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Tofacitinib Suppresses Antibody Responses to Protein Therapeutics in Murine Hosts

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Immunogenicity remains the “Achilles’ heel” of protein-based therapeutics. Anti-drug Abs produced in response to protein therapeutics can severely limit both the safety and efficacy of this expanding class of agent. In this article, we report that monotherapy of mice with tofacitinib (the JAK inhibitor) quells Ab responses to an immunotoxin derived from the bacterial protein Pseudomonas exotoxin A, as well as to the model Ag keyhole limpet hemocyanin. Thousand-fold reductions in IgG1 titers to both Ags were observed 21 d post immunization. In fact, suppression was evident for all IgG isotypes and IgM. A reduction in IgG3 production was also noted with a thymus-independent type II Ag. Mechanistic investigations revealed that tofacitinib treatment led to reduced numbers of CD127+ pro–B cells. Furthermore, we observed fewer germinal center B cells and the impaired formation of germinal centers of mice treated with tofacitinib. Because normal Ig levels were still present during tofacitinib treatment, this agent specifically reduced anti-drug Abs, thus preserving the potential efficacy of biological therapeutics, including those used as cancer therapeutics. The Journal of Immunology, 2014, 193: 48–55.

Recombinant immunotoxins (RITs) have been developed for the treatment of cancer (1). These therapeutic proteins are composed of a 38-kDa truncated form of Pseudomonas exotoxin A (PE38) fused to the variable fragment (Fv) of an Ab. The binding moiety of the Fv targets Ag+ cells that are then killed by the cytotoxic activity of the toxin moiety (2). Three PE-based RITs are currently being evaluated in clinical trials. One of these, moxetumomab pasudotox (HA22), targets CD22 expressed by B cell malignancies. In phase I trials, HA22 produced a high rate of complete remission in patients with drug-resistant hairy cell leukemia and objective responses in acute lymphoblastic leukemia (3, 4). The CD25 targeting RIT anti–Tac(Fv)-PE38 (LMB-2) has shown antitumor activity in patients with hairy cell leukemia and other hematological malignancies (5). Another RIT being tested in phase I studies is the antimesothelin immunotoxin, SS1(dsFv)-PE38 (SS1P). As a monotherapy, SS1P produced only minor responses in patients with mesothelioma (6, 7). However, in preclinical testing, combinations of SS1P with chemotherapy produced more promising results than SS1P alone, suggesting a path forward for clinical trials (8, 9).

Importantly, one major factor limiting the efficacy of these RITs is their immunogenicity. The PE38 domain can lead to Ab responses in treated patients (5, 10). Like other biologicals, protein therapeutics frequently contain immunogenic epitopes with the potential to activate the immune system, including T cells and B cells. This activity can result in the production of anti-drug Abs (ADAs) and the loss of a therapeutic response. In patients with hematological malignancies, the risk for developing ADAs is low. These patients typically present with an immune system impaired by their disease or by chemotherapy, which protects them from ADAs during repeated treatment cycles of RITs. In contrast, in patients with solid tumors, such as mesothelioma or ovarian cancer, the immune system is still functional and the risk for developing ADAs is $>75\%$, as observed after one cycle of treatment with SS1P (7).

Several approaches have been proposed to reduce the immunogenicity of protein therapeutics. One way is silencing major B cell epitopes of protein therapeutics by masking them with polyethylene glycol (PEG) or by introducing mutations (11–14). Another approach is to use purine analog–based immune depletion regimens. For example, pentostatin acts synergistically with cyclophosphamide to deplete host lymphoid cells with a minimum effect on myeloid cells. A regimen of pentostatin plus cyclophosphamide abrogated murine host capacity to form anti-RIT Abs (15). Traditional immunosuppressants like azathioprine or methotrexate have been reported to lower the risk for ADAs directed against other biologicals like TNF antagonists (16). However, the use of such general immunosuppressants or chemotherapeutics is often limited by metabolic side effects, such as hepato- or nephrotoxicity. For more specific and precise control of T cell– and B cell–dominated immune responses, novel inhibitors targeting JAK may prove a more powerful tool.

JAK3 is an intracellular tyrosine kinase that associates with the common γ-chain (γc) of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (17). Signal transduction mediated by JAK3 is obligatory for lymphocyte activation, differentiation, and homeostasis.

