann–Whitney U test, $p = 0.03$) ($t_{1/2}$ could not be accurately calculated because of the rapid clearance of LMB-100 from the blood). This demonstrates that the $t_{1/2}$ of LMB-100 can be maintained by preventing ADAs with MTX.

MTX prevents the memory recall response but does not induce tolerance in mice with pre-existing Abs

Patients with pre-existing Abs to RITs have been excluded from participation in immunotoxin clinical trials (1). We therefore investigated the tolerogenic efficacy of MTX in mice with pre-existing Abs to LMB-100 from previous immunization with LMB-100 (Fig. 3A). Mice were immunized with six doses of 50 μg LMB-100 i.v. on weeks 1 and 3, and rested for 7 wk. On week 11, mice were challenged with three doses of LMB-100, LMB-100 with 1 mg/kg MTX, MTX alone, or PBS. Mice challenged with LMB-100 showed a spike in anti–LMB-100 Ab titers from a mean of 665 at week 9 to 9808 at week 12, characteristic of memory B cell activation in the immune recall response. However, mice challenged with the combination of LMB-100+MTX had a slight decrease in titer, with an average of 665 at week 9 to an average of 371 at week 12 (paired t test, $p = 0.25$) (Fig. 3A). No difference was observed in titers between mice challenged with LMB-100+MTX and mice challenged with PBS (Mann–Whitney U test, $p = 0.3$) (Fig. 3B). Challenge with LMB-100+MTX prevented the recall response.

To determine if these mice could mount an Ab response when MTX was stopped, we gave a second challenge of LMB-100 alone in week 13, which resulted in a spike in ADAs detected at week 14 (Supplemental Fig. 1C). Therefore, challenge of LMB-100+MTX prevents the recall response but does not induce tolerance to subsequent challenge with LMB-100.

MTX-induced tolerance is Ag-specific

We next evaluated the specificity of MTX-induced tolerance. Mice were injected with twice-weekly doses of 40 μg LMB-100 i.v. with or without 1 mg/kg MTX i.p. 0, 24, and 48 h for weeks 1–3.

