Targeted Disruption of the Leukotriene B₄ Receptor in Mice Reveals Its Role in Inflammation and Platelet-activating Factor-induced Anaphylaxis

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Abstract

Leukotrienes are derived from arachidonic acid and serve as mediators of inflammation and immediate hypersensitivity. Leukotriene B₄ (LTB₄) and leukotriene C₄ (LTC₄) act through G protein–coupled receptors LTB₄ receptor (BLTR) and Cys-LTR, respectively. To investigate the physiological role of BLTR, we produced mice with a targeted disruption of the BLTR gene. Mice deficient for BLTR (BLTR−/−) developed normally and had no apparent hematopoietic abnormalities. Peritoneal neutrophils from BLTR−/− mice displayed normal responses to the inflammatory mediators C5a and platelet-activating factor (PAF) but did not respond to LTB₄ for calcium mobilization or chemotaxis. Additionally, LTB₄ elicited peritoneal neutrophil influx in control but not in BLTR−/− mice. Thus, BLTR is the sole receptor for LTB₄-induced inflammation in mice. Neutrophil influx in a peritonitis model and acute ear inflammation in response to arachidonic acid was significantly reduced in BLTR−/− mice. In mice, intravenous administration of PAF induces immediate lethal anaphylaxis. Surprisingly, female BLTR−/− mice displayed selective survival (6 of 9; P < 0.002) relative to male (1 of 11) mice of PAF-induced anaphylaxis. These results demonstrate the role of BLTR in leukotriene-mediated acute inflammation and an unexpected sex-related involvement in PAF-induced anaphylaxis.

Key words: arachidonic acid • neutrophil influx • knock-out • sex-related • chemotaxis

Introduction

Leukotriene B₄ (LTB₄) is a potent chemoattractant for neutrophils, eosinophils, and macrophages and also activates the respiratory burst and granule release from neutrophils (1). Leukotriene C₄ (LTC₄) activates smooth muscles and is a potent bronchoconstrictor (2, 3). G protein–coupled receptors LTB₄ receptor (BLTR) and Cys-LTR mediate the activity of LTB₄ and LTC₄, respectively (4, 5). BLTR is expressed in spleen and on leukocytes, whereas Cys-LTR is expressed mainly in smooth muscle and spleen and on leukocytes and pulmonary macrophages. BLTRs activate the G family of G proteins to mediate chemotaxis but use other G proteins as well to mediate cytotoxic activities (4, 6). Leukotrienes play a major role in the pathophysiology of asthma and other pulmonary diseases (7, 8). Several inhibitors of leukotriene biosynthesis and activity have been developed for clinical use (9). Studies on mouse models and antagonists of LTB₄ suggested a role for BLTR in rheumatoid arthritis, skin inflammation, and acute septic peritonitis (10–12). Mice with targeted disruption of 5-LO and 5-LO activating protein (FLAP), and, more recently, LTA₄ hydrolase allowed the determination of the role of leukotrienes in inflammation and hypersensitivity (13–16). Nonenzymatic formation of LTB₄ receptor agonists have been demonstrated (17), and the nuclear receptor peroxisome proliferator–activated receptor α was shown to mediate several of the effects of LTB₄ (18). Therefore, to determine the in vivo biological functions of BLTR and the potential consequences of chronic BLTR deficiency, we used gene targeting in embryonic stem (ES) cells to disrupt the mouse BLTR gene. The results demonstrate that BLTR deficiency blocks cellular responses to LTB₄ and diminishes acute inflammatory responses. The data also disclosed an unexpected role for BLTR in sex-dependent leukotriene effects in mediating hypersensitivity to platelet-activating factor (PAF).
Materials and Methods