Abbreviations used in this article: ADA, anti-drug Ab; γc, γ-chain; Fv, variable fragment; KLH, keyhole limpet hemocyanin; PE38, truncated form of Pseudomonas exotoxin A; PEG, polyethylene glycol; RIT, recombinant immunotoxin; SS1P, SS1(dsFv)PE38; TI-II, thymus-independent type II; TNP, trinitrophenyl; XSCID, X-linked SCID.
After binding of the IL to its specific type I or II receptor, JAKs will associate with the receptor and activate downstream proteins, STATs (18). Activated STATs control gene expression (19). Although JAKs are typically present in many tissues, JAK3 expression is largely restricted to hematopoietic cells. Thus, JAK3 may be an excellent target for silencing immune responses and reducing ADA production against protein therapeutics without affecting other organs (20).

The kinase inhibitor tofacitinib (originally CP-690, 550) was initially reported as a selective JAK3 inhibitor (21–23). However, more recent data indicate that tofacitinib binds JAK3 and JAK1 with roughly equivalent affinity, and JAK2 to a much lesser extent. Therefore, some of the efficacy of tofacitinib in immune responses is likely due to the combined inhibition of JAK3 and JAK1 (24). Tofacitinib has mainly been investigated in a variety of preclinical models of autoimmunity and inflammation. Tofacitinib has demonstrated efficacy and safety in rheumatoid arthritis, thus leading to its approval by the Food and Drug Administration. Clinical trials are ongoing in various arthropathies, psoriasis, and other autoimmune diseases (25, 26). Its exact mode of action on B cell–mediated Ab responses has not been clarified. However, we assumed that a potent inhibitor of the JAK3/JAK1-utilizing cytokines IL-4, IL-7, and IL-21 would have a major impact on B cell responses. Therefore, we investigated the effects of tofacitinib on B cell responses in a mouse model of RIT-induced immunogenicity. Our hypothesis contended that a selective therapeutic regimen, including JAK3/JAK1 inhibition, would abrogate the ADA production and preserve the efficacy of biological protein therapeutics in a cancer setting. We have evaluated the potency of tofacitinib for specifically reducing ADA production and studied the mechanism that controls Ab production in mice receiving RIT.

Materials and Methods

Materials

Female BALB/c mice (6–8 wk old) were obtained from the Frederick Cancer Research Facility (Frederick, MD), and JAK3 knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were treated with an approved animal protocol (LMB-005) and maintained in a specific pathogen–free facility. Experiments were performed according to the National Institutes of Health guidelines for the use of live animals.

Reagents

Tofacitinib was synthesized at the National Center for Advancing Translational Sciences using a published protocol (27). Tofacitinib was resuspended at 100 mg/ml in PEG 300 for in vivo studies or in DMSO for in vitro use. SS1P was manufactured in the National Cancer Institute Laboratory of Molecular Biology according to previous methods (28). Hemocyanin from Megathura crenulata keyhole limpet was purchased from Sigma-Aldrich (St. Louis, MO).

Drug treatment and immunizations

Mice received tofacitinib in PEG 300 (100 mg/ml) or vehicle alone (PEG 300) by osmotic pump infusion (Alzet Model 2004, 0.25 µl/h, 28 d; Durect, Cupertino, CA). At 4 d prior to immunization, mice were anesthetized and their dorsal surface was shaved. A 1-cm incision was made on the back to create an s.c. pocket and insert the pump. The incision site was closed with wound clips. Mice were injected weekly (i.p.) with SS1P recombinant immunotoxin (RIT; 5 µg per mouse) beginning on day 0; control mice received injections of saline alone. Every week before SS1P or vehicle immunization, ~50 µl blood was drawn to obtain serum samples. Sera were stored at –80°C until analyzed.

Thymus-independent type II Ag immunization

To characterize responses to a thymus-independent type II (TH-II) Ag, 10 µg trinitrophenyl (TNP)–Ficoll (in PBS) was injected i.p., and TNP-specific Ab titers were determined 5 d later by ELISA. ELISA plates were coated overnight with 1 µg/ml TNP-BSA (50 µl per well). Several dilutions of both pre- and postimmunization sera were added. Isotypespecific Abs were determined with an HRP-conjugated goat anti-mouse Ig (H+L) Ab (Southern Biotechnology, Birmingham, AL) and a TMB (tetramethylbenzidine) Substrate Kit (Thermo Scientific, Rockford, IL).