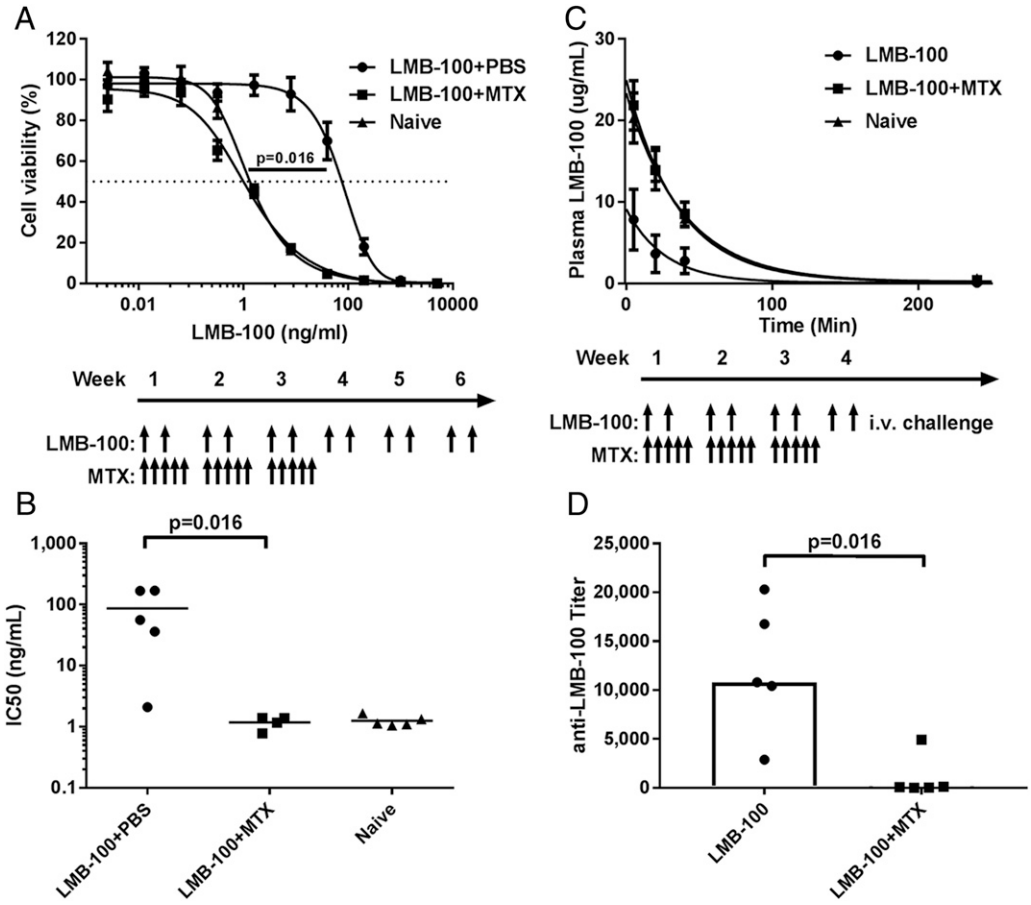

FIGURE 2. MTX prevents the formation of neutralizing ADAs and prevents ADA-mediated clearance of LMB-100. (**A** and **B**) Plasma from week 6 of five mice per group from Fig. 1A was incubated with LMB-100 and applied to KLM-1 cells for 72 h. (A) Cell viability was assessed by WST-8 assay, four replicas per sample. (B) IC_{50} values for each replicate. (**C** and **D**) Mice were immunized with two doses of 40 μg LMB-100 i.p. in weeks 1 through 3, with 1 mg/kg MTX given i.p. 0, 24, and 48 h after each LMB-100 dose. Mice were challenged with a single dose of 20 μg LMB-100 i.v. in week 4, and blood samples were collected 5, 20, and 40 min and 4 and 8 h after injection. (C) Plasma LMB-100 was quantified by ELISA ($n = 5$). (D) Anti–LMB-100 titers were determined using plasma collected 24 h after challenge. Titer is reported as dilution factor. Data points are mean, error bars are SEM. Each experiment was repeated at least once.

During weeks 4–6 after MTX was stopped, both groups of mice were injected i.v with 40 μg LMB-100 and 40 μg OVA. We found that MTX treatment suppressed the mean anti–LMB-100 Ab titer by 6-fold from 5917 at week 6 in mice treated with LMB-100 alone to 915 in mice treated with the combination (Fig. 4A). In contrast, MTX did not suppress the immune response against the

