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Complete List of Authors:	Lok, Laurence; University of Cambridge Medicine, Medicine Farahi, Neda; University of Cambridge Medicine, Medicine Juss, Jatinder; University of Cambridge Medicine, Medicine Loutsios, Chrystalla; University of Cambridge Medicine, Medicine Solanki, Chandra; Addenbrooke's Hospital, Department of Nuclear Medicine Peters, A. Michael; Brighton Sussex Medical School, Donaldson, Francis; Roche Products Ltd Porter-Brown, Benjamin; Roche Products Ltd Chilvers, Edwin; University of Cambridge, dept of medicine
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EFFECTS OF TOCILIZUMAB ON NEUTROPHIL FUNCTION AND KINETICS

Authors: Laurence SC Lok MB BChir,[1] Neda Farahi PhD,[1] Jatinder K Juss PhD,[1] Chrystalla Loutsios MBBS,[1] Chandra K Solanki MSc,[2] A Michael Peters DSc,[3] Francis Donaldson PhD,[4] Benjamin Porter-Brown MBBS,[4] and Edwin R Chilvers PhD ScD[1]

Affiliations: [1]Department of Medicine, University of Cambridge, United Kingdom; [2]Department of Nuclear Medicine, Cambridge University Hospitals NHS Foundation Trust, United Kingdom; [3]Division of Clinical and Laboratory Investigation, Brighton and Sussex Medical School, United Kingdom; [4]Roche Products Ltd., Welwyn Garden City, United Kingdom

Corresponding Author: Professor Edwin R Chilvers PhD, ScD, FMedSci

Address and requests for reprints: Department of Medicine, University of Cambridge, Level 5 Box 157, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, United Kingdom

E-mail: erc24@cam.ac.uk

Phone: +44 1223 762007

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ABSTRACT

Background: Decreases in circulating neutrophils (polymorphonuclear leukocytes, PMNs) have been reported in patients treated with the anti-interleukin-6 receptor (IL-6R) antibody tocilizumab (TCZ); the mechanism for this is unclear. We hypothesize that TCZ reduces circulating neutrophils by affecting margination and / or bone marrow trafficking without affecting neutrophil function or apoptosis.

Materials and methods: 18 healthy subjects were randomized to single intravenous dose of TCZ 8 mg/kg (n = 12) or placebo (n = 6) on day 0. On day 4, each subject had autologous indium-111-labeled neutrophils re-injected, and their kinetics quantified with longitudinal profiling in a whole body gamma-counter. TCZ-treated subjects were divided into two groups according to the extent of reduction in neutrophil count.

Results: Mean day 4 neutrophil counts, as % baseline, were 101.9%, 68.3% and 44.2% in the placebo, TCZ-PMN-'high' and TCZ-PMN-'low' groups, respectively ($p < 0.001$). Following TCZ, neutrophil function, activation and apoptosis *ex vivo* were all unaffected. *In vivo*, there were no differences in early blood recovery or margination to liver / spleen and bone marrow; however, later neutrophil re-distribution to bone marrow was markedly reduced in the TCZ-PMN-low group (peak pelvic count as % day 4 count on: day 5, 188% placebo *vs* 127% TCZ-PMN-low, $p < 0.001$; day 10, 180% placebo *vs* 132% TCZ-PMN-low, $p < 0.01$), with a trend towards higher liver / spleen neutrophil retention.

Conclusions: We have demonstrated for the first time in humans that IL-6R blockade affects neutrophil trafficking to the bone marrow without influencing neutrophil functional capacity.

Key words: neutrophil; interleukin-6; tocilizumab; trafficking

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INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine with a central role in the pathogenesis of a number of inflammatory and autoimmune disorders [1]. Neutrophils (polymorphonuclear leukocytes, PMNs) are involved in many inflammatory diseases, in part through the release of cytokines and chemokines, and through antigen presentation [2]. Activated neutrophils are a source of soluble interleukin-6 receptor (sIL-6R), initiating 'trans-signaling' in cell types that do not express membrane-bound IL-6R (mIL-6R), thus driving chronic inflammation [3-5].

Tocilizumab (TCZ), a humanized monoclonal antibody against both forms of the IL-6R, is licensed for the treatment of rheumatoid arthritis, systemic juvenile idiopathic arthritis and polyarticular juvenile idiopathic arthritis [6-8]. Decreases in circulating neutrophil levels have been reported after initiation of TCZ in clinical trials. Despite this, the rate of serious infections is low (4.7 per 100 patient-years) [9] and no temporal association has been described between decreased neutrophil counts and the occurrence of such infections [6-10].

Data from animal studies on the effects of IL-6 on neutrophil function have, to date, been inconsistent. IL-6 administration in rabbits results in reduced expression of surface CD62L on circulating and bone marrow neutrophils [11]. IL-6 administration in monkeys, however, results in reduced expression of CD162 whilst CD11b and CD62L expression is unchanged; this process is inhibited by TCZ [12]. In humans, *in vitro* exposure of healthy neutrophils to IL-6 does not affect neutrophil survival or functional capacity [13], although additional lipopolysaccharide (LPS) stimulation following TCZ exposure *in vitro* has been reported to result in differences in oxidative burst and phagocytic capacity under hypoxic conditions

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[14]. Studies on neutrophils isolated from patients with rheumatoid arthritis 28 days after administration of TCZ, however, show no effect on neutrophil function or apoptosis [13].