Targeting construct and generation of BLTR-deficient mice. To target the mouse BLTR gene was isolated as BAC clones from a genomic library from 129/SvJ strain from R Research Genetics using an EST clone for mouse BLTR (19, 20). The BAC clones were mapped by restriction analysis, and a 10-kb BamHI fragment (see Fig. 1 A) was subcloned into two BamHI–SacII fragments into pBluescript. The knockout construct was made in a vector (pPNT) containing both PGK-neo and PGK-tk cassettes (21). A SpeI linker was added to the SacII end of the 3′ BamHI–SacII clone. A 1.5-kb SpeII–KpnI fragment from this subcloned was excised into XbaI–KpnI sites in pPNT vector in between the neo and tk cassettes. The 5′ BamHI–XhoI fragment was excised from the pBluescript clone as a NotI–XhoI fragment and cloned into the same sites of the pPNT already containing the 3′ SacII (SpeII–KpnI) fragment. The resulting final knockout construct pHB–BLTR was 13 kb in length. A mock construct prepared by inserting the 3′ 4.5-kb SacII–BamHI fragment into the same sites of pPNT served as a positive control for PCR screening of the ES cell clones. AK7 (1295S4/SvJaeSor) ES cells (107) were electroporated with 25 μg of NotI-linearized pH-B–BLTR DNA. The transfected cells were grown in DMEM media with 200 μg/ml G418 and 2 × 10–6 M gancyclovir for 10 d. Surviving clones (95) were tested for recombination using a neomycin-coding sequence primer, tcgcagcgcatcgccttctatcg, and a primer from the 3′ end of BLTR gene external to the knockout construct, gctggactcatacaagcact. Of the 25 positive clones, 15 were expanded, and the genotypes were confirmed by Southern blot analysis with 10–20 kb band, and the homozygous lanes (BLTR–/–) show the 6.5-kb band, and the homozgyous lanes (–/–) show the 6.5-kb mutant band. The heterozygous (+/–) lanes show both the wild-type and mutant bands. Mice deficient for BLTR were born at the expected Mendelian ratios, and were fertile. A three-primer PCR (Fig. 1 C) was used to establish the mouse colonies. Genotyping was performed using Southern blot analysis with the same probe indicated above or more routinely using a three-primer PCR reaction with the primers (I) tccgagcatcgcttgactgct, (II) tccgagcatcgcttgacctg, and (III) tccgagcatcgcttcat. All mice were housed in a specific pathogen-free barrier facility. Mice were 8–12 wk old at the time of use. All studies and procedures were approved by the Animal Care and Use Committee of Duke University Medical Sector.

Zymosan-induced peritoneal inflammation. Zymosan (Sigma-Aldrich) was prepared in PBS to a final concentration of 1 mg/ml as described (15), and 1.0 ml was injected intraperitoneally. Mice were killed by CO2 asphyxiation, and a peritoneal lavage was performed, and the genotypes were confirmed by Southern blot analysis with the same probe (BglII–SacII fragment) external to the 3′ end of the knockout construct (see Fig. 1 A). Six undifferentiated clones were individually microinjected into C57BL/6 blastocysts and transferred into pseudopregnant C57BL/6 mice. Chimeric mice generated from two individual cell clones resulted in immediate germline transmission, and the F1 (C57BL/6 and 129SvJ) offspring were used to establish the mouse colonies. Genotyping was performed using Southern blot analysis with the same probe indicated above or more routinely using a three-primer PCR reaction with the primers (I) tccgagcatcgcttgactgct, (II) tccgagcatcgcttgacctg, and (III) tccgagcatcgcttcat. All mice were housed in a specific pathogen-free barrier facility. Mice were 8–12 wk old at the time of use. All studies and procedures were approved by the Animal Care and Use Committee of Duke University Medical Sector.

Zymosan (Sigma-Aldrich) was prepared in PBS to a final concentration of 1 mg/ml as described (15), and 1.0 ml was injected intraperitoneally. Mice were killed by CO2 asphyxiation, and a peritoneal lavage was performed, 0, 4, 18, or 72 h after injection, with 9 ml of ice cold RPMJ medium containing 2% FCS and 2 mM EDTA. Total cell counts were determined with a hemocytometer, 50 μl of lavage fluid was cytospun and stained with Diff-Qwik, and the percentages of macrophages, neutrophils, eosinophils, and lymphocytes were determined from a count of at least 200 cells. These percentages were multiplied by the total cell number to obtain the number of peritoneal neutrophils and macrophages. In some experiments, levels of LT Bα in peritoneal lavage fluids were measured using an EIA kit from Cayman Chemicals following the manufacturer’s protocol.