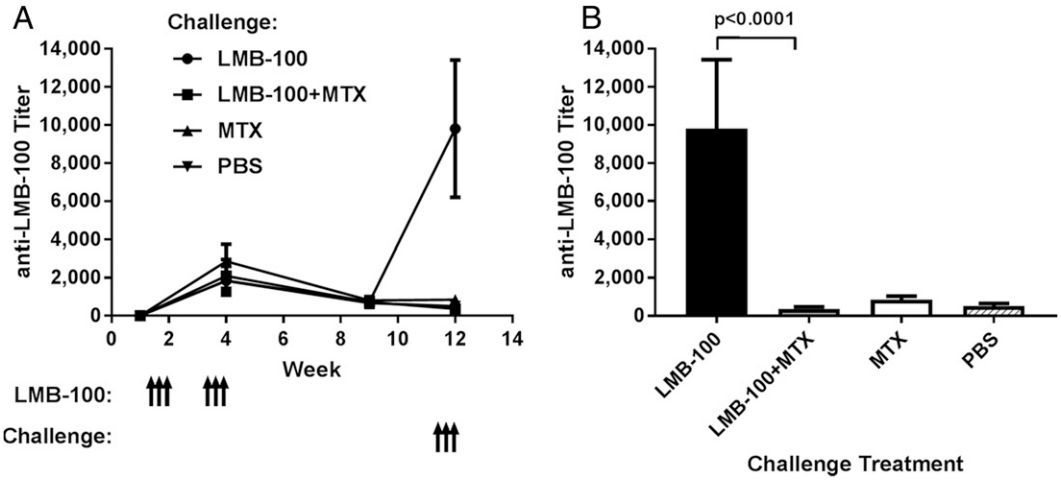

FIGURE 3. MTX prevents the immune memory response. (**A**) Mice were immunized with three doses of 50 μg LMB-100 i.v. in weeks 1 and 3 and challenged in week 11 with either LMB-100 (50 μg i.v.), LMB-100+MTX (1 mg/kg i.p), MTX, or PBS. MTX was given 0, 24, and 48 h after LMB-100. Plasma were collected and analyzed for anti–LMB-100 Abs by ELISA ($n = 11$). (**B**) Week 12 titers. Titer is reported as dilution factor. Data points are mean, error bars are SEM.

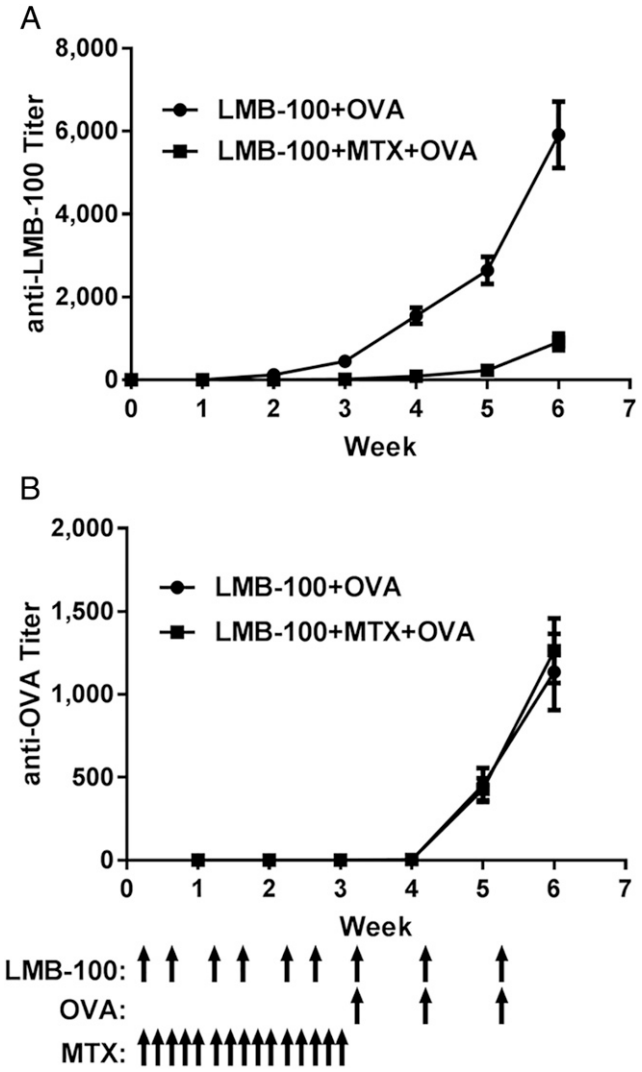

FIGURE 4. MTX-induced tolerance is Ag specific. Mice were immunized with two doses of 40 μg LMB-100 i.v. in weeks 1 through 3 or in combination with 1 mg/kg MTX i.p given 0, 24, and 48 h after each LMB-100 dose. In weeks 4–6 mice were challenged with 40 μg LMB-100 i.v. plus 40 μg OVA i.v. Plasma samples were collected and analyzed for anti–LMB-100 (**A**) and anti-OVA (**B**) Abs by ELISA (n = 14). Titer is reported as dilution factor. Data points are mean, error bars are SEM. Data compiled from two separate experiments.

second Ag. Mice treated with MTX had a similar average anti-OVA Ab titer at week 6 compared with mice not receiving MTX (AUC 1264 and 1136 respectively, Mann–Whitney U test, p = 0.28) (Fig. 4B). This indicates that mice tolerized with MTX can generate normal immune responses against other Ags.