Administration of recombinant human IL-6 in rats [15] and rabbits [16] results in a biphasic blood neutrophilia, with an early peak resulting from leukocyte mobilization from the intravascular marginated pool, and a late peak due to neutrophil release from the post-mitotic bone marrow pool. IL-6 contributes to granulopoiesis in granulocyte colony-stimulating factor (G-CSF)- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-deficient mice [17]. In humans, strenuous exercise similarly causes a biphasic neutrophilia [18] that has been shown to correlate with an increase in serum IL-6 [19], although this correlation has not been replicated in all studies [20].

Broader aspects of neutrophil function and apoptosis at the expected neutrophil nadir 4 days following TCZ administration have not been assessed, and no studies have been undertaken to examine the effects of IL-6 modulation on *in vivo* whole body neutrophil kinetics in humans. Likewise, to date there is no mechanistic understanding of the decrease in circulating neutrophils following treatment with TCZ. Such data are also required to comply with European Medicines Agency requests on providing further safety information on the use of TCZ. Therefore, an exploratory study was conducted in healthy subjects to investigate the hypothesis that IL-6R blockade affects whole body neutrophil kinetics by affecting neutrophil margination and / or re-uptake by the bone marrow, without affecting neutrophil function and survival.

METHODS

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Study subjects

This single-blind, randomized study (ClinicalTrials.gov identifier NCT01991990) enrolled healthy male subjects aged 18-65 years. Subjects had a body mass index between 18 kg/m^2 and 30 kg/m^2, were non-smokers, and did not receive any medication other than paracetamol. The study was approved by the East Midlands Nottingham Research Ethics Committee (Reference 13/EM/0350). All subjects provided written informed consent in accordance with the Declaration of Helsinki and local laws and regulations.

Study design

Subjects were randomized to receive a single intravenous dose, blinded to subjects only, of TCZ 8 mg/kg or saline placebo (2:1 randomization) on day 0 (Figure 1A). No subjects experienced clinically significant Common Terminology Criteria (CTC) grade 3 or 4 neutropenia (neutrophil count below 1×10^9/L). However, there was a wide variation in the relative reduction in blood neutrophil count following TCZ; therefore in the analysis TCZ-treated subjects were divided into two equal-sized groups to examine the effects of IL-6R blockade based on the extent of the decrease in their neutrophil count. This grouping was result-driven and not pre-defined.

Neutrophil isolation

Neutrophils were isolated from peripheral blood using dextran sedimentation and discontinuous plasma-Percoll (Fisher Scientific, Loughborough, UK) gradient centrifugation, as previously described [21].

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Neutrophil function and apoptosis ex vivo

Neutrophil function and apoptosis were assessed *ex vivo* on day 0 (pre-dose) and day 4 (post-dose). Respiratory burst was measured in neutrophils, primed with tumor necrosis factor-alpha (TNFα, 20 ng/mL; R&D, UK) or phosphate-buffered saline (PBS) control for 30 minutes, as the peak in N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich, UK)-stimulated, horseradish peroxidase (HRP; Sigma-Aldrich, UK)-catalyzed luminol (Sigma-Aldrich, UK) chemiluminescence [22]. Phagocytosis was assessed in neutrophils equilibrated at 4°C or 37°C for 1 hour followed by addition of heat-killed, eFluor670 (eBioscience, UK)-labeled *Streptococcus pneumoniae* (ratio 1 PMN : 29 *S. pneumoniae*) for 1 hour, and quantified by flow cytometry [23].

For assessment of apoptosis, neutrophils were incubated for 20 hours with vehicle control or a pro-survival factor, either GM-CSF (1 ng/mL; R&D, UK) or TNFα (20 ng/mL), and apoptosis was quantified by annexin V-FITC and propidium iodide (PI) (BD Biosciences, UK) staining, with early apoptotic cells being annexin V^+/PI^- and late apoptotic cells annexin V^+/PI^+. Cytospin slides were also generated and stained with Diff-Quik staining (Gamidor, UK), and neutrophil morphology examined in a minimum of 300 neutrophils per slide using oil immersion light microscopy with the assessor blinded to treatment conditions. Apoptotic cells were identified by their darkly stained, pyknotic nuclei [24].

Surface markers were measured in fresh (0 min), PBS control (30 min) and fMLP-stimulated (30 min) neutrophils incubated for 30 minutes with the anti-human antibodies CD11b-BV421, CD16-FITC, CD62L-APC and CD162-PE, or isotype controls (BD Biosciences, UK). Expressions were quantified as median fluorescence intensities (MFI) by flow

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cytometry. Shape change was assessed by median forward scatter (FSC). Data were analyzed using FlowJo software.

Neutrophil kinetics in vivo

Autologous neutrophils were isolated from peripheral blood by Hetastarch (Grifols, UK) sedimentation and discontinuous plasma-Percoll gradient centrifugation. Neutrophils were harvested and labeled with indium-111 (^{111}In; Mallinckrodt, UK) chelated to tropolone (Ipswich Pharmacy Manufacturing Unit, UK) by incubation at room temperature for 10 minutes, subsequently washed twice in platelet-poor plasma and re-injected (maximum 2.5 MBq) into the subjects [25,26].

Blood was sampled 45 minutes post re-injection to determine the percentage recovery of radiolabeled neutrophils, as previously described [26]. Longitudinal whole body profiles were generated in a fully shielded whole body gamma-counter at 45 minutes (day 4), 1 day (day 5) and 6 days (day 10) following re-injection. For each scan, the collimated anterior and posterior detectors moved from head downwards at 14 cm/min for 10 minutes (Figure 5A). Counts were background and decay corrected, and geometric means normalized to day 4 counts.