Characterization of B cell differentiation and proliferation

Splenocyte and bone marrow cell suspensions were prepared from BALB/c mice, and total cells were counted. Cells (1 × 10⁶) were stimulated with various combinations of the following anti-murine Abs (BD Biosciences, San Jose, CA): CD3, B220, CD43, IgM, Fas, GL-7, CD24, BP-1, CD127, or IgG1 conjugated with FITC, PE, or allophycocyanin (BD Biosciences) and analyzed on a FACS Calibur flow cytometer (BD Biosciences). A total of ≥10,000 live events were acquired. For assessment of in vitro B cell proliferation, CD43+ splenocytes were MACS (Miltenyi Biotec, San Diego, CA) purified and labeled with 1 µM CFSE (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Labeled cells were activated for 48 h with 25 µg/ml LPS (Escherichia coli 0111: B4; Sigma-Aldrich) and 5 ng/ml IL-4 in the presence of 0, 0.1, 0.3, or 1.0 µM tofacitinib. Following culture, cells were washed, surface stained, and examined by flow cytometry (FACS Calibur; BD Biosciences).

ELISA

An ELISA format was used to measure anti-PE38 Abs. Briefly, microwell plates (MaxiSorp, Sigma-Aldrich) were coated with 100 ng per well of SS1P or keyhole limpet hemocyanin (KLH) in PBS overnight at 4°C. After adding blocking buffer (25% DMEM, 5% FBS, 25 mM HEPES, 0.5% BSA, and 0.1% azide in PBS), plates were washed with PBS containing 0.05% Tween 20. Then serially diluted sera were transferred to individual wells (50 µl per well). ADSs were detected by rabbit anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-mouse Ig isotype-HRP (Southern Biotechnology) and tetramethylbenzidine (TMB Substrate Kit; ThermoScientific). Each plate contained serial dilutions of the anti-PE38 mAb, IP30, as a binding control standard (29). For the measurement of serum Ig isotype studies, ELISA plates were directly coated with goat anti-mouse Ig (H+L) (Southern Biotechnology), before adding the sera samples and secondary Abs-HRP, as described above. Serum Ab concentrations were determined by ELISA using mouse isotype control Abs (BD Biosciences).

Tissue staining

Spleens were removed after immunization and fixed/paraffin-embedded, and organ sections were stained with H&E (Pathology/Histotechnology Laboratory, SAIC-Frederick, National Cancer Institute−Frederick, MD).

Statistics

For comparisons between the experimental groups in the immunogenicity study, the Mann–Whitney nonparametric test was used. The p values < 0.05 were considered statistically significant.

Results

Ag-specific Ab response

To examine the effect of JAK3/JAK1 inhibition on Ab responses, we implanted Alzet osmotic mini-pumps (on day −4) delivering tofacitinib, for a calculated duration of 28 d, or vehicle (PEG solution) to BALB/c mice. On day 0 mice were immunized with SS1P as a protein Ag. Beginning on day 7 after immunization, the serum concentration of ADA (anti-SS1P Abs) was determined weekly. In vehicle-treated mice, ADAs became detectable on day 28. A difference of 1000- to 2000-fold in titers to SS1P was apparent from days 21 through 35, respectively (Fig. 1A). Of interest, animals that were treated with tofacitinib showed a significantly lower production of ADAs compared with PEG-treated control mice (for 5 wk after initial immunization, p < 0.01, n = 8). Moreover, ADAs became detectable earliest on day 28. A difference of 1000- to 2000-fold in titers to SS1P was apparent from days 21 through 35, respectively (Fig. 1A). To demonstrate that the suppressive effect of pharmacological JAK3/JAK1 inhibition on ADA development was not restricted to SS1P Ag, we next immunized mice with KLH. Mice injected with KLH, compared with SS1P, generated a more rapid Ab response (Fig. 1B). Yet, the administration of tofacitinib reduced anti-KLH
titers compared with controls ($p < 0.05$ on day 21, $p < 0.01$ on day 28, respectively, $n = 5$). Reductions in titers ranged from 5000- to 250-fold from days 21 through 28, respectively. To confirm the relevance of pharmacological JAK3 inhibition for ADA development, we next used a genetic model of JAK3 deficiency. Wild-type or JAK3 knockout mice were immunized with SS1P, and specific Ab titers were analyzed after 5 wk. As expected, no ADAs were detected in JAK3 knockout mice that were immunized with SS1P (Fig. 1C). From these results we conclude that the JAK3/JAK1 pathway is important for the generation of relevant titers of Abs to immunized proteins.