MTX suppresses all Ab isotypes against LMB-100

To evaluate the effects of MTX on ADA class switching, plasma samples from mice treated with LMB-100 twice a week for 6 wk with or without 3 wk of MTX were analyzed for IgG1, IgG2a, IgG2b, IgG3, and IgM anti–LMB-100 Abs by ELISA (Fig. 5A). Anti–LMB-100 Ab titers of each isotype were lower in mice treated with MTX (Fig. 5A). This demonstrates that MTX suppresses all isotypes in a similar manner and suggests MTX is acting at an early step.

We also investigated the change in IgM titer over time (Fig. 5B). Mice treated with LMB-100 had a continuous increase in IgM titer. However, mice treated with MTX had a delayed IgM response during the 6 wk experiment. This suggests that MTX interferes with IgM B cell differentiation.

Adoptive transfer of splenocytes from tolerized mice confers tolerance in naive animals

To investigate the mechanism by which MTX induces tolerance, we investigated if adoptive transfer of whole splenocytes from tolerized mice could confer tolerance to LMB-100 in naive mice. Donor mice were injected with three doses of LMB-100 with or without MTX on weeks 1 and 3. On week 4, donor mice were sacrificed and splenocytes were isolated and transferred to naive recipient mice by i.v. injection. Recipient mice were then challenged with three doses of 50 μg LMB-100 in weeks 4 and 6, and anti–LMB-100 Abs were measured in week 7. MTX significantly suppressed ADA formation in donor mice (Fig. 6A); recipient mice receiving splenocytes from donor mice tolerized with LMB-100+MTX had diminished anti–LMB-100 titers compared with recipient mice receiving splenocytes from LMB-100 only–treated donors (Mann–Whitney U test, p = 0.03) or naive donors (Mann–Whitney U test, p = 0.02) (Fig. 6B). This indicates that the immune tolerance induced by MTX can be transferred by the transfer of splenocytes, and suggests immune tolerance induction at a cellular level in the spleen.

MTX does not have a major effect on immune cell subpopulations

To investigate the effect of MTX on B cells and other immune cell types, we performed complete blood count analyses, performed immunophenotyping of mouse splenocytes by flow cytometry, and detected IL-10–secreting B cells by ELISPOT (Fig. 7, Supplemental Fig. 2). We compared the relative cell counts of B cells ($CD19^+$), CD4 T cells ($CD3^+$, $CD4^+$), regulatory T cells ($CD3^+$, $CD4^+$, $Foxp3^+$), CD8 T cells ($CD3^+$, $CD8^+$), monocytes ($Ly6C^+$, $Ly6G^+$), and dendritic cells ($IAIE^+$, $CD11c^+$) (Supplemental Fig. 3). Mice received one dose of 40 μg LMB-100 i.v., with or without 1 mg/kg MTX 0, 24, and 48 h after each dose. Blood samples and spleens were collected after 72 h. Cell populations were compared with those of naive mice. MTX treatment alone or with LMB-100 did not induce a significant change in blood neutrophils, lymphocytes, or monocytes (Fig. 7A); a 2.4-fold increase in blood eosinophils (Dunn multiple comparisons, p = 0.002 and p = 0.018, respectively) was observed. MTX caused a 60% decrease in blood basophils, and the combination of LMB-100+MTX produced a 78% decrease in basophils but these decreases are not significantly different. MTX alone did not affect the relative composition of immune cell types in the spleen; however, LMB-100 alone or with MTX caused a 2-fold increase in $Ly6C^+$ $Ly6G^+$ monocytes (Fig. 7B). Interestingly, there was no change in IL-10–secreting B cells in the spleen. These data demonstrate that MTX does not deplete immune cell subpopulations in either blood or spleen.

Discussion

In these studies, we provide evidence that adding MTX to treatment of mice with LMB-100 is safe and effective in preventing the formation of ADAs against LMB-100. LMB-100 contains a portion of *Pseudomonas* exotoxin A, which is a potent immunogen, and preventing Ab formation to this foreign protein is a difficult challenge. We found that treatment of LMB-100 and MTX for 3 wk induced immune tolerance that prevented ADA formation against subsequent challenges of LMB-100. ADA formation was dramatically suppressed for over 12 wk of LMB-100 challenge. Suppression of the immune response prevented the decrease in blood levels of LMB-100 because of ADAs and prevented LMB-100 neutralization.