Safety

Safety was assessed by physical examination, vital signs, and monitoring of adverse events and laboratory parameters. All subjects completed all study and safety visits.

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Statistical analysis

All *ex vivo* assays were performed in triplicate. Data are presented as mean ± SEM of (n) independent experiments, and analyzed using GraphPad Prism version 6. Treatment groups were compared using one-way ANOVA and unpaired t-tests with Holm-Sidak correction for multiple comparisons.

RESULTS

Study subjects

A total of 18 subjects were treated with a single intravenous dose of TCZ 8 mg/kg (n = 12) or saline placebo (n = 6) on day 0. There was a significant reduction in day 4 neutrophil count in TCZ-treated ($p < 0.001$) but not placebo-treated subjects (Figure 1B-C). In the TCZ-treated subjects, splitting the groups evenly resulted in 6 subjects with < 51.3% relative reduction in day 4 neutrophil count (TCZ-PMN-'high' group) and 6 subjects with > 51.3% relative reduction in day 4 neutrophil count (TCZ-PMN-'low' group). No subjects experienced CTC grade 3 or 4 neutropenia. Mean neutrophil counts at day 4 as % baseline were 101.9% in the placebo group, 68.3% in the TCZ-PMN-high group, and 44.2% in the TCZ-PMN-low group ($p < 0.001$; Figure 1D).

Neutrophil function and apoptosis ex vivo

Respiratory burst activity was intact at days 0 and 4 in neutrophils isolated from placebo, TCZ-PMN-low, and TCZ-PMN-high groups; mean peak chemiluminescence did not show statistically significant differences between the unprimed groups, or between the TNFα-primed groups (Figure 2A,D-E). Rates of phagocytosis of heat-killed *S. pneumoniae* were

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also similar in all groups, indicating intact phagocytic activity (Figure 2B-C,F-G). Neutrophil survival following 20 hours of *ex vivo* culture was unaffected by TCZ, with apoptosis rates showing no differences between placebo and TCZ groups under either untreated conditions or in the presence of the pro-survival factors GM-CSF or TNFα (Figure 3). Cell surface markers of neutrophil activation, including CD11b, CD62L, CD162, and shape change [27-29], were likewise unaffected by TCZ in both control and fMLP-stimulated neutrophils (Figure 4A-B,D-G). Expression of CD16, a marker expressed on mature neutrophils, and declines on aging neutrophils [30], was also unaffected by TCZ (Figure 4A,C).

Neutrophil kinetics in vivo

For analysis of the *in vivo* kinetic data, one subject from the TCZ-PMN-high group was excluded because of external (skin) contamination, which affected the profiling data. The mean (SEM) 45-minute blood recovery values of ^{111}In-labeled neutrophils at day 4 as % total body count were 26.7 (4.7) in the placebo group (n = 6), 28.4 (2.5) in the TCZ-PMN-high group (n = 5) and 29.9 (5.9) in the TCZ-PMN-low group (n = 6) (Figure 5B); these were not significantly different, indicating a similar extent of early neutrophil intravascular margination in all groups. Consistent with these data, in TCZ-treated subjects, there were no differences in the distribution of radiolabeled neutrophils in the liver / spleen or pelvic marrow pools 45 minutes post re-injection (Figure 5C-D). These data indicate again that the kinetics of early neutrophil margination are unaffected following TCZ.

However, neutrophil re-distribution to, and uptake in, the pelvic marrow region (which could be used as an index of generalized bone marrow activity) on days 5 and 10 (1 and 6 days following ^{111}In-labeled neutrophil re-injection, respectively) was considerably lower in the

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TCZ-PMN-low group (Figure 5E-F); peak pelvic count, as % day 4 count, on day 5 was 188% in the placebo group, 171% in the TCZ-PMN-high group, and 127% in the TCZ-PMN-low group ($p < 0.001$, placebo *vs* TCZ-PMN-low), whereas on day 10, it was 180% in the placebo group, 168% in the TCZ-PMN-high group, and 132% in the TCZ-PMN-low group ($p < 0.01$, placebo *vs* TCZ-PMN-low) (Figure 5H). This was associated with a clear trend towards higher neutrophil retention in the liver / spleen on days 5 and 10 in the TCZ-PMN-low group (Figure 5G), and a correlation between day 10 liver / spleen peak radioactivity and day 4 neutrophil count ($r^2 = 0.38$, $p < 0.05$; Figure S1C).

Safety

There were no adverse events leading to study withdrawal. Overall, 8 subjects experienced 12 adverse events, and all resolved without sequelae (Table S1). Of the 9 adverse events in the TCZ-treated subjects, all were mild or moderate in severity and were consistent with the known safety profile of TCZ.

DISCUSSION

Anti-IL-6R therapy is an important intervention in inflammatory diseases, and has been used successfully in diseases such as rheumatoid arthritis [8,31]. However, concerns have been raised about its effects on circulating neutrophil numbers and function. This study is the first to assess the pharmacodynamic effects of anti-IL-6R on human neutrophil kinetics *in vivo* and the first to comprehensively investigate the effects of IL-6R blockade on neutrophil function and apoptosis *ex vivo* at the expected nadir of blood neutrophil count.