Next we examined responding titers to SS1P by assaying Ag-specific isotypes to determine whether the effect of tofacitinib was general or was restricted to a particular Ab isotype. After four injections, most responding Abs were from the IgG1 subclass, followed by IgG2b (Fig. 2A). No apparent increase in SS1P-specific IgG2a and IgG3 titers was observed when using this immunization protocol and SS1P as Ag. Treatment with tofacitinib completely blocked the anti-SS1P responses by IgG1 and IgG2b subclasses ($p < 0.001$, $p < 0.05$, respectively; $n = 8$; Fig. 2A). The IgM titer to SS1P was reduced by tofacitinib treatment ($p < 0.05$; Fig. 2A). To show that tofacitinib activity was not restricted to a particular thymus-dependent Ag, we used a similar immunization protocol with the model Ag KLH. Mice treated with or without tofacitinib were immunized weekly with KLH. After three injections, sera were analyzed for the presence of KLH-specific Igs. This time all subclasses of IgG, including IgG2a and IgG3, showed increased titers to KLH (Fig. 2B). Similar to SS1P, the titers of all subclasses of responding IgGs were reduced in the tofacitinib-treated mice ($p < 0.01$; $n = 5$, Fig. 2B), confirming that inhibition is not restricted to SS1P but apparently is general for thymus-dependent Ags. These results suggest that tofacitinib treatment effectively interferes with Ag-specific immune responses mediated by T and B cell interaction.

Typically, TI-II Ags stimulate the production of IgG3 Abs (30). Therefore, we studied the effects of JAK3/JAK1 inhibition...
on IgG3 responses to the TI-II Ag TNP-Ficoll. Mice received TNP-Ficoll i.p., and 5 d later TNP-specific IgM and IgG3 Ab levels were determined. Although the IgM response in mice treated with tofacitinib was similar to that in control mice, the TNP-specific IgG3 responses were reduced by ∼3-fold in tofacitinib-treated mice (Fig. 3). Thus, tofacitinib inhibits T cell–independent Ab production of the IgG3 subclass. Taken together, the results with SS1P, KLH, and TNP immunization suggest that tofacitinib affects B cell differentiation and/or inhibits B cell proliferation. To address these issues, we decided to study the overall effects of tofacitinib treatment on the lymphoid cells of the immune system. Specifically, we analyzed the proportion of B and T cell subsets in spleen and bone marrow and measured serum Ig content.

**B cell numbers**

In agreement with previous reports, tofacitinib-treated mice thrived and showed no signs of toxicity (31). Of interest, when immunized with KLH, mice treated with tofacitinib showed a 3- to 4-fold reduction in total splenic T cells (p = 0.008) and B cells (p = 0.004), compared with sham-treated controls (Fig. 4). However, the relative proportions of B and T cells were minimally affected (Supplemental Fig. 1A). Because JAK3 knockout mice have been reported to have some development and signaling defects within B and T cell lineages (32, 33), we decided to test whether treatment with tofacitinib produced a similar phenotype. Although no reduction in the percentage of splenic B220+ CD19+ IgMlow CD43+ cells (mature B cell phenotype; Supplemental Fig. 1B) was observed, we detected a reduction in the percentage of splenic Fas+ GL-7+ germinal center B cells in KLH-immunized mice treated with tofacitinib. This resulted in a significant reduction of total germinal center B cells upon treatment with tofacitinib (Fig. 4, Supplemental Fig. 1C, p = 0.01).