Nautrophil calcium mobilization and chemotaxis. The 4-h zymosan-elicited peritoneal lavage cells contained >80% neutrophils and were used in functional assays. For calcium mobilization, 3 × 106 cells were washed and loaded with 1.0 μM IN D0–1 AM for 30 min at room temperature as previously described (6). Calcium traces were recorded in a Perkin-Elmer fluorescence spectrometer (model 650-19) with an excitation wavelength of 355 nm and an emission wavelength of 405 nm. Chemotaxis of peritoneal exudate cells was measured by a 48-well microchemotaxis chamber technique as described (6).

A radiodynamic A2a-adherent ear inflammation. Mice were intravenously injected with PBS containing 0.5% Evans blue and indomethacin (Sigma-Aldrich) at final amounts of 10 mg/kg body weight to minimize the contribution from cyclooxygenase products (14). The inside of the right ear of each mouse was painted with 20 μl of arachidonic acid (AA; 100 μg/ml in acetone; Sigma-Aldrich), and the left ear was painted with acetone alone. Ear thickness was measured at 0 and 90 min after AA application using a calibrated thickness gauge. Mice were killed at 0.5 ml of formamide at 55°C for 45 min. Evans blue extravasation was determined by measuring the absorbance at 610 nm in a spectrophotometer. Histological sections of the ears were stained with hematoxylin and eosin.

Results

Generation of BLTR-deficient mice. To selectively ablate BLTR, a targeting vector that deleted the coding region of BLTR between amino acids 81 and 316 and replaced it with the PGK-neomycin (PGK-Neo) cassette was constructed (Fig. 1 A). After electroporation into ES cells, screening of 95 individual neomycin- and gancyclovir-resistant colonies by PCR yielded 25 positive recombinant targeted clones. A 3′ probe was selected for Southern blots screening of 15 of these clones, and the results indicated that they were all correctly targeted (data not shown). Of the six clones injected into C57BL/6 blastocysts, all yielded chimeric mice; four of the males succeeded in efficient germline transmission. All of the animals in this study consisted of offspring derived from the crossings of F1 heterozygous mice. Fig. 1B shows a Southern blot of BamHI and XhoI-digested DNA from the three genotypes. The wild-type littermate lanes (+/+), the heterozygous (+/–), and the homozygous lanes (–/–) show the 5.2-kb band, and the homozygous lanes (–/–) show the 6.5-kb mutant band. The heterozygous (+/–) lanes show both the wild-type and mutant bands. Mice deficient for BLTR were born at the expected Mendelian ratios, showed no overt developmental or morphological abnormalities, and were fertile. A three-primer PCR (Fig. 1 C) was routinely used to determine the genotypes from the DNA isolated from tail biopsies.

To confirm that the mutation disrupted BLTR expression and not other chemoattractant receptors, zymosan-elicited peritoneal exudate cells (>80% neutrophils) were analyzed for calcium mobilization and chemotaxis. LT Bα C5a, PAF all induced calcium mobilization in cells.
from littersmate wild-type animals (Fig. 2 A). In contrast, cells from the BLTR<sup>−/−</sup> animals showed no calcium mobilization in response to LT<sub>B</sub><sup>4</sup> but showed equivalent responses to C5a and PAF compared with cells from BLTR<sup>+/−</sup> mice. In addition, no calcium response was observed even at 1.0 μM LT<sub>B</sub><sup>4</sup> in cells from BLTR<sup>−/−</sup> animals (data not shown). Hemotaxis assays also showed no response to LT<sub>B</sub><sup>4</sup> by BLTR<sup>−/−</sup> cells, whereas cells from both BLTR<sup>−/−</sup> and BLTR<sup>+/−</sup> animals showed similar levels of migration to C5a (Fig. 2 B). Peritoneal injection of LT<sub>B</sub><sup>4</sup> led to a rapid neutrophil influx in BLTR<sup>+/−</sup> mice that was completely abrogated in BLTR<sup>−/−</sup> mice (Fig. 2 C; P < 0.05). No statistically significant differences were observed in macrophage numbers at this time point.

Analysis of lymphoid tissues found no gross alterations in the size of the thymus, spleen, or lymph nodes between BLTR<sup>−/−</sup> and BLTR<sup>+/−</sup> littersmates. The number and distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes or B220<sup>+</sup> B lymphocytes found within the spleen, peripheral lymph nodes, mesenteric lymph nodes, or within the blood were similar in BLTR<sup>−/−</sup> and BLTR<sup>+/−</sup> animals (data not shown). In addition, no significant differences were found in numbers of circulating lymphocytes, monocytes, neutrophils, or eosinophils or in serum IgG and IgM levels between the BLTR<sup>−/−</sup> and BLTR<sup>+/−</sup> mice (data not shown).

