Journal of Biological Methods | **2023** | **Vol**. 10 | e99010008
DOI: 10.14440/jbm.2023.408

ARTICLE

Utilizing a human TLR selective ligand in a humanized immune system mouse model to investigate human TLR4 signaling

Rachel Twomey[1,*], Sean Graham[1], Joseph S. Spina[1], Xiaoming Wu[1], Philip E. Dubé[2], Courtney Ferrebee[2], William Housley[1]

[1]AbbVie Bioresearch Center, 100 Research Drive, Worcester, MA 01605
[2]Taconic Biosciences, Inc., 5 University Place, Rensselaer, NY 12144

***Corresponding authors:** Rachel Twomey, e-mail: Rachel.Twomey@abbvie.com.

Abbreviation used: AGP, aminoalkyl glucosaminide 4-phosphate; APC, antigen presenting cell; CD, cluster of differentiation; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; GLA, glucopyranosyl lipid A; HSC, CD34+ hematopoietic stem cell; IL, interleukin; IRAK4, interleukin 1 receptor associated kinase 4; IC_{50}, median inhibition concentration; IFN, interferon; IP, intraperitoneally; IRF, interferon regulatory factor-3; kg, kilogram; LPS, lipopolysaccharide; MD2, myeloid differentiation factor-2; mg, milligram; mL, milliliter; MPL, monophosphoryl lipid A; MyD88, myeloid differentiation primary response 88; NFkB, nuclear factor kappa b; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; pg, picogram; PO, *per os*/by mouth, PRR, pattern recognition receptor; SEM, standard error of the mean; TLR, toll-like receptor; TNFα, tumor necrosis factor-alpha; TRIF, TIR domain-containing adaptor inducing interferon-beta; µg, microgram; µL, microliter

Received August 7, 2023; Revision received October 19, 2023; Accepted October 23, 2023; Published November 20, 2023

ABSTRACT

Mouse models with humanized immune systems are becoming increasingly prevalent in pharmaceutical research as a platform for preclinical testing with potential for greater translatability to clinical applications. However, the presence of both mouse and human cells that respond to TLR ligands poses a challenge for investigating therapeutic modalities targeting TLR signaling. AZ617 is a human TLR4 agonist, which has been shown *in vitro* to preferentially induce human cytokines via the TLR4 signaling pathway. We sought to examine the ability of AZ617 to preferentially induce human cytokines in CD34+ stem cell-engrafted NOG-EXL mice (huNOG-EXL), to determine its suitability as an *in vivo* human functional readout. AZ617 elicited a strong human TNFα and IL-6 response *in vivo* that demonstrated a 10- and 5-fold preference, respectively, over the mouse TNFα and IL-6. To assess efficacy of inhibiting a key protein in the TLR4 signaling pathway, PF-06650833, a small molecule inhibitor of IRAK4, was used as a tool molecule. PF-0660833 was found to effectively inhibit AZ617-induced human TNFα release *in vitro*. Likewise, PF-06650833 reduced AZ617-induced human TNFα in the huNOG-EXL mouse model, with a weaker effect on human IL-6. A longitudinal study tracking functionality of monocytes revealed that the ability of monocytes to respond to *ex vivo* stimuli was increased by 21 weeks after engraftment. Taken together, our data suggests that human selective TLR ligands could preferentially drive cytokine production from human cells in huNOG-EXL mice. This model will allow for investigation of pharmacological inhibition of human TLR signaling pathways in an *in vivo* model system.

Keywords: AZ617, humanized mouse, huNOG-EXL, human hematopoietic stem cell, toll-like receptor, human cytokine induction, steroid, IRAK4

INTRODUCTION

Pre-clinical mouse models for immunological research are well-characterized and widely used to test agents targeting various aspects of the immune response. However, a common hurdle in the development of therapeutics for autoimmune indications is the differences in relevant immunological signaling cascades between human and model species [1,2]. Screening candidate molecules in rodents provides critical pharmacokinetic (PK) data regarding systemic absorption and metabolism, and pharmacodynamic (PD) changes that the drug elicits along the target pathway, which aid in decision-making in drug discovery. While many canonical innate immune features are conserved between mice and humans, even minor divergences can result in over- or under-estimation of the effects of a pharmacological intervention on humans [3-5]. In addition, some targets of interest are only expressed on human cells, limiting our capability to assess these targets pre-clinically in mouse models [6]. Development of translatable humanized models for immunological research

How to cite this article: Twomey R, Graham S, Spina JS, Wu XM, Dubé PE, Ferrebee C, Housley W. Utilizing a human TLR selective ligand in a humanized immune system mouse model to investigate human TLR4 signaling. *J Biol Methods* 2023;10:e99010008. DOI: 10.14440/jbm.2023.408

is important for the successful advancement of drug candidates for clinical use.

Recently, murine models in which super-immunodeficient mice are reconstituted with human immune cells [hematopoietic stem cells (HSC) or peripheral blood mononuclear cells (PBMC)], have emerged as a strategy for characterizing drug candidates in mice while ensuring that the on-target activity and mechanism of action are validated on human cells [1,7]. Much of the current research in humanized mice is in immuno-oncology, in well-characterized models where patient-derived tumor xenografts (PDX) are implanted into mice bearing a humanized immune system, to better capture the human anticancer immune response [8,9]. The ability for human immune cells to infiltrate the tumors in these humanized PDX models has allowed for improved ways of pre-clinically assessing targeted immune therapy and immune checkpoint blockade in a human-relevant environment [10-12]. Treatment response in humanized PDX models has been shown to correlate with clinical data [13], thereby supporting the utility of humanized models in cancer research and strengthening the notion that humanized models for immunological targets could provide translatable pre-clinical data.