**B cell development**

To investigate whether JAK inhibition by tofacitinib treatment directly affects early B cell development, we isolated and analyzed bone marrow for the presence of cells expressing B220, CD24, and CD43. As defined (34), prepro–B cells lack or minimally express CD24, whereas pro–B cells typically express CD24. As shown in Fig. 5A, a small decrease in B220+CD43+CD24− B lymphocytes (pro–B cells) and a similar percentage of B220+CD43+CD24− B cells (prepro–B cells) were observed in the tofacitinib treatment group, indicating that the partial developmental block mediated by JAK3/JAK1 inhibition appeared after the prepro–B cell stage. Bone marrow cells were also analyzed for the expression of CD127 (IL-7R) and B220. As depicted in Fig. 5B, the majority of cells expressing IL-7R were positive for B220, indicative of a pro–B cell and pre–B cell population. Tofacitinib treatment strongly reduced the percentage of CD127+ cells. This feature of reduced CD127+ cells resembles the defect in B cell development reported in JAK3-deficient mice (35, 36). Our results suggested that the suppression within the B cell population in tofacitinib-treated mice was likely caused by impaired expression of IL-7R and IL-7R signaling. During B cell development, IL-7Rα is expressed by common lymphoid precursors, which can generate both T and B cells. This induction of IL-7Rα expression might be in response to stimulation by the FLT3 ligand, which has been shown to induce IL-7Rα expression in vitro, and expression of both FLT3 and IL-7Rα occurs at the same developmental stage (37). Tofacitinib may suppress the FLT3 ligand expression by an unknown mechanism. Presumably, JAK3/JAK1 inhibition prevents further differentiation of B cells from the prepro–B cell stage through intervention with the IL-7R/STAT5 signaling pathway (38, 39).

**B cell proliferation**

We next performed functional studies on splenic B cells by culturing them in the presence or absence of tofacitinib. The effects of JAK3/JAK1 inhibition on B cell proliferation were investigated by labeling the cells with CFSE and stimulation with LPS and IL-4, a cytokine that, like IL-7, signals through JAK3 and JAK1. After 48 h the cells were studied by flow cytometry and proliferation was

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**FIGURE 3.** Effects of tofacitinib on responses to TI-II Ag. Mice were either pretreated with tofacitinib (△) or vehicle control (○) and immunized with TNP-Ficoll. After 5 d, TNP-specific IgM and IgG3 responses were measured by ELISA. Symbols represent average values of five mice. The □ and △ indicate Ab levels in the preimmune serum, whereas ■ and ▲ represent titers from TNP-Ficoll-immunized mice. Starting at 1:100, 3-fold dilutions were made and analyzed for reactivity to TNP. Data are expressed as the mean ± SD. **p < 0.01.

**FIGURE 4.** Effect of tofacitinib on the number of splenocytes. Spleens were harvested from KLH-immunized mice with (●) or without (○) tofacitinib treatment. The cell numbers are calculated from the results of flow cytometry. **p < 0.01.
evaluated by CFSE dilution. Tofacitinib had no negative effects on B cell proliferation by LPS/IL-4 activation at concentrations ranging from 0.1 to 1.0 μM (Fig. 6A). In addition, we studied the IL-4–dependent expression of IgG1 by proliferating B cells. Even though B cell proliferation in vitro by LPS was not negatively affected by tofacitinib, the IL-4–dependent expression of IgG1 was suppressed in a dose-dependent manner (Fig. 6B, 6C, *p*, 0.01).

### Serum Ig levels

To investigate whether the decrease in splenic B cell numbers and the block in B cell development by pharmacological JAK3/JAK1 inhibition correlated with a reduction of total serum Ig levels, we followed Ig isotypes in mice immunized with SS1P (Fig. 7) or KLH (Supplemental Fig. 2). As shown in Supplemental Fig. 2, serum IgG1 and IgG2a levels were suppressed in mice treated with tofacitinib compared with sham-treated control mice during the treatment period. In contrast, the serum levels of other Ig isotypes were not affected by tofacitinib treatment. This finding indicates that in vivo significant numbers of Ig-producing plasma cells were still induced in mice treated with tofacitinib, but that Ag-specific IgG1 and IgG2a production was diminished (Fig. 2). As shown in Supplemental Fig. 2, when KLH was used as an immunogenic Ag, we had similar findings as observed with SS1P immunization (Fig. 7). IgG1 and IgG2a levels were suppressed with tofacitinib treatment, and the serum levels of other Ig isotypes were generally not affected.