Peritoneal Inflammation Induced by Zymosan. To assess the role of BLTR in neutrophil and macrophage recruitment in response to a “nonspecific” inflammatory stimulus, zymosan was injected into the peritoneum of BLTR<sup>+/−</sup> and BLTR<sup>−/−</sup> mice. In uninjected mice, there were no significant differences in the resident peritoneal leukocyte populations in the BLTR<sup>+/−</sup> versus BLTR<sup>−/−</sup> mice (Fig. 3). In contrast, 4 and 18 h after zymosan injection, significantly fewer neutrophils were recovered from BLTR<sup>−/−</sup> relative to BLTR<sup>+/−</sup> mice (Fig. 3 A; P < 0.05). Likewise, significantly lower numbers of macrophages were recovered from BLTR<sup>−/−</sup> relative to BLTR<sup>+/−</sup> mice at 18 h after injection (Fig. 3 B; P < 0.01). However, at 72 h after injection, no differences were seen in the recovery of either cell type from BLTR<sup>−/−</sup> or BLTR<sup>+/−</sup> mice. Levels of LT<sub>B</sub><sup>4</sup> in peritoneal lavage fluids at 4 h ranged from 1.2 ± 0.3 ng/ml in BLTR<sup>+/−</sup> animals to 1.0 ± 0.2 ng/ml in BLTR<sup>−/−</sup> animals.

AA-induced Ear Inflammation. When applied topically, AA induces an acute inflammatory reaction involving both vascular leakage and cellular components that is highly dependent on leukotrienes (13–15). To determine which if
any of these inflammatory reactions were mediated by BLTR, the BLTR +/+ and BLTR −/− animals were treated with AA and edema, and vascular permeability was measured by changes in swelling, ear weight, and Evans blue dye extravasation. BLTR +/+ mice showed significant ear inflammation 90 min after AA application (Fig. 4). Specifically, the AA-treated ears showed a 60% increase in weight, 270% increase in leakage of Evans blue, and 63% increase in thickness (data not shown) compared with vehicle-only-treated ears. In contrast, AA-treated ears from BLTR −/− animals showed no significant increase in thickness or weight and only a 118% increase in Evans blue leakage compared with control ears. Histological examination of the tissue showed neutrophil infiltration only in BLTR +/+ and not in BLTR −/− mice (Fig. 4, C and D).

**PAF-induced Anaphylaxis.** PAF is an important endogenous mediator of systemic anaphylactic shock in mice (13). Intravenous injection of PAF leads to hypotension, increased vascular permeability, vasodilation, bronchospasm, and endothelial adhesion and activation of neutrophils, macrophages, and eosinophils, resulting in death within an hour. Previous studies indicated that leukotrienes are important if not sole mediators of this response (13–16). PAF, when administered at a dose of 200 μg/kg body weight, resulted in the death of 16 of 20 BLTR +/+ animals. This was not significantly different from the death rate of 13 of 20 BLTR −/− animals. However, a significant difference was observed in the survival of BLTR −/− animals when the results were segregated by sex. As shown in Table I, the female BLTR −/− animals displayed a selective advantage, with 6 of the 9 animals surviving PAF treatment, whereas only 1 of 11 male BLTR −/− animals survived the treatment. By contrast, the littermate BLTR +/+ male and female animals did not show any significant differences in their survival.

**Discussion**

The deletion of the BLTR gene reported in this study reveals the critical role this receptor plays in acute inflammation and immediate hypersensitivity. The BLTR −/− mice were viable, developed and reproduced normally, and displayed no overt behavioral or morphological defects. The number and the development of lymphocyte subpopulations was normal in BLTR −/− mice. Although the BLTR −/− mice exhibited no obvious phenotype in a specific pathogen-free environment, exposure to inflammatory challenges revealed significant defects in neutrophil and macrophage recruitment and conferred a survival advantage in females to PAF-induced anaphylaxis.