Conveniently, some humanized models are commercially available and ready-to-use, removing certain challenges associated with the development of stably engrafted humanized mice in-house. NOG mice are super-immunodeficient due to the *Prkdc*^{scid} mutation on an NOD background plus a targeted mutation in the gamma chain of the IL-2 receptor (*Il2rg*^{tm1Sug}), resulting in lack of development of murine immune cells due to deficient cytokine signaling and defects in V(D)J recombination [14,15]. Moreover, NOG-EXL mice transgenically express human GM-CSF and IL-3, which supports the expansion of a human myeloid compartment in addition to the lymphoid compartment, resulting in a more complete humanized immune system following human HSC engraftment [1,14]. Toll-like receptors (TLRs) are evolutionarily conserved receptors which play a crucial role in innate immunity. TLR4, the most widely studied of the TLR family, is a cell-surface receptor that recognizes bacterial lipopolysaccharide (LPS) and upon engagement, signals the release of pro-inflammatory cytokines and chemokines [4,16,17]. Even though TLR4 functions similarly across species, species-specific differences exist and need to be considered when using pre-clinical pharmacological models. Several groups have suggested that differences in myeloid differentiation factor-2 (MD-2), a molecule which associates with TLR4 and is required for signaling, may play a role in immune response differences between species [18-20]. It is possible that species-specific MD-2 recognizes different LPS ligands. For example, LPS-A is easily recognized by human and mouse, but LPS-IVa and Taxol are only recognized by mouse [18,19,21]. Given this information, using a human-specific TLR4 agonist in humanized mice may result in more robust agonism of human TLR4 without contribution from murine TLR signaling.

Marshall and colleagues have developed a new class of small molecule TLR4 agonists, known as the Ugi compounds, which display preferential activity for human over mouse TLR4 [21]. Using HEK cells transfected with combinations of human or mouse TLR4 or MD2, they found that the Ugi compounds had the highest activity on cells transfected with both human TLR4 and MD2. Moreover, they found that cells transfected with hTLR4 in the absence of MD2 did not respond to stimulation, suggesting that MD2 is required for the event. The highest observed responses to the Ugi compounds were in cells expressing hMD2, regardless of the TLR4 species, suggesting that the MD2 component is critical for human-specific activation of the TLR4 pathway [21]. Of the Ugi compounds tested, AZ617 potently induced human, but not mouse cytokines and is easily solubilized in aqueous solution, rendering it an ideal candidate for *in vivo* studies [21].

In this study, we characterized the ability of AZ617 to preferentially induce human cytokines *in vitro* using human PBMCs and mouse splenocytes. We then examined whether administration of AZ617 to huNOG-EXL mice resulted in preferential induction of human cytokines *in vivo*. We hypothesized that AZ617 would induce human-specific cytokines in huNOG-EXL mice, and that pharmacological intervention with a small molecule IRAK4 inhibitor would decrease or prevent cytokine release, thus providing a straightforward immunological PD model in which human-specific drug candidates can be tested and possibly provide greater clinical translatability.

MATERIALS AND METHODS

Mice

All animal work was performed under the guidelines of AbbVie's Institutional Animal Care and Use Committee. Female C57BL/6NTac (C57BL/6) mice and human CD34+ hematopoietic stem cells (HSC)-engrafted female NOG-EXL [NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}Tg(SV40/HTLV-IL3,CSF2)10-7Jic/JicTac] mice (huNOG-EXL) used for *ex vivo* stimulation studies were purchased from Taconic Biosciences (Rensselaer, NY). Female huNOG-EXL mice used for *in vivo* stimulation studies were provided by Taconic. Mice were housed 3-5/cage and given Teklad 2914-irradiated rodent diet and sterile water *ad libitum*. Housing rooms were maintained at temperature and humidity consistent with the IACUC guidelines. It is recommended that immunodeficient mice are housed in sterilized cages containing sterilized bedding, food, and water. huNOG-EXL mice are often anemic, so if multiple blood sampling is required, extra care should be exercised to collect only the minimum amount of blood required and to provide recovery and hydration support.

Cells and reagents

Human LEUKOMAX products were purchased from BioIVT (Westbury, NY) and peripheral blood mononuclear cells (PBMCs) were isolated via magnetic negative selection with a MultiMACS Cell24 Separator (Miltenyi, Gaithersburg, MD) prior to cryo-

preservation. Human whole blood was collected from healthy volunteer donors (Sanguine, Waltham, MA) into CPT tubes (BD Biosciences, 362753) and centrifuged to isolate PBMCs to be used fresh without cryopreservation. These healthy donor samples were taken from consented and deidentified volunteers at AbbVie Bioresearch Center under IRB approved protocol SAN-05252 through a service agreement with Sanguine Biosciences. Donor demographic information can be found in **Table S2**. Naïve mouse splenocytes were isolated from C57BL/6 mouse spleens [14] that were crushed through 100 µm filters (Falcon, 352360) and subjected to red blood cell lysis using 1X eBioscience RBC Lysis Buffer, multi-species (eBioscience, 00-4300-54). In all *in vitro* studies, cells were resuspended in RPMI 1640 (Thermo Fisher, 11875-085) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher, 10438-026), 1% HEPES (Thermo Fisher, 15630-080), and 1% penicillin-streptomycin (Thermo Fisher, 10378-016). AZ617 (AOBIOUS, AOB8299) was resuspended in 100% DMSO (Sigma, D2650), aliquoted, and frozen at -20ºC for long-term storage.

In vitro studies

For titration studies, AZ617 was titrated on 500,000 cells of either species per well containing DMSO with a final concentration of 0.5% and incubated at 37ºC and in 5% CO_2 for 24 hours. For TLR4-dependency studies, human PBMCs were pre-incubated with 12.5 µg/mL anti-human TLR4 blocking antibody (Invivogen, mabg-htlr4) for 1 hour at 37ºC prior to stimulation with 50 ng/mL AZ617 for an additional 24 hours. For inhibition studies, cells ± dexamethasone or PF-06650833 (both AbbVie) dose responses were pre-incubated at 37ºC for 30 minutes prior to stimulation with 50 ng/mL AZ617 for an additional 24 hours. For all *in vitro* experiments, supernatants were pulled and secreted cytokines were quantified using Meso Scale Discovery (MSD) assays according to the manufacturer's instructions. Dose-response curves were generated by using GraphPad Prism version 9 and fit to a logistic, four-parameter function to identify the IC_{50}. Unpaired *t*-tests were performed using GraphPad Prism version 9.