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Results of this exploratory study in healthy subjects demonstrated a reduction in neutrophil count 4 days after administration of intravenous TCZ, representing the previously defined neutrophil nadir following TCZ administration [32]. Neutrophil respiratory burst and phagocytic activity remained intact at day 4, suggesting that the capacity of circulating neutrophils to respond to pathogens was unaffected at the time point when TCZ has its greatest effect on the numbers of circulating neutrophils. These data support the clinical trial experience of TCZ use in that no temporal relationship has been demonstrated between reductions in neutrophil counts and risks of serious infection [6-10,33].

Neutrophils were not activated at baseline after TCZ infusion and responded to *ex vivo* fMLP activation to a similar degree to neutrophils from placebo subjects. Rates of apoptosis in *ex vivo* culture were also unaffected, suggesting that TCZ does not affect the survival of circulating neutrophils, whose half-life has been estimated in other *ex vivo* and *in vivo* labeling studies ranging from several hours to 3.8 days [34-36]. The systemic administration of TCZ makes differential functional effects on neutrophils in the circulating and marginated pools unlikely. The current results are consistent with a recent study in healthy subjects and patients with rheumatoid arthritis, which showed no effect of TCZ on *ex vivo* neutrophil function and rates of apoptosis, when measured 4 and 12 weeks after multiple doses of TCZ [13].

Assessment of whole body kinetics showed that early neutrophil margination was unaffected by TCZ, with no difference in neutrophil distribution in the blood, liver / spleen and pelvic marrow regions 45 minutes following re-injection of radiolabeled neutrophils. However, neutrophil trafficking at later times was significantly different in the TCZ-PMN-low group,

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as demonstrated by reduced neutrophil re-uptake to the pelvic marrow region, with a trend towards higher neutrophil retention in the liver / spleen region on days 5 and 10. These findings indicate that TCZ treatment reduces bone marrow re-uptake of neutrophils, either directly, or indirectly via a mechanism involving enhanced retention by the liver and spleen. The former mechanism would be consistent with the previously defined role of IL-6 in the release of neutrophils from the bone marrow compartment in animal models [15,16]. No subjects experienced CTC grade 3 or 4 neutropenia. However, the significant relative reduction in neutrophil count in TCZ-treated subjects and the differences in neutrophil kinetics in the TCZ-PMN-low group demonstrate the biological effects of TCZ even in the absence of clinically significant neutropenia.

The study has several limitations. The control subjects in our study received saline placebo rather than irrelevant immunoglobulin G (IgG). Previous studies have shown that IL-6-induced STAT-3 phosphorylation is effectively blocked by TCZ but not by IgG in neutrophils from healthy subjects [13]; nevertheless, we cannot rule out the possibility of non-specific IgG effects on neutrophils in our results.

Neutrophil release from the bone marrow was not directly examined in the current model, and as such, the effects of TCZ on the release of neutrophils from the bone marrow post-mitotic pool in response to a stimulus were not formally assessed. Further investigation is required to demonstrate definitively whether IL-6R blockade affects neutrophil de-margination and / or release of these cells from the bone marrow.

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The 45-minute blood recovery values of ^{111}In-labeled neutrophils in the current study were slightly lower than previously demonstrated values. This can indicate a degree of cellular damage during the isolation and radiolabeling process. However, there was no evidence of abnormally high margination of radiolabeled neutrophils to the liver / spleen in the placebo or TCZ groups, which would be observed in the case of cellular damage. In addition, it has been demonstrated previously that the current method of neutrophil isolation and radiolabeling does not cause *ex vivo* priming or activation of neutrophils, hence allowing neutrophil kinetics to be studied under physiological conditions [26].

The whole body profiling data demonstrated the effects of TCZ treatment on neutrophil kinetics *in vivo*, suggesting reduced neutrophil re-uptake to the pelvic bone marrow and some evidence of persistent retention of neutrophils in the liver / spleen either through a failure of de-margination or enhanced neutrophil destruction at these sites. In humans healthy neutrophils are removed from the circulation in approximately equal proportions in the liver, spleen and bone marrow. This occurs in a seemingly random and age-independent fashion, and does not reflect intravascular apoptosis, which in health is extremely rare. This situation contrasts somewhat to the mouse where aged neutrophils are preferentially removed by the bone marrow and this involves up-regulation of CXCR4 [37]. Following removal from the circulation neutrophils then undergo apoptosis and are efferocytosed by splenic marginal zone and bone marrow stromal macrophages; this later process has been shown to regulate blood neutrophil levels via the production of G-CSF [38,39]. While the role of the IL-6/IL-6R axis in these processes is uncertain, marrow sampling was not part of our study.

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Overall, these data suggest that the reduction in circulating neutrophils results in a relative rather than absolute neutropenia in patients treated with TCZ. This observation may help explain why no temporal relationship between CTC grade 3 or 4 neutropenia and serious infection has been observed with TCZ treatment. Further work exploring the release of neutrophils from the bone marrow in the presence of TCZ is warranted.

In summary, we have demonstrated for the first time in humans that IL-6R blockade by a single intravenous dose of tocilizumab affects *in vivo* neutrophil trafficking to the bone marrow without influencing the *ex vivo* functional capacity, activation and apoptosis of these cells.

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AUTHORSHIP

Contribution: L.S.C.L., N.F., J.K.J., C.L., and C.K.S. performed experiments; L.S.C.L. analyzed results and made the figures; A.M.P., F.D., B.P-B., and E.R.C. designed the research; all authors contributed to the writing of the manuscript.