### Failure of germinal center formation by tofacitinib

To investigate whether JAK3/JAK1 inhibition affects germinal center formation, a requirement for efficient Ab responses, we prepared histological sections from spleens of mice on day 17 after immunization with KLH and treatment with tofacitinib or control PEG. The tissue sections were stained with H&E. In sham-treated mice immunized for KLH, we could readily detect germinal centers in the spleen on day 17 after immunizations. In contrast, in mice treated with tofacitinib we could not detect germinal center formation on day 17 after immunization with KLH (Supplemental Fig. 3). Thus, our data suggest that mechanisms affecting germinal center formation, B cell development, and Ig class switching are potentially responsible for the suppression of specific Ab responses by pharmacological JAK3/JAK1 inhibition.

### Tofacitinib did not prevent the specific cytotoxicity of immunotoxin on cancer cells

To study whether tofacitinib affected the specific cytotoxicity of immunotoxin on a cancer cell line, we measured the cytotoxic activity of 1 ng/ml of immunotoxin, HB21(Fv)-PE40 (HB21 is an Ab that targets human transferrin receptor), on several cancer cell lines (DLD1 cells are a colorectal adenocarcinoma, KB-3-1 cells are derived from cervical carcinoma, and Raji cells are a Burkitt lymphoma) with different amounts of tofacitinib (0–60 μM). As most growing cells express transferrin receptors, we can estimate the activity of this immunotoxin on a variety of cancer cell types. There was no difference in the cytotoxic effect of 1 ng/ml HB21 when tofacitinib-treated cells were compared with nontreated cells.

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**FIGURE 5.** Altered B cell profile in bone marrow cells after tofacitinib administration. Bone marrow cells were harvested from KLH-immunized mice with/without tofacitinib. (A) Cells were stained with anti-CD43, anti-CD24, and anti-B220 Abs. (B) Cells were stained with anti-CD127 and anti-B220 Abs. Expression was analyzed by flow cytometry. Numbers next to the gates in the dot plots indicate percentage of gated cells.

**FIGURE 6.** Tofacitinib blocks IgG1 expression by B cells. Isolated B cells were labeled with 1 μM CFSE and stimulated in vitro with LPS and IL-4 in the presence of indicated doses of tofacitinib. (A and B) B cell proliferation was determined by CFSE dilution. (C) IgG1 expression was determined by Ab staining and flow cytometry. *n* = 5, ***p* < 0.001.
Thus, tofacitinib, by inhibiting JAK3/JAK1, could improve the therapeutic index of immunotoxins by decreasing ADA response without blocking antitumor effects.

### Discussion

We have analyzed the effects of tofacitinib on the murine immune response to two thymus-dependent Ags and one thymus-independent Ag. Tofacitinib reduced Ag-specific Ig responses to each of these Ags, sometimes by as much as 5000-fold. Further, tofacitinib reduced the number of B and T cells, leading to a small reduction in total serum IgG levels. Tofacitinib treatment regimens were generally well tolerated with no apparent side effects. Specifically in mice, in which the production of ADA against SS1P was induced by weekly immunizations, tofacitinib significantly reduced ADA both in time to response and in overall titer.

Although protein therapeutics constitute a promising class of drugs, their efficacy can be reduced considerably when they elicit an Ab response. Certain ADAs work by neutralization, whereas others reduce drug efficiency by hastening clearance or preventing access to target tissue. In this study, we have used an in vivo model to show that tofacitinib-mediated inhibition of JAK3/JAK1 reduced immunogenicity even in the face of repetitive immunotoxin dosing. These results establish a proof-of-principle for combining immunotoxin treatments with immune-modifying tofacitinib treatment. It is noteworthy that the therapeutic effect in the current study was achieved with continuous infusion. It is likely that tofacitinib treatment, producing very low anti-immunotoxin responses, relates to reduced immunogenicity of *Pseudomonas* exotoxin. These observations have broad applicability not only for protein therapeutics per se but also for gene therapy strategies in which wild-type or “corrected” proteins are expressed from engineered vectors. Further, for immunotoxins specifically, our results will likely inform the next generation of immunotoxin clinical trials. Traditional immunosuppressants like methotrexate or azathioprine have also been shown to reduce ADA during concomitant treatment with biologicals. However, those drugs can induce severe immunosuppression and accompanying toxicities. As tofacitinib suppresses Ig production more specifically than other immunosuppressants, clinical tolerance by cancer patients will be easier and safer.