In vivo dose response experiments

huNOG-EXL mice were used at approximately 16 weeks post-engraftment (WPE) of human HSC (approximately 21-22 weeks of age). On average, mice weighed approximately 20 grams and dose calculations were based on this average weight. Approximately 2-4 days prior to the beginning of the study, a small blood sample was collected by puncture of the retro-orbital sinus under oxygen/isoflurane anesthesia and analyzed by flow cytometry for human CD45+ cell frequencies. %CD45+ values were used to normalize the treatment groups, and mice were enrolled appropriately. LPS (Sigma, L4130) and AZ617 were formulated in sterile 1X phosphate-buffered saline (PBS), and bath sonicated for dissolving prior to dosing. Mice were challenged intraperitoneally [22] with LPS at 10 mg/kg, or AZ617 at 50, 150, 300, or 500 µg/mouse. At 1, 3 and 6-hours post-challenge, approximately 100-150 µl of blood was harvested by retro-orbital puncture under isoflurane/oxygen anesthesia into K2EDTA Microtainer tubes (BD Biosciences) for cytokine measurement and flow cytometry (6 h only). Mice were humanely euthanized by isoflurane overdose after the 6-hour time point, and spleens were collected into sterile PBS for flow cytometric analysis.

Efficacy experiments

Studies were performed at approximately 16 WPE, and pre-study engraftment checks and normalization of treatment groups were performed as previously described. Vehicle for LPS and AZ617 was sterile 1X PBS. Vehicles used for the IRAK4 inhibitor (PF-06650833) were 10:30:60 EtOH:PEG400:Phosal50PG (experiment 1) and 5:10:10:30:25:20 DMSO:PEG400:PG:MaisineCC:KolliphorEL:LabrafilM1944CS (experiment 2). PF-06650833 was administered at 100 mg/kg orally [22], 30 minutes prior to challenge. At the time of challenge, LPS was administered IP at 5 mg/kg (200 µl of a 0.5mg/mL solution), and AZ617 was given IP at 500 µg/mouse. Sample collections were performed as described for the dose response experiments, except spleens were not collected for flow cytometry.

Longitudinal assessment of functional response

Three mice each reconstituted with CD34+ HSCs from 3 different donors were followed longitudinally to assess functional response of myeloid cells to *ex vivo* stimuli over time. Blood was collected every 2 weeks from each mouse by retro-orbital sinus puncture as previously described, starting at 17-18 WPE (22-24 weeks of age) and ending at 23-24 WPE (28-30 weeks of age). After each bleed, 2 ml of lactated Ringer's solution was administered subcutaneously to support recovery and hydration. Additionally, Hydrogel (ClearH_2O, 70-01-5022) was provided on the cage bottoms throughout the duration of the study. At each time point, blood samples were processed by lysing red blood cells in a 1X solution of RBC Lysis Buffer, multi-species for 15 minutes at room temperature, followed by addition of 1X PBS to neutralize buffer and by centrifugation at 300 g for 5 minutes to pellet cells. One sample per donor was flow cytometrically assessed for presence and frequencies of human CD45+ leukocytes and CD14+ monocytes. Total leukocyte pellets (n=1-3/donor, depending on the time point) were stimulated overnight with 10 ng/ml LPS (Sigma, L5293) with 10 ng/ml recombinant human IFNγ (PeproTech, 300-02), and supernatants were collected for measurement of human cytokines (TNFα, IL-6, IL-1β, IL-8) by MSD immunoassay (Meso-Scale Discovery, K15053D).

Flow cytometry

50-200 µL aliquots of blood were pipetted either into individual 15 ml conical tubes or into individual wells of a 96-well 2 ml assay block. 5 ml (dose response and experiment 1) or 1 ml (experiment 2 and longitudinal study) of a 1X solution of RBC lysis buffer, multi-species (diluted from 10X stock in dH_2O) was

added to each tube/well, and samples were incubated at room temperature for 10-15 minutes. 10 mL of 1X PBS was added to neutralize the lysis buffer and centrifuged at 300 g for 5 minutes to pellet the cells. Supernatants were decanted and samples were resuspended in 200 µL of FACS buffer, then moved to a 96-well non-treated, round-bottom plate for staining. Plate was centrifuged at 300 g for 5 minutes and supernatants were poured off. Cells were resuspended in pre-diluted live/dead Near-IR (Life Technologies) and incubated at room temperature for 5-10 minutes, protected from light. Samples were washed with FACS buffer and centrifuged as described to remove live/dead dye. Samples were resuspended in 50 µL total volume blocking solution, containing 2 µL each of Human TruStain FcX (Biolegend) and rat anti-mouse CD16/32 (BD or eBioscience) Fc receptor block and incubated for 10 minutes at room temperature, protected from light. 50 µL of a surface marker staining cocktail was added and incubated on ice or at 4ºC for 20-30 minutes, protected from light. Surface markers used were anti-huHLA-DR BUV395 [BD], and anti-huCD16 BV421, anti-huCD20 BV510, anti-huCD19 BV711, anti-huCD45 BV785 or PE, anti-huCD14 FITC or BV421, anti-CD11c PE-Cy7, anti-huCD3 AF700, anti-huCD11b PE Dazzle 594, and anti-muCD45 BV605 or AF647 (Biolegend). Complete information for all staining antibodies can be found in **Table S1**. After staining, the cells were washed twice with FACS buffer, with then 200 µL PBS added, and acquired on a BD LSR Fortessa. Data were analyzed using FlowJo version 10 and graphed using GraphPad Prism version 9. Statistical analyses were performed in GraphPad.

Cytokine measurement in plasma samples from *in vivo* studies

After pulling blood aliquots for FACS staining from the *in vivo* studies, the remaining blood was centrifuged at top speed on a benchtop microcentrifuge to separate plasma. Plasma was carefully pipetted off and placed in 96-well round-bottom plates for assessment of human and mouse cytokine panels. Pre-configured panels from Meso-Scale Discovery (MSD) were used, including V-Plex Proinflammatory Panel 1 Human Kit (IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα) and U-Plex TH1/TH2 Combo Mouse (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, KC/GRO, TNFα). An individual MSD kit was used to measure mouse IL-6. For *ex vivo* LPS stim studies, a pre-configured panel from MSD containing TNFα, IL-1β, IL-6, and IL-8 was employed to measure human cytokine production.

RESULTS

AZ617 *in vitro* characterization

To confirm the species specificity of the TLR4 agonist AZ617, naïve healthy human PBMCs and naïve mouse splenocytes were incubated for 24 hours with dose titrations of compound. Concentration-dependent secretion of human, but not mouse TNFα, IL-6, IL-1β, and IFNγ were detected by MSD cytokine assays from the culture supernatants (**Fig. 1A**). AZ617 did not induce expression of IFNα in either human or mouse cells (**Fig. S1**), suggesting the activity was primarily NFκB-mediated. Pre-treating human PBMCs with a human-specific TLR4 blocking antibody prior to stimulation with AZ617 resulted in a significant reduction of secreted human TNFα and human IL-6, supporting that AZ617 is a TLR4-dependent agonist (**Fig. 1B**).