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FIGURE LEGENDS

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Figure 1. Effect of intravenous TCZ administration on blood neutrophil count. (A) Study design. (B-C) Day 0 (pre-dose) and day 4 (post-dose) blood neutrophil counts in placebo-treated subjects (n = 6) and TCZ-treated subjects (n = 12). # $p < 0.001$. (D) Day 4 neutrophil count, as % day 0 count, in placebo (n = 6), TCZ-PMN-high (n = 6) and TCZ-PMN-low (n = 6) groups. * $p < 0.05$, # $p < 0.001$ *vs* placebo; # $p < 0.001$ across three groups. Data shown as mean ± SEM.

Figure 2. *Ex vivo* neutrophil respiratory burst and phagocytic capacity following TCZ. (A) Representative results of respiratory burst measurement by luminol-HRP chemiluminescence. RLU, relative light units. (B) Representative flow cytometry results demonstrating phagocytosis as *S. pneumoniae*-eFluor670 fluorescence. (C) Light microscopy example of a neutrophil containing phagocytosed *S. pneumoniae* (arrow). Original magnification ×100. (D-E) Respiratory burst activity, quantified as (D) absolute and (E) relative peak chemiluminescence, in unprimed (a) and TNFα-primed (b) neutrophils. * $p < 0.05$, † $p < 0.01$, # $p < 0.001$ *vs* a; ns, non-significant across treatment groups (a and b). (F-G) Phagocytosis of heat-killed eFluor670-labeled *S. pneumoniae*, quantified as (F) MFI and (G) % eFluor670-positive neutrophils, in neutrophil only (a), 4°C (b) and 37°C (c) treatment groups. # $p < 0.001$ *vs* b; ns, non-significant across treatment groups (c). Placebo, n = 6; TCZ-PMN-high, n = 6; TCZ-PMN-low, n = 6. Data shown as mean ± SEM.

Figure 3. *Ex vivo* neutrophil apoptosis following TCZ. (A) Representative flow cytometry plots of neutrophils following 20 hours of culture showing annexin V and PI staining. (B) Light microscopy example of viable (black arrow) and apoptotic (red arrows) neutrophils. Original magnification ×100. (C-D) Apoptosis following 20 hours of culture, quantified as

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(C) % annexin V-positive cells by flow cytometry and (D) % apoptotic cells by microscopic morphology, under untreated (a), GM-CSF-treated (b) and TNFα-treated (c) conditions. * $p < 0.05$, † $p < 0.01$, # $p < 0.001$ *vs* a; ns, non-significant across treatment groups (a, b and c). Placebo, n = 6; TCZ-PMN-high, n = 6; TCZ-PMN-low, n = 6. Data shown as mean ± SEM.

Figure 4. *Ex vivo* neutrophil activation following TCZ. (A) Representative flow cytometry plots of CD11b, CD16, CD62L, CD162 and forward scatter (FSC) in neutrophils stimulated with fMLP *ex vivo*. (B-G) Flow cytometry quantification of surface markers of activation, including (B) CD11b MFI, (C) CD16 MFI, (D) CD62L MFI, (E) CD162 MFI, and shape change, including (F) forward scatter and (G) % shape changed cells, at 0 minute control (a), 30 minute control (b) and 30 minute fMLP (c). * $p < 0.05$, # $p < 0.001$ *vs* a; ns, non-significant across treatment groups (a and c). Placebo, n = 6; TCZ-PMN-high, n = 6; TCZ-PMN-low, n = 6. Data shown as mean ± SEM.

Figure 5. *In vivo* whole body neutrophil kinetics following TCZ. (A) Schematic of whole body profiling. (B-D) Early neutrophil distribution in (B) blood, (C) liver / spleen, and (D) pelvic bone marrow compartments, at 45 minutes post re-injection of autologous ^{111}In-labeled neutrophils on day 4. ns, non-significant across groups. (E-F) Representative longitudinal whole body profiles from a placebo subject and a TCZ-PMN-low subject. Radioactivity from pelvic bone marrow marked by black arrows. (G) Peak liver / spleen radioactivity, normalized to day 4 count, on days 4, 5 and 10 (45 minutes, 1 day and 6 days post re-injection, respectively). ns, non-significant across placebo groups and across TCZ-PMN-low groups. (H) Peak pelvic bone marrow radioactivity, normalized to day 4 count, on days 4, 5 and 10. # $p < 0.001$, placebo day 4 *vs* placebo day 5 and placebo day 4 *vs* placebo day 10; # p

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< 0.001, placebo day 5 *vs* TCZ-PMN-low day 5; † $p < 0.01$, placebo day 10 *vs* TCZ-PMN-low day 10. Placebo, n = 6; TCZ-PMN-high, n = 5; TCZ-PMN-low, n = 6. Data shown as mean ± SEM.

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Figure 1.