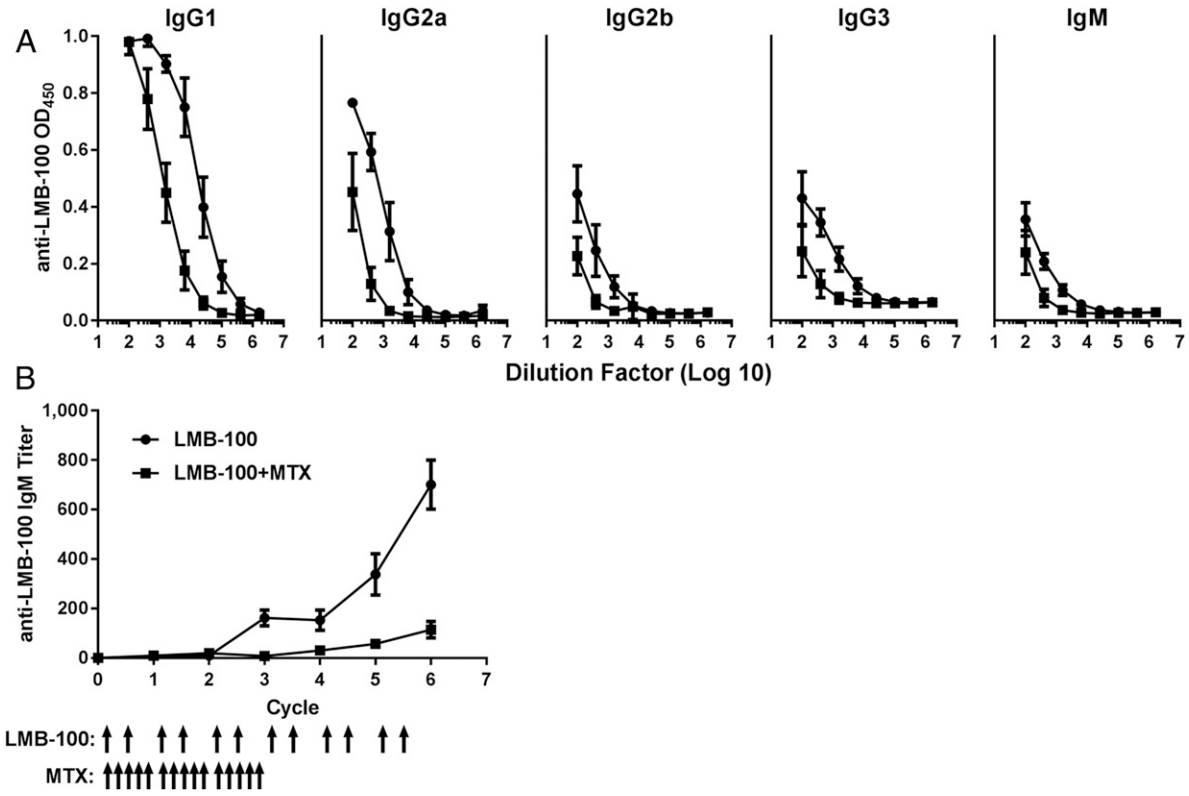

FIGURE 5. Anti–LMB-100 Ab class switching is not affected by MTX treatment. Female BALB/c mice were treated with 20 μg LMB-100 i.v. twice a week for 6 wk. One group received 1 mg/kg MTX i.p. 0, 24, and 48 h after LMB-100 for the first 3 wk. (**A**) ELISA plates were coated with LMB-100 and 1 μl of plasma was diluted 1:100 and then by four down the plate. Anti–LMB-100 Abs in plasma samples from week 6 were detected with anti-isotype secondary Abs (n = 4). Circle: LMB-100 treatment; square: LMB-100+MTX. (**B**) Anti–LMB-100 IgM Abs were detected in plasma samples from mice treated with 40 μg LMB-100 i.v. and 1 mg/kg MTX (n = 8). Titer is reported as dilution factor. Data points are mean, error bars are SEM. Assays were repeated once.