AZ617-induced TNFα release was inhibited by the pharmacological agents dexamethasone and PF-06650833, a small molecule inhibitor of interleukin 1 receptor-associated kinase 4 (IRAK4), with an IC_{50} of 0.0036 and 0.015 µM, respectively (**Fig. 2**).

AZ617 *in vivo* dose response

Treatment with AZ617 resulted in a dose-dependent increase of human TNFα and IL-6 *in vivo* in huNOG-EXL mice (**Fig. 3**) and demonstrated a selectivity towards human TLR4 agonism. Human TNFα was detectable by 3 h post-challenge with both 300 and 500 µg doses, resulting in 15-fold or 10-fold higher levels, respectively of human TNFα compared to mouse TNFα (**Fig. 3A**). Interestingly, the 500 µg, but not the 300 µg dose, resulted in continued elevation of TNFα for up to 6 hours, where an approximate 15-fold greater level of human TNF versus mouse TNF was maintained (**Fig. 3A**). Similarly, human IL-6 was induced to a greater degree than mouse IL-6 at both the 300 and 500 µg doses at the 3 and 6 h time points (**Fig. 3B**). The 300 µg dose resulted in a greater window between human and mouse IL-6 levels vs. the 500 µg dose at both 3 and 6 h, demonstrating a 10-fold or 25-fold window, respectively (**Fig. 3B**).

In vivo efficacy using IRAK4 inhibitor

Consistent with observations from the dose response experiment (**Fig. 3**), treatment with 500 µg AZ617 resulted in significantly higher levels of circulating human TNFα at 3 h as compared to mouse TNFα, and oral pre-treatment with 100 mg/kg of PF-06650833 30 minutes prior to challenge resulted in significant reduction of human TNFα (**Fig. 4A**). AZ617 resulted in elevated levels of human IL-6 over mouse IL-6, the difference being non-significant, and PF-06650833 did not significantly reduce circulating human IL-6 (**Fig. 4B**). The difference in circulating human cytokines between AZ617 and LPS was not significant for both TNFα and IL-6. Conversely, LPS resulted in mouse cytokine induction to a significantly greater degree than AZ617 (**Fig. 4**).

These data recapitulate what was captured *in vitro* with human PBMCs and mouse splenocytes, where AZ617 demonstrated a specificity towards human TNFα and IL-6 induction (**Fig. 1A**). Although human IL-6 levels were not significantly elevated over mouse IL-6 *in vivo* due to variability in the response, there was a trend towards greater human IL-6 induction, mimicking what was observed *in vitro* (**Fig. 1A**). Additionally, LPS demonstrated

greater induction of mouse cytokines *in vivo* compared to human cytokines, supporting the need for a human-specific TLR4 agonist to robustly induce human cytokines.

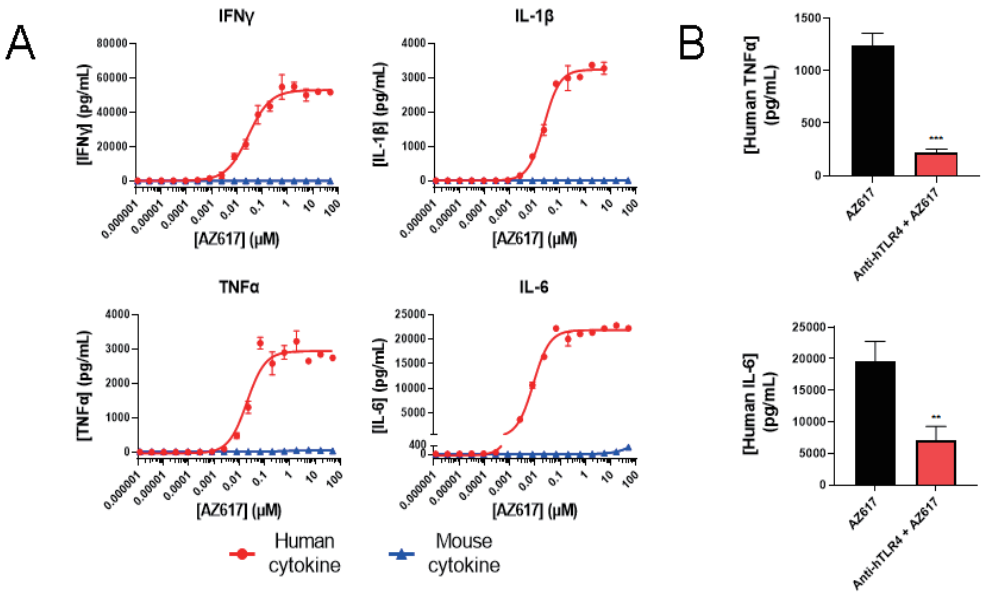
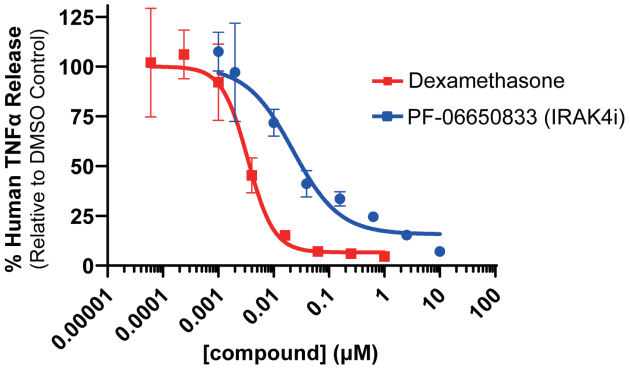


Figure 1. AZ617 is a human-specific, TLR4-dependent agonist that activates NFκB. A. Stimulation of human PBMCs, but not mouse splenocytes, with AZ617 for 24 hours effected a concentration-dependent release of NFκB-related cytokines IFNγ, IL-1β, TNFα, and IL-6. Representative graphs generated from 4 independent experiments. **B.** AZ617 activity was reduced when human PBMCs were pre-treated with an anti-human TLR4 blocking reagent for one hour prior to stimulation. Representative graphs derived from 2 independent experiments. Unpaired t-test **p<0.01, ***p<0.001. Error bars represent standard error of the mean (SEM).