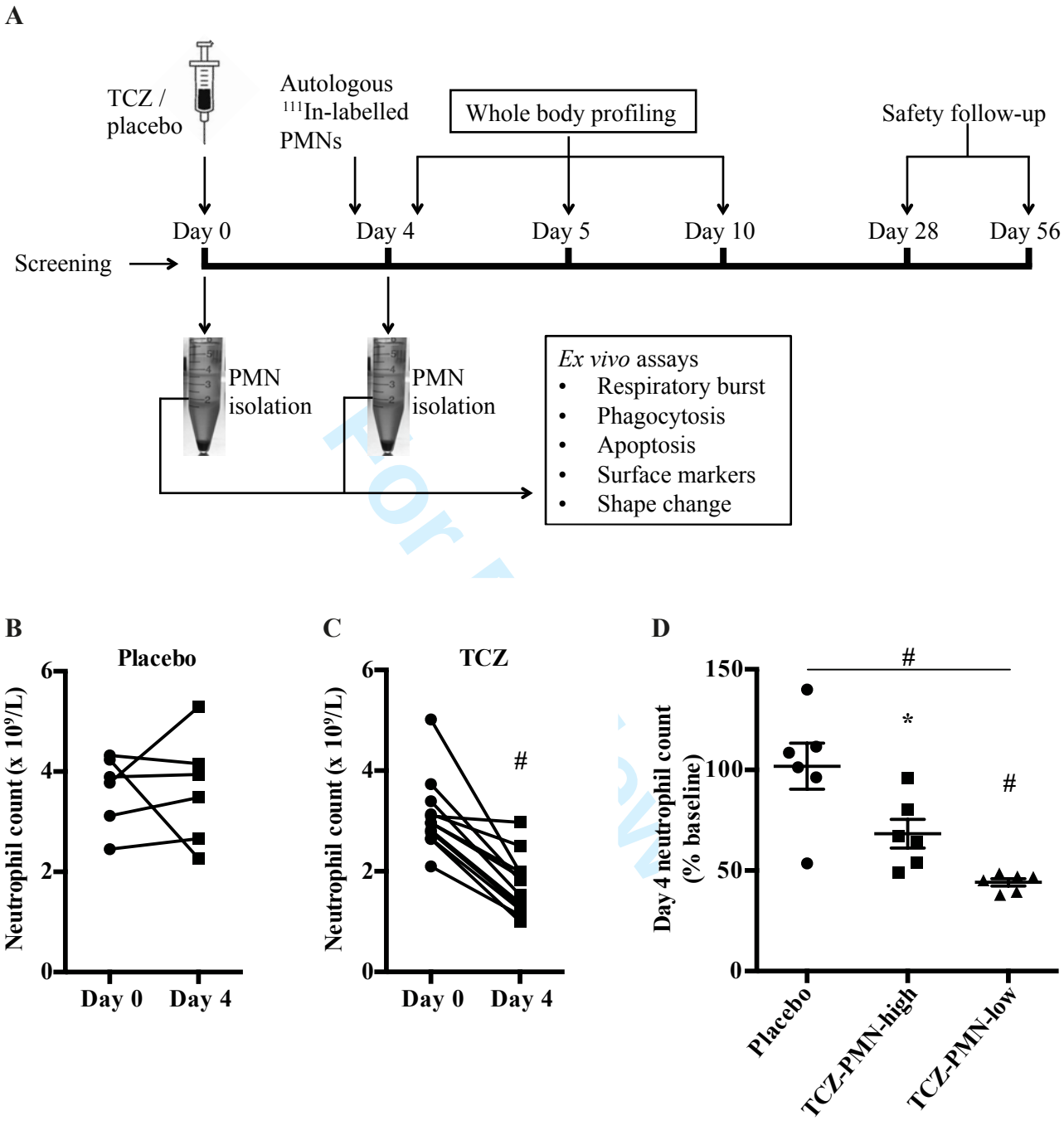
A
TCZ / placebo
Autologous ^{111}In-labelled PMNs
Whole body profiling
Safety follow-up
Day 0
Day 4
Day 5
Day 10
Day 28
Day 56
Screening
PMN isolation
PMN isolation
Ex vivo assays
• Respiratory burst
• Phagocytosis
• Apoptosis
• Surface markers
• Shape change
B
Placebo
Neutrophil count (x 10⁹/L)
Day 0
Day 4
C
TCZ
Neutrophil count (x 10⁹/L)
#
Day 0
Day 4
D
Day 4 neutrophil count (% baseline)
#
*
#
Placebo
TCZ-PMN-high
TCZ-PMN-low

Figure 2.

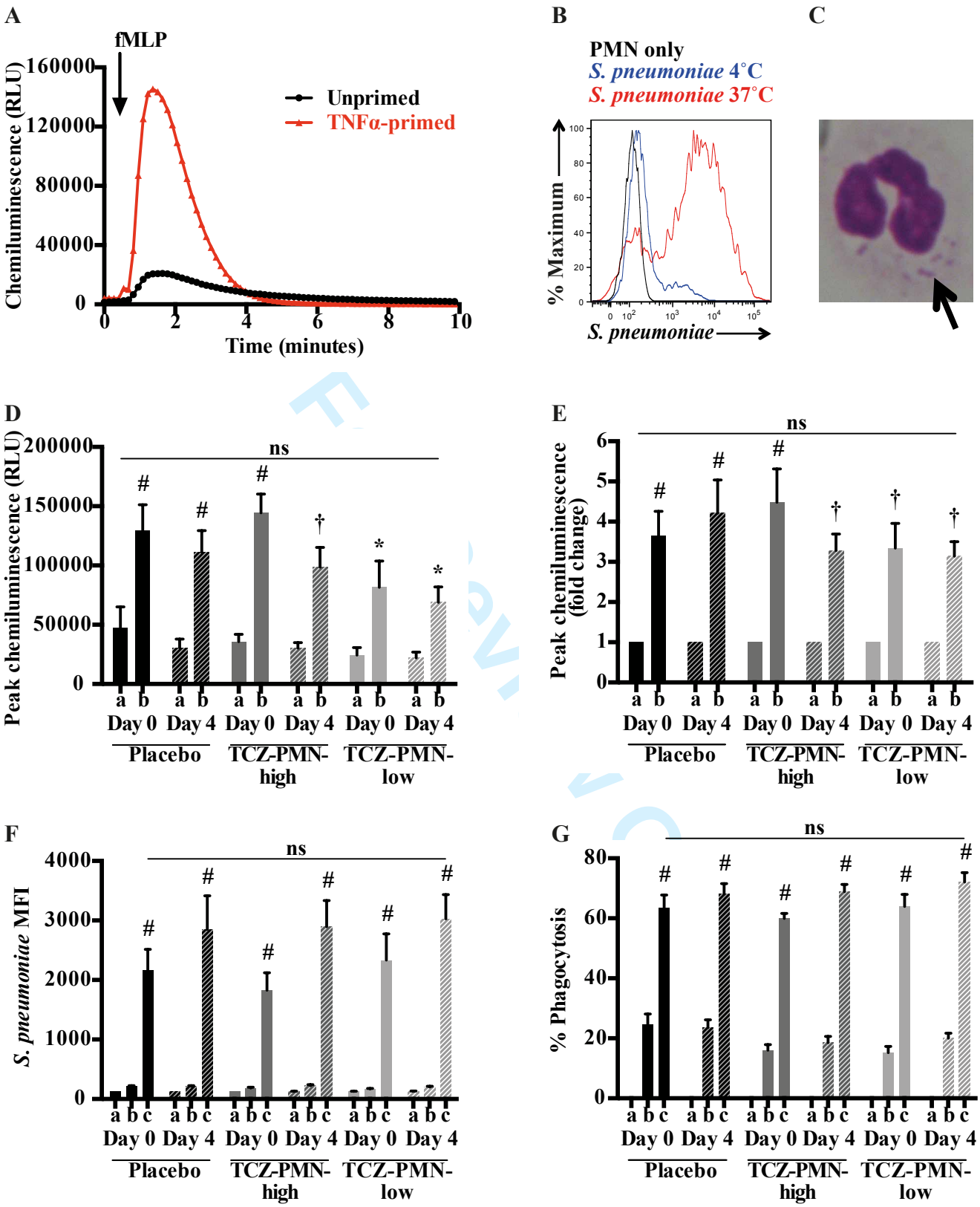
A
fMLP
Chemiluminescence (RLU)
160000
120000
80000
40000
0
Unprimed
TNFα-primed
0
2
4
6
8
10
Time (minutes)
B
PMN only
S. pneumoniae 4°C
S. pneumoniae 37°C
% Maximum
S. pneumoniae
C
D
Peak chemiluminescence (RLU)
200000
150000
100000
50000
0
ns
Day 0
Day 4
Placebo
TCZ-PMN-high
TCZ-PMN-low
E
Peak chemiluminescence (fold change)
F
S. pneumoniae MFI
4000
3000
2000
1000
G
% Phagocytosis
80
60
40
20

Figure 3.