Our experiments also point to the importance of maintenance tofacitinib treatment to prevent immunogenicity during prolonged immunotoxin dosing. Indeed, we observed that the continuous infusion of tofacitinib was required for complete protection against ADA formation during five weekly challenges with immunotoxin. In the data from healthy volunteers, tofacitinib is rapidly absorbed after oral administration, with peak plasma concentrations of tofacitinib attained within 0.5–1.0 h (40). The oral bioavailability of the tofacitinib after a single oral 10-mg dose was 74% (40). For clinical application, it is possible that the continuous infusion regimen used in our mice experiments could be replaced by a daily oral administration regimen. It should be noted that our experiments were carried out in a tumor-free model. Further evaluation of combination between immunotoxin and tofacitinib treatment may be warranted in experiments that incorporate tumor cells, such as xenograft models incorporating a mesothelin-expressing tumor cell line (41). Yet, additional treatment with tofacitinib had no impact on the cytotoxicity of an immunotoxin directed to DLD1, KB3-1, and Raji cells in vitro (Supplemental Fig. 4). As the constitutive activation of JAKs and STATs is a common feature of a variety of cancers, JAK inhibitors are being tested in malignancies. For example, the constitutive activation of JAK1 has been recently reported in mesothelioma cell lines and in primary malignant mesothelioma (42). Thus, inhibition of JAKs may even be beneficial in mesothelioma treatment as well as blocking ADA production.

We have identified that the percentage of CD127 (IL-7R)⁺ pro-B cells in tofacitinib-treated mice was greatly reduced. IL-7 is an essential pre-B cell growth factor in mice (43). Prepro-B cells...
were not diminished in IL-7R-deficient mice, but the pro-B cell population and pre-B cell population were reduced compared with these populations in control mice (38). Our findings, which highlight a partial developmental block at the pro-B cell stage, suggest that the reduced number of B cells in tofacitinib-treated mice was likely caused by lower expression of the IL-7R. However, this suppression was weak, and we can detect serum Igs in the tofacitinib-treated mice to some extent. This finding suggested that immune impairment by tofacitinib is limited and that a remarkable number of T and B cells were still present in tofacitinib-treated mice. Tofacitinib-treated mice also showed about half the amount of serum IgG1 and IgG2a, but identical amounts of other serum Igs (Fig. 7, Supplemental Fig. 2). Ag-specific IgG1 responses, however, were remarkably diminished in the tofacitinib-treated mice. This observation can be explained by the inhibition of IL-4 signaling, which is important for IgG1 response (44). In addition, during tofacitinib treatment, all mice appeared to retain full health without obvious side effects caused by immunosuppression.

In animal models of organ engraftment, tofacitinib treatment prolonged allograft survival in both heart and kidney transplant models. Recently, the results of clinical trials of tofacitinib to treat patients with refractory rheumatoid arthritis have led to drug approval in the United States. Treatment with tofacitinib resulted in significant dose-dependent improvements in patients’ responses. After 6-wk treatments, disease activity was significantly reduced in the 5 mg, 15 mg, and 30 mg twice daily treatment groups (25). The incidences of adverse events in the tofacitinib 5 mg twice daily and placebo groups were similar. This kind of progress together with low toxicity suggests that it may be possible to develop a safe and effective treatment regimen for combining tofacitinib and immunotoxin therapy.

We have shown that tofacitinib treatments successfully reduced the immunogenicity of an immunotoxin and other immunogenic agents in a mouse model. Remarkably, this result was achieved with a compound that does not induce neutropenia. However, several additional considerations are important when translating these findings from mice to humans. It is still unclear whether IL-7 is an important factor in B cell homeostasis in mice. The role of Jak3 in B cell homeostasis may differ in mice and humans; however, the effect of tofacitinib on function may very well be the same. JAK3 in B cell homeostasis may differ in mice and humans; how-