	IC$_{50}$ (μM) ± SEM
Dexamethasone	0.0036 ± 0.0016
PF-06650833 (IRAK4i)	0.015 ± 0.0038

Figure 2. AZ617 activity was pharmacologically inhibited by dexamethasone and IRAK4 inhibitor PF-06650833. Human PBMCs were pre-incubated with compounds for 30 minutes prior to stimulation with 50 ng/mL AZ617 for 24 hours. Values are presented as a percent of DMSO control TNFα release. Representative graph was generated from 4 independent experiments. Table shows geometric mean ± standard error of the mean (SEM).

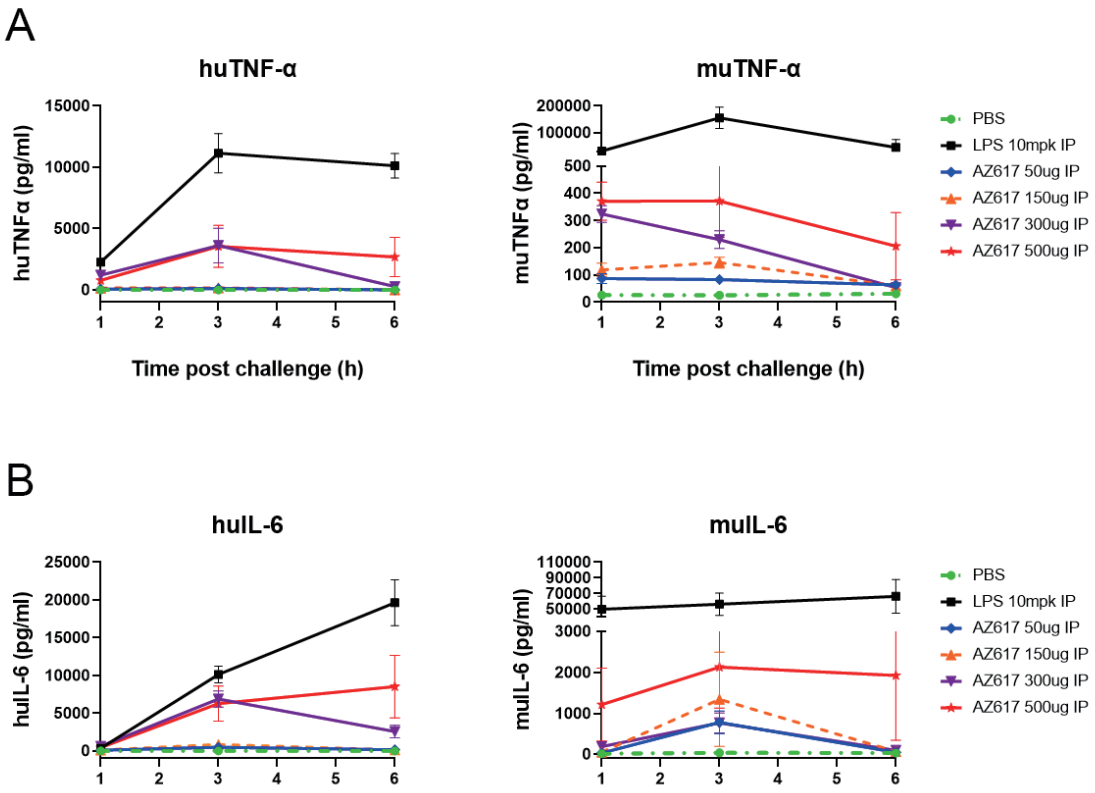


Figure 3. ***In vivo*** **treatment with AZ617 resulted in a dose-dependent increase of human TNFα and IL-6 in huNOG-EXL mice.** AZ617 and controls were administered IP, and blood was sampled at 1, 3, and 6 h post challenge via the retro-orbital sinus. **A.** Human TNFα was observable by 3 h post-challenge at both the 300 and 500 µg doses of AZ617 (left). Mouse TNFα levels were approximately 15-fold and 10-fold lower, respectively, at the 300 µg and 500 µg doses at 3 h (right). **B.** Human IL-6 was observable by 3 h post challenge at both the 300 and 500 µg doses (left). Mouse IL-6 was approximately 10-fold and 3-fold lower, respectively, at the 300 µg and 500 µg doses at 3 h. Error bars represent standard error of the mean (SEM). Data were from one independent experiment, n=5-6 mice/group.

Longitudinal *ex vivo* assessment of myeloid functional response

To assess the functional ability of human myeloid cells over time in the huNOG-EXL model, and to look at variability in functional response related to CD34+ cell donor, three small huNOG-EXL mouse cohorts generated from 3 separate CD34+ HSC donors were tracked over time for *ex vivo* cytokine production in response to LPS. Human CD14+ monocytes were present in each donor cohort at each time point and appeared to stabilize around 21-22 WPE. However there existed a marked variability in monocyte frequencies between donors (**Fig. 5A**). After *ex vivo* stimulation with LPS, there was an increase in human IL-6 and TNFα, respectively, beginning at 21-22 WPE (**Fig. 5B**-C) and consistent with the time at which the CD14+ population stabilized.

Similarly, there was a considerable variability in the cytokine response that is likely related to donor differences. Taken together, these data suggest that cohorts should be pre-screened for myeloid cell engraftment and that optimal engraftment occurs later than 21 WPE.

DISCUSSION

In the current study, we sought to develop a novel pre-clinical humanized mouse model that enables the profiling of therapeutics targeting proteins in the TLR4 pathway. huNOG-EXL mice contain primarily, but not exclusively, human myeloid and lymphoid lineage cells along with some residual murine immune cells. By challenging them with the human-specific TLR4 ligand AZ617, we aimed to generate a more specific and robust human cytokine response by minimizing contribution from murine TLR4 signaling. The resulting signaling cascade and effector functions of TLR pathway activation can then be inferred to be due to activation of cells of human, but not mouse origin, and drug treatments can then be studied in the target cells of interest.