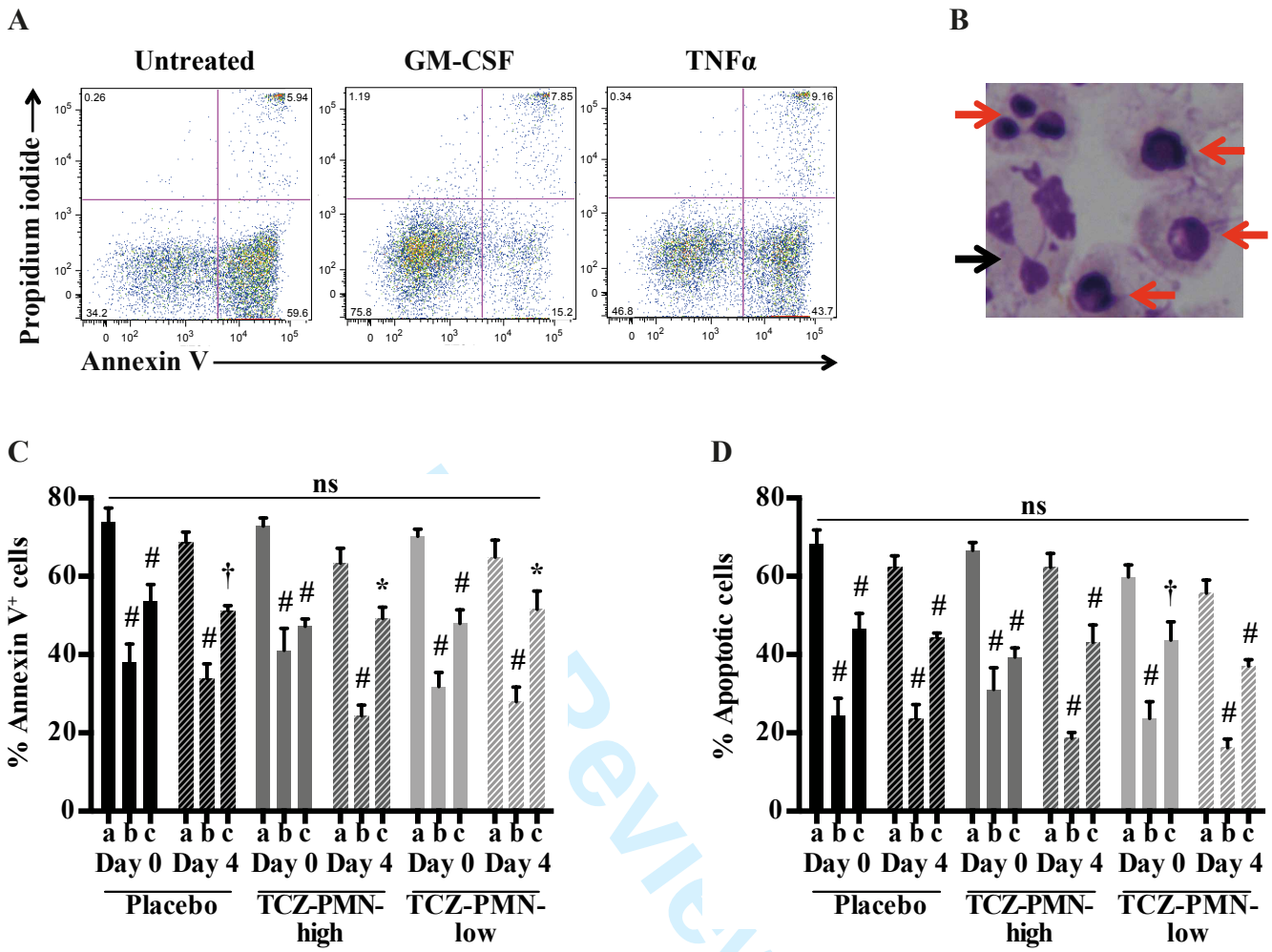

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Figure 4.

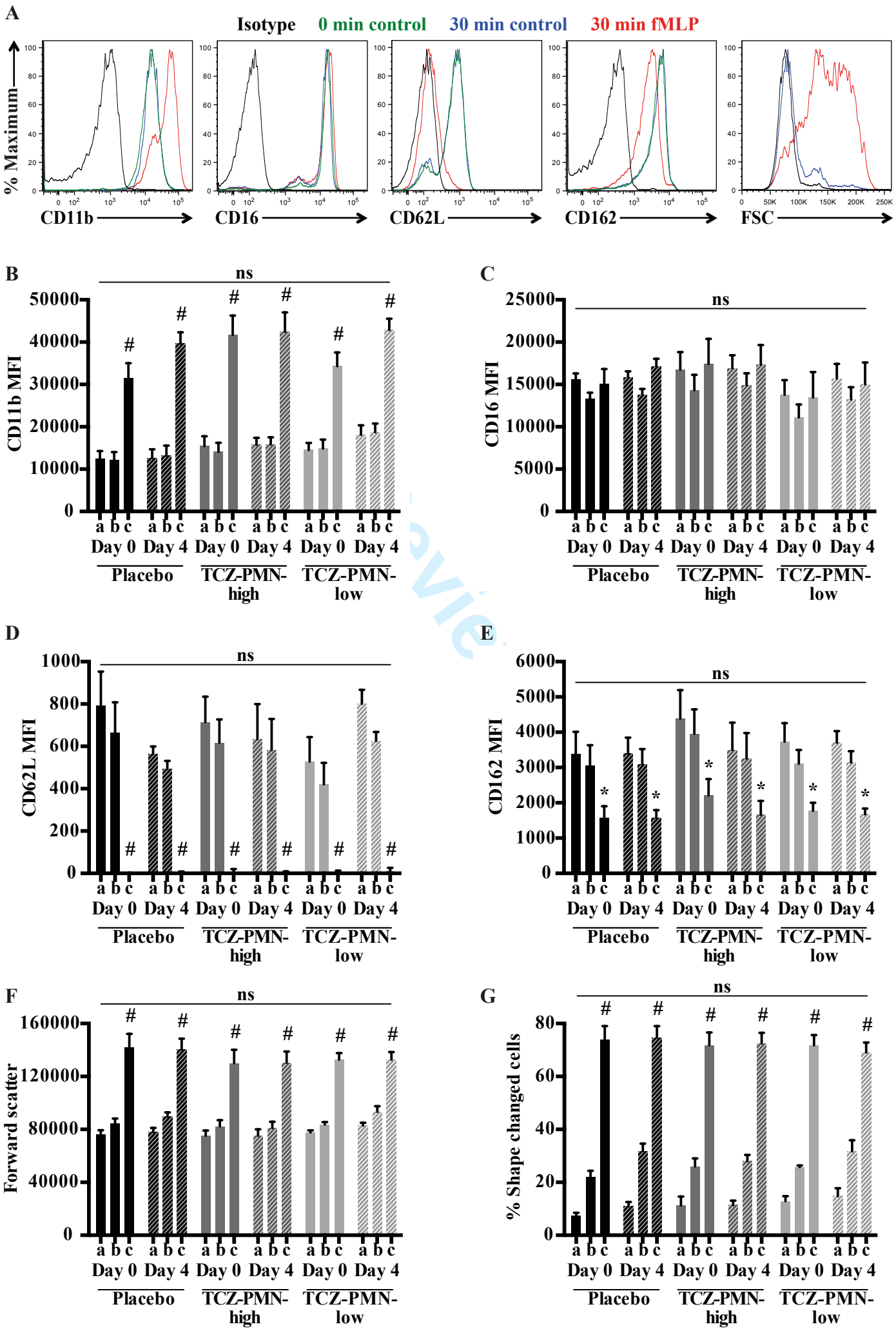