The lack of developmental defects correlates with the observation that mice deficient for 5-LO, FLAP, or LTA4 hydrolase all develop normally, indicating that neither enzymatic production of leukotrienes nor their action on specific receptors is essential for growth or development (13–16). Similarly, no developmental defects have been observed in mice deficient for C5a or N-formylpeptide receptors, indicating the nonessential nature of individual neutrophil chemoattractant receptors (22, 23). The observation that elicited peritoneal neutrophils did not display any calcium mobilization or chemotaxis to LTBA4 and the complete absence of neutrophil accumulation to peritoneal LTBA challenge in the BLTR −/− mice indicates that BLTR is likely the sole LTBA4-responsive G protein-coupled receptor mediating inflammatory responses in mice.

Recent studies demonstrated that transgenic mice overexpressing human BLTR displayed a profound increase in neutrophil recruitment in skin inflammation, peritonitis, and reperfusion-initiated second organ injury (24). These results are in agreement with the current studies that neutrophil infiltration is critical for neutrophil accumulation to peritoneal LTBA challenge in the BLTR −/− mice. This was demonstrated by the observation that mice deficient for 5-LO, FLAP, or LTA4 hydrolase all develop normally, indicating that neither enzymatic production of leukotrienes nor their action on specific receptors is essential for growth or development (13–16). Similarly, no developmental defects have been observed in mice deficient for C5a or N-formylpeptide receptors, indicating the nonessential nature of individual neutrophil chemoattractant receptors (22, 23). The observation that elicited peritoneal neutrophils did not display any calcium mobilization or chemotaxis to LTBA4 and the complete absence of neutrophil accumulation to peritoneal LTBA challenge in the BLTR −/− mice indicates that BLTR is likely the sole LTBA4-responsive G protein-coupled receptor mediating inflammatory responses in mice.

**R eferences**

1. Recent studies demonstrated that transgenic mice overexpressing human BLTR displayed a profound increase in neutrophil recruitment in skin inflammation, peritonitis, and reperfusion-initiated second organ injury (24). These results are in agreement with the current studies that neutrophil influx was reduced at early times (4 and 18 h) in zymosan-induced peritonitis. In addition, a reduction in the 18-h recruitment of macrophages suggests a role for LTBA4 in the early times of monocyte/macrophage migration during peritoneal inflammation. Zymosan activates complement to produce C5a, resulting in neutrophil influx (25). The data from the studies in this paper indicate that LTBA4 and BLTR also play a role in this process. Indeed, previous studies indicated that LTBA4 is the major AA metabolite produced by the entering neutrophils in murine zymosan-induced peritonitis (25). LTBA4 likely produced by the resident macrophages and/or early accumulating neutrophils may be involved in an autocrine loop of neutrophil accumulation during inflammation. The early reduction of neutrophil influx in BLTR −/−
mice may have resulted in reduced synthesis of other mediators for further monocyte influx. However, the similar macrophage levels at 72 h after zymosan treatment in the BLTR\(^{-/-}\) and BLTR\(^{+/+}\) mice indicate the existence of as yet unknown compensatory mechanisms.

Previous studies with mice deficient in leukotriene synthesis showed decreased inflammatory responses to AA (14–16). Although the relative contributions of LT\(_B_4\) and LTC\(_4\) were not clear, both protein extravasation and cellular infiltration were affected. Intermediate responses were observed in LT\(_A_4\) hydrolase–deficient mice relative to 5-LO– and FLAP-deficient mice. However, the LT\(_A_4\) hydrolase–deficient mice produced larger amounts of LTC\(_4\) relative to control mice. Current studies with BLTR\(^{-/-}\) mice indicate that both edema and cellular components of inflammation were decreased, suggesting a role for BLTR in both vascular leakage and neutrophil accumulation.