In line with recent reports, our studies found that AZ617 is a human-specific TLR4-dependent agonist (**Fig. 1**) [21]. The activity of AZ617 was pharmacologically inhibited by dexamethasone, a broad immunosuppressive agent targeting the glucocorticoid receptor, as well as by PF-06650833, a specific inhibitor of IRAK4, currently in development for the treatment of rheumatoid

arthritis and other autoimmune indications/conditions (**Fig. 2**) [23]. These data suggest that AZ617 is a relevant human-specific agonist for profiling modulators of the TLR4 pathway.

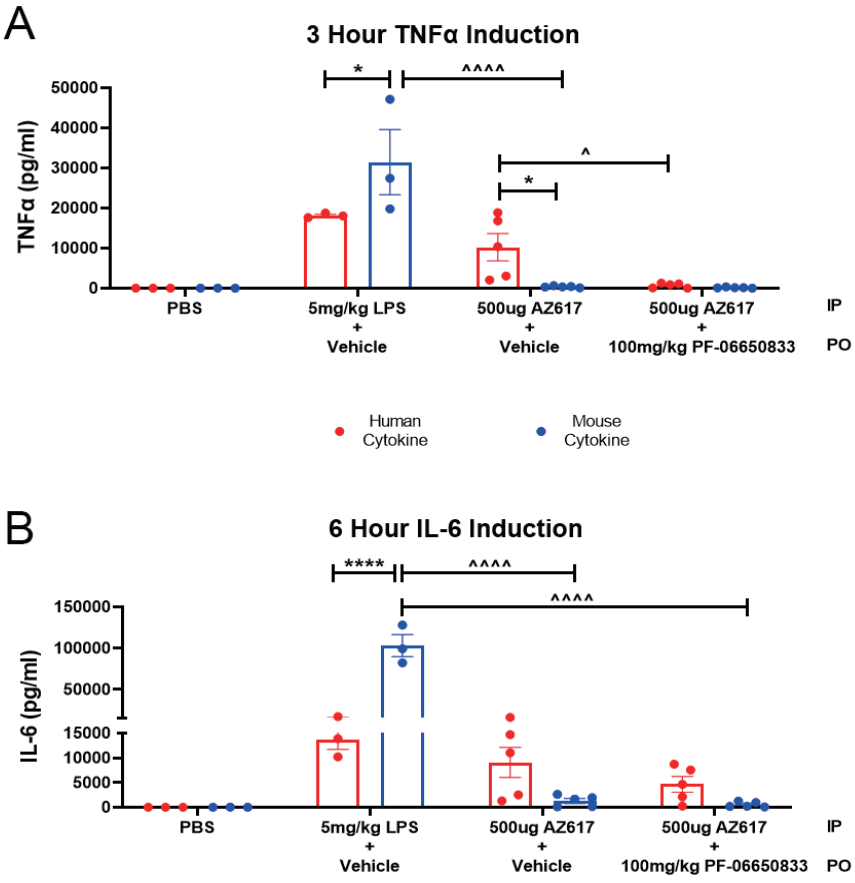


Figure 4. Inhibiting IRAK4 significantly reduced human TNFα following *in vivo* challenge with AZ617. Mice were pre-dosed with vehicle control or PF-06650833 30 minutes before IP administration of LPS or AZ617. Blood was collected for cytokine measurement via the retro-orbital sinus. **A.** AZ617 induced higher human TNFα compared to mouse TNFα, and treatment with PF-06650833 significantly reduced circulating human TNFα. **B.** Levels of human and mouse IL-6 induced by AZ617 were not significantly different, and treatment with PF-06650833 did not impact circulating human IL-6. Statistical results were as follows: human vs. mouse by two-way ANOVA and Šidák's multiple comparisons, where *p<0.05 and ****p<0.0001. Human vs. human or mouse vs. mouse by two-way ANOVA and Tukey's multiple comparisons, where ^p<0.05 and ^^^^p<0.0001. Error bars represent standard error of the mean (SEM). Data were from one independent experiment, n=3-5/group.

It was determined that AZ617 could strongly induce human IL-6 and TNFα *in vivo* in huNOG-EXL mice by 3 hours after challenge at 300 and 500 µg doses (**Fig. 3**). Unexpectedly, mouse IL-6 and TNFα were secreted to a detectable range as well, although to a much lesser extent than the human cytokines, a finding not observed in an *in vitro* co-culture study with C57BL/6 mouse splenocytes (**Fig. S2**). It is possible that, *in vivo*, the AZ617-induced human cytokines are exerting an indirect effect on residual mouse immune cells throughout the body, resulting in subsequent immune signaling and secretion of mouse cytokines, which cannot be successfully captured by simple co-culture experiments using only splenocytes. Detection kits were also confirmed to be species-specific (**Fig. S3**). Nonetheless, the AZ617 huNOG-EXL model and resulting human cytokine induction proved sufficient to characterize drug candidates whose inhibitory potencies on mouse cells are inferior to those on human cells.

To validate this hypothesis, IRAK4 inhibitor PF-06650833 was tested in the model and was shown to significantly lower AZ617-induced human TNFα at the optimal 3-hour time point (**Fig. 4**). By 6 hours post challenge, the significance was lost, suggesting that 3 hours is the appropriate time point for measuring human TNFα in this system (**Fig. S4**). Human IL-6 at the optimal 6-hour time point was not significantly inhibited by treatment with PF-06650833 due to inter-animal variability in response to

AZ617. Additionally, the vehicle-treated huNOG-EXL mice did show trace mouse IL-6 levels in response to AZ617, whereas mouse TNFα levels were much closer to baseline. Based on our data demonstrating a greater window of human TNFα induction over mouse as compared to IL-6, and in combination with the lack of significant induction of several other human cytokines (**Fig. S5**), we propose that human TNFα is the preferred acute readout of this model for profiling human responses.