The MTX dose of 1 mg/kg used in our study did not cause weight loss or lymphopenia in the mice. A murine daily dose of 1 mg/kg is equivalent to a human dose of 5.7 mg/d (for a 70 kg man) (24) and is within the range of 5–25 mg/wk used to treat patients with rheumatoid arthritis (25). Higher doses of 0.4 mg/kg were safely used in prophylactic settings to prevent immunogenicity of enzyme replacement therapy in infantile Pompe disease (12).

In a clinical trial in mesothelioma patients with the anti-mesothelin immunotoxin SS1P, immunosuppressive cyclophosphamide and pentostatin, used to prevent ADA formation, resulted in grade 4 lymphopenias in 100% of patients (4). In contrast, low-dose MTX has not been found to cause lymphopenia in humans (26). Our findings in mice showed that low doses of MTX induced only very minor changes in immune cell populations in the blood and spleen. We found an increase in blood eosinophils in mice

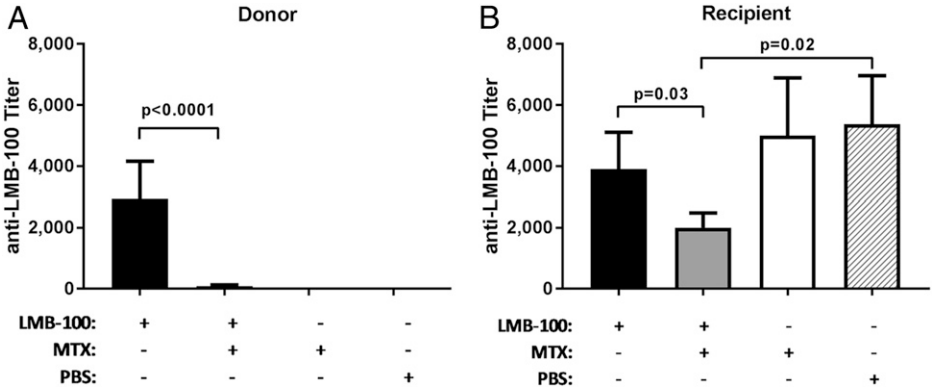

FIGURE 6. Adoptive transfer of whole splenocytes from tolerized mice confers LMB-100 tolerance to naive recipients. (**A**) Female BALB/c mice were immunized with three doses of 40 or 50 μg LMB-100 i.v. in weeks 1 and 3, with 1 mg/kg MTX given i.p. 0, 24, and 48 h after each LMB-100 dose. Blood samples were collected in week 4 prior to sacrifice, and anti–LMB-100 Abs were detected by ELISA (n = 8–12). (**B**) In week 4 donor mice were sacrificed, splenocytes were isolated, and 10×10^6 to 25×10^6 splenocytes were injected i.v. into naive recipient mice. Recipient mice were challenged with three doses of LMB-100 in weeks 5 and 7. Plasma was collected in week 8 and analyzed for anti–LMB-100 Abs by ELISA (n = 8–34). Data shown is a compilation of two separate experiments; table below indicates treatment of donor splenocytes. Titer is reported as dilution factor. Bars show mean, error bars are SEM.