A surprising observation from the current studies in this paper is the relative resistance of female BLTR\(^{-/-}\) mice to PAF-induced immediate hypersensitivity. Previous studies with 5-LO– and FLAP-deficient animals showed a strong protection from PAF-induced lethality (13–15). However, sex differences in survival, if any, were not reported. These studies show clear differences in the survival of female versus male BLTR\(^{-/-}\) mice to challenge with PAF. Several autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) display sex-dependent predilection, with females being most severely affected (26, 27). In autoimmune disease–prone MRL-\(lpr/lpr\) mice, females are more susceptible to SLE-like pathology, whereas males appear to express increased inflammatory responsiveness to leukotrienes (28). Studies with 5-LO–deficient mice on the MRL-\(lpr/lpr\) background showed that males lost their survival advantage and became as susceptible to disease as females, suggesting that leukotrienes protect against SLE-like disease (28). The relevance, if any, of the sex-related difference in PAF anaphylaxis in BLTR\(^{-/-}\) mice to autoimmune diseases is unknown at this time. The loss of male advantage against SLE in 5-LO–deficient MRL-\(lpr/lpr\) mice needs to be placed in context with our results of gain of a female advantage in BLTR\(^{-/-}\) mice. We postulate that a threshold level of leukotriene responsiveness provides protection against autoimmune disease but produces susceptibility to PAF-mediated anaphylaxis. If so, males may have greater leukotriene responsiveness than females. The mice with 5-LO or BLTR deficiency cross these thresholds to result in either male susceptibility to SLE or female protection to PAF anaphylaxis, respectively. Clearly, PAF-induced anaphylaxis is a complex process, as all 5-LO–deficient mice are protected but the male BLTR\(^{-/-}\) mice are not. Further studies on hormonal effects will likely delineate the sex-dependent leukotriene effects in these mice.

### Table I. Sex-related Differences in PAF-Induced Anaphylaxis

| Genotype: BLTR\(^{+/+}\) | BLTR\(^{-/-}\) |
|------------------------|--------------|
| Sex                    | Male | Female | Male | Female |
| Survivors/total         | 3/11 | 1/9    | 1/11 | 6/9    |

Mice were injected intravenously with PAF at a dose of 200 \(\mu g/\text{kg body weight}\) and observed for 24 h. The total number of animals tested for each genotype and sex is shown separately with the number of surviving animals. The difference in mortality between female and male BLTR\(^{-/-}\) mice is statistically significant (\(P = 0.002\), Fisher’s exact test). PAF-induced immediate hypersensitivity. Previous studies with 5-LO– and FLAP-deficient animals showed a strong protection from PAF-induced lethality (13–15). However, sex differences in survival, if any, were not reported. These studies show clear differences in the survival of female versus male BLTR\(^{-/-}\) mice to challenge with PAF. Several autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) display sex-dependent predilection, with females being most severely affected (26, 27). In autoimmune disease–prone MRL-\(lpr/lpr\) mice, females are more susceptible to SLE-like pathology, whereas males appear to express increased inflammatory responsiveness to leukotrienes (28). Studies with 5-LO–deficient mice on the MRL-\(lpr/lpr\) background showed that males lost their survival advantage and became as susceptible to disease as females, suggesting that leukotrienes protect against SLE-like disease (28). The relevance, if any, of the sex-related difference in PAF anaphylaxis in BLTR\(^{-/-}\) mice to autoimmune diseases is unknown at this time. The loss of male advantage against SLE in 5-LO–deficient MRL-\(lpr/lpr\) mice needs to be placed in context with our results of gain of a female advantage in BLTR\(^{-/-}\) mice. We postulate that a threshold level of leukotriene responsiveness provides protection against autoimmune disease but produces susceptibility to PAF-mediated anaphylaxis. If so, males may have greater leukotriene responsiveness than females. The mice with 5-LO or BLTR deficiency cross these thresholds to result in either male susceptibility to SLE or female protection to PAF anaphylaxis, respectively. Clearly, PAF-induced anaphylaxis is a complex process, as all 5-LO–deficient mice are protected but the male BLTR\(^{-/-}\) mice are not. Further studies on hormonal effects will likely delineate the sex-dependent leukotriene effects in these mice.
In summary, this study has shown that deletion of BLT R reduced the vascular and cellular components of acute inflammatory responses in mice. In addition, these data suggest a role for BLT R in mediating systemic anaphylaxis, with female mice being more dependent on this pathway than males. Inhibition of BLT R function may provide a target for therapeutic intervention in certain human inflammatory conditions. Further studies with expression of the BLT R deficiency on different strain backgrounds will allow investigation of the role of BLT R in asthma, rheumatoid arthritis, lupus, and colitis as well as infectious diseases.

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