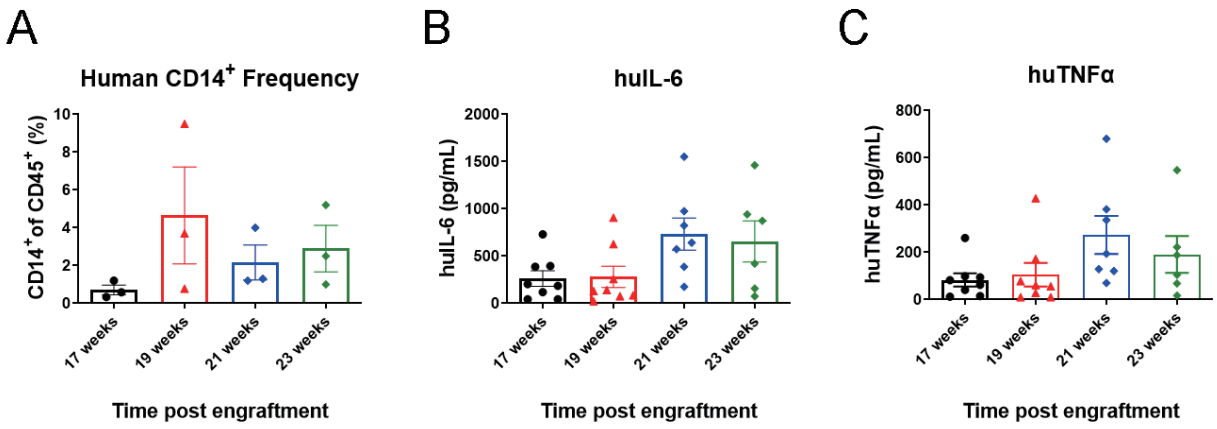


Figure 5. Myeloid cell responsiveness increased after 19 weeks post-engraftment. Three cohorts of huNOG-EXL mice (n=3), each generated from a different CD34+ HSC donor, were bled every 2 weeks by retro-orbital sinus puncture. **A.** Red blood cells were lysed and a small aliquot of the resulting white blood cell pellet was flow cytometrically assessed for frequencies of CD14+ human monocytes. Monocyte populations appeared to stabilize around 21 WPE. **B-C.** White blood cell pellets from each mouse (n=1-3, depending on the time point) were treated overnight with 1 ng/ml LPS, and secreted cytokine was measured from the supernatants. Both human IL-6 and TNFα were detected, with an observable increase over the first two time points at around 21 WPE, consistent with the time at which the CD14+ populations stabilized. Error bars represent standard error of the mean (SEM).

One potential way to address the induction of mouse IL-6 would be to reduce the AZ617 dose from 500 µg to 300 µg. The dose-response data revealed that, with a 300 µg dose of AZ617 at the 6-hour time point (optimal for measuring IL-6), there was a 25-fold induction of human vs. mouse IL-6 (compared to a 5-fold window with the 500 µg dose). Furthermore, for TNFα, 300 µg resulted in a 15-fold induction of human vs. mouse cytokine (compared to a 10-fold window with the 500 µg dose) at the optimal 3-hour time point. It is possible that reducing the AZ617 dose could deliver robust agonism of human TLR4 whilst reducing murine TLR4 signaling. However, more experiments are warranted to test this hypothesis.

Donor-to-donor variability, engraftment differences, and expression levels of relevant TLR pathway proteins are inherent limitations of huNOG-EXL mice which must be considered. In longitudinal studies, LPS, a stronger inducer of cytokine responses in huNOG-EXL mice than AZ617 (**Fig. 3**), was found to track human TLR responses better over time. Studies performed with mice around 17 weeks and longer post-engraftment provided more robust IL-6 and TNFα levels with less variability than younger mice (**Fig. 5**). Use of mice at later time points after engraftment could help reconcile the noise observed in the AZ617 huNOG-EXL model we have established.

We have demonstrated that AZ617 preferentially induced human cytokines both *in vitro* and *in vivo* in a humanized immune system mouse model. At the AZ617 and LPS doses tested *in vivo*, human cytokines were induced with similar potency, suggesting that AZ617 is a viable agonist of human TLR4 that yields comparable results to a non-preferential agonist like LPS. Furthermore, LPS far outperformed AZ617 in inducing mouse cytokines, supporting the hypothesis that AZ617 is selective for the human receptor. Altogether, we have described a novel, acute system useful for profiling drug candidates with inadequate cross-species potency properties for which standard mouse models would be insufficient.

Acknowledgements

The authors would like to thank Nancy Crosbie and Carolyn Grande of the AbbVie assay lab for their assistance with the MSD cytokine assays.

Disclosures

Rachel Twomey, Joseph Spina, Xiaoming Wu, and William Housley are employees of AbbVie. Sean Graham was an employee of AbbVie at the time this project was initiated. Philip Dubé is an employee of Taconic Biosciences, and Courtney Ferrebee was an

employee of Taconic at the time of the study. The experimental designs were agreed upon by AbbVie and Taconic, and financial support for this research was provided by AbbVie. Mice used in this study were provided, free of charge, by Taconic Biosciences. AbbVie and Taconic collaboratively participated in the interpretation of data, review, and approval of the publication.