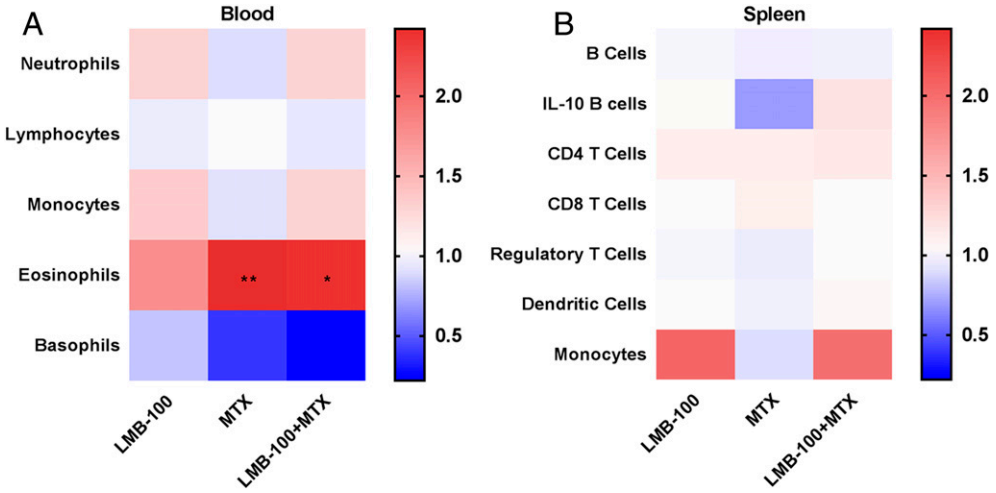

FIGURE 7. MTX treatment does not alter blood counts or splenocyte cell populations. (**A**) Blood was collected from mice treated with one dose of 40 μg LMB-100 i.v. and 1 mg/kg MTX i.p. 0, 24, and 48 h after LMB-100 and analyzed on a hematology analyzer ($n = 4–6$). (**B**) Spleens were harvested 3 d after the last MTX dose. Relative cell populations were analyzed by flow cytometry ($n = 3$). IL-10–secreting B cells were analyzed by ELISPOT assay. Color scale represents fold change compared with naive mice. Dunn multiple comparisons test, $*p < 0.05$, $**p < 0.005$.

treated with either MTX or LMB-100+MTX compared with naive mice, a phenomenon which has been documented in chronic arthritis patients treated with low-dose MTX (27). Furthermore, MTX induced an Ag-specific immune tolerance as indicated by an intact Ab response to OVA challenge. These findings suggest that low-dose MTX will be better tolerated by patients than immune suppression by cyclophosphamide and pentostatin.

Many mechanisms have been proposed for the anti-inflammatory efficacy of MTX. Joly et al. (16) reported an increase in IL-10–secreting B cells in MTX-treated mice using flow cytometry, implicating induction of B-10 regulatory B cells as a potential mechanism for MTX-induced tolerance. However, in our study we did not observe and increase in IL-10–secreting splenic B cells using ELISPOT. This discrepancy can be explained by differing methods of analysis. Nevertheless, our finding that adoptive transfer of splenocytes from donor mice, tolerized with LMB-100 and MTX, suppressed ADA formation in recipient mice challenged with LMB-100 and suggests the involvement of a regulatory cell subset, which persists beyond the duration of MTX treatment and does not interfere with immune responses to other Ags.

Patients with pre-existing Abs to immunotoxins have been excluded from immunotoxin clinical trials. Thus, we investigated the tolerogenic efficacy of MTX in the context of pre-existing Abs against LMB-100. MTX coadministered with LMB-100 prevented the rise in ADAs associated with the immune memory recall response in mice previously immunized with LMB-100. Indeed, MTX was effective in reducing high levels of anti-infliximab ADAs and restoring the anti-TNF activity in 11 patients with inflammatory bowel disease (28, 29). Nevertheless, a subsequent challenge with LMB-100 alone induced the memory response. MTX can therefore prevent the memory response but not induce tolerance in the context of pre-existing immune memory. The combination of MTX with immunotoxins should be useful for patients with pre-existing Abs with or without prior immunotoxin therapy.

The findings reported here provide a strong preclinical rationale for using low-dose MTX therapy to prevent formation of Abs to immunotoxins, maintaining their blood concentration, allowing more treatment cycles, and consequently enhancing their clinical efficacy. Moreover, MTX can be used not only for patients beginning immunotoxin therapy, but also for patients with pre-existing Abs or patients who were previously excluded from clinical trials because of neutralizing ADA formation. MTX is approved by the Food and Drug Administration, and its clinical side effects are well documented (30). Beyond immunotoxins, this dosing regimen of MTX could be used to tolerize and suppress ADAs against immunogenic therapies for other disease applications.

Disclosures

The authors have no financial conflicts of interest.

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