References

1. Allen TM, Brehm MA, Bridges S, Ferguson S, Kumar P, Mirochnitchenko O, et al. Humanized immune system mouse models: progress, challenges and opportunities. Nature Immunology. 2019;20(7):770-4. https://doi.org/10.1038/s41590-019-0416-z
2. Perkins DJ, Vogel SN. Inflammation: Species-specific TLR signalling -- insight into human disease. Nat Rev Rheumatol. 2016;12(4):198-200. Epub 2016/03/24. https://doi.org/10.1038/nrrheum.2016.36 PMID: 27006311 PMCID: PMC4955569
3. Scheer N, Wilson ID. A comparison between genetically humanized and chimeric liver humanized mouse models for studies in drug metabolism and toxicity. Drug Discov Today. 2016;21(2):250-63. https://doi.org/10.1016/j.drudis.2015.09.002
4. Vaure C, Liu Y. A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front Immunol. 2014;5:316. Epub 2014/07/30. https://doi.org/10.3389/fimmu.2014.00316 PMID: 25071777 PMCID: PMC4090903
5. Zuberi A, Lutz C. Mouse Models for Drug Discovery. Can New Tools and Technology Improve Translational Power? ILAR J. 2016;57(2):178-85. Epub 2017/01/06. https://doi.org10.1093/ilar/ilw021 PMID: 28053071 PMCID: PMC5886322
6. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol. 2004;172(5):2731-8. Epub 2004/02/24. https://doi.org/10.4049/jimmunol.172.5.2731 PMID: 14978070
7. Hasgur S, Aryee KE, Shultz LD, Greiner DL, Brehm MA. Generation of Immunodeficient Mice Bearing Human Immune Systems by the Engraftment of Hematopoietic Stem Cells. Methods Mol Biol. 2016;1438:67-78. Epub 2016/05/07. https://doi.org/10.1007/978-1-4939-3661-8_4 PMID: 27150084 PMCID: PMC5268072
8. Liu Y, Wu W, Cai C, Zhang H, Shen H, Han Y. Patient-derived xenograft models in cancer therapy: technologies and applications. Signal Transduct and Target Ther. 2023;8(1):160. https://doi.org/10.1038/s41392-023-01419-2
9. Yan C, Nebhan CA, Saleh N, Shattuck-Brandt R, Chen S-C, Ayers GD, et al. Generation of Orthotopic Patient-Derived Xenografts in Humanized Mice for Evaluation of Emerging Targeted Therapies and Immunotherapy Combinations for Melanoma. Cancers. 2023;15(14):3695. https://doi.org/10.3390/cancers15143695
10. Chen L, Morris DL, Vyse TJ. Genetic advances in systemic lupus erythematosus: an update. Curr Opin Rheumatol. 2017;29(5):423-33. Epub 2017/05/17. https://doi.org/10.1097/BOR.0000000000000411 PMID: 28509669
11. Liu WN, Fong SY, Tan WWS, Tan SY, Liu M, Cheng JY, et al. Establishment and Characterization of Humanized Mouse NPC-PDX Model for Testing Immunotherapy. Cancers (Basel). 2020;12(4). Epub 2020/04/26. https://doi.org/10.3390/cancers12041025 PMID: 32331230 PMCID: PMC7225949
12. Zhao Y, Shuen TWH, Toh TB, Chan XY, Liu M, Tan SY, et al. Development of a new patient-derived xenograft humanised mouse model to study human-specific tumour microenvironment and immunotherapy. Gut. 2018;67(10):1845-54. Epub 2018/04/01. https://doi.org/10.1136/gutjnl-2017-315201 PMID: 29602780 PMCID: PMC6145285
13. Abdolahi S, Ghazvinian Z, Muhammadnejad S, Saleh M, Asadzadeh Aghdaei H, Baghaei K. Patient-derived xenograft (PDX) models, applications and challenges in cancer research. Journal of Translational Medicine. 2022;20(1):206. https://doi.org/10.1186/s12967-022-03405-8
14. huNOG-EXL SA (Standard Access) Humanized Immune System Mouse Model: Taconic Biosciences, Inc.; 2023 [2023 January 25]. Available from: https://www.taconic.com/mouse-model/hunog-exl-sa-standard-access.
15. Radaelli E, Hermans E, Omodho L, Francis A, Vander Borght S, Marine JC, et al. Spontaneous Post-Transplant Disorders in NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac (NOG) Mice Engrafted with Patient-Derived Metastatic Melanomas. PLoS One. 2015;10(5):e0124974. Epub 2015/05/23. https://doi.org/10.1371/journal.pone.0124974 PMID: 25996609 PMCID: PMC4440639
16. Gaudino SJ, Kumar P. Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. Front Immunol. 2019;10:360. Epub 2019/03/22. https://doi.org/10.3389/fimmu.2019.00360 PMID: 30894857 PMCID: PMC6414782
17. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol. 2008;9(4):361-8. Epub 2008/02/26. https://doi.org/10.1038/ni1569 PMID: 18297073 PMCID: PMC4112825
18. Akashi S, Nagai Y, Ogata H, Oikawa M, Fukase K, Kusumoto S, et al. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. Int Immunol. 2001;13(12):1595-9. Epub 2001/11/22. https://doi.org/10.1093/intimm/13.12.1595 PMID: 11717200
19. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Involvement of TLR4/MD-2 complex in species-specific lipopolysaccharide-mimetic signal transduction by Taxol. J Endotoxin Res. 2001;7(3):232-6. Epub 2001/10/03. PMID: 11581576
20. Oblak A, Jerala R. The molecular mechanism of species-specific recognition of lipopolysaccharides by the MD-2/TLR4 receptor complex. Mol Immunol. 2015;63(2):134-42. Epub 2014/07/20. https://doi.org/10.1016/j.molimm.2014.06.034 PMID: 25037631
21. Marshall JD, Heeke DS, Rao E, Maynard SK, Hornigold D, McCrae C, et al. A Novel Class of Small Molecule Agonists with Preference for Human over Mouse TLR4 Activation. PLoS One. 2016;11(10):e0164632. Epub 2016/10/14. https://doi.org/10.1371/journal.pone.0164632 PMID: 27736941 PMCID: PMC5063506 AstraZeneca. This does not alter our adherence to PLOS ONE policies on sharing data and materials.
22. Ripoll E, de Ramon L, Draibe Bordignon J, Merino A, Bolanos N, Goma M, et al. JAK3-STAT pathway blocking benefits in experimental lupus nephritis. Arthritis Res Ther. 2016;18(1):134. Epub 2016/06/10. https://doi.org/10.1186/s13075-016-1034-x PMID: 27278657 PMCID: PMC4898357
23. Winkler AS, W; De, S; Jiao, A; Sharif, MN; Symanowicz, PT; Athale, S; Shin, JH; Wang, J; Jacobson, BA; Ramsey, SJ; Dower,

K; Andreyeva, T; Liu, H; Hegen, M; Homer, BL; Brodfuehrer, J; Tilley, M; Gilbert, SA; Danto, SI; Beebe, JJ; Barnes, BJ; Pascual, V; Lin, L; Kilty, I; Fleming, M; Rao, VR. The Interleukin-1 Receptor-Associated Kinase 4 Inhibitor PF-06650833 Blocks Inflammation in Preclinical Models of Rheumatic Disease and in Humans Enrolled in a Randomized Clinical Trial. Arthritis Rheumatol. 2021;73(12):2206-18.

Supplementary Information

Table S1. Staining antibodies used for flow cytometry.

Table S2. Demographic information for human PBMC donors.

Figure S1. AZ617 does not induce IFN alpha secretion.

Figure S2. Human PBMCs and mouse splenocytes do not cross-talk *in vitro* upon AZ617 stimulation.

Figure S3. Human and mouse TNF alpha and IL-6 detection kits do not cross-react.

Figure S4. Comparison of mouse and human TNF alpha and IL-6 outside of ideal time points.

Figure S5. Profiles of additional human and mouse cytokines after AZ617 challenge.

Supplementary information of this article can be found online at https://polscientific.com/jbm/index.php/jbm/article/view/408/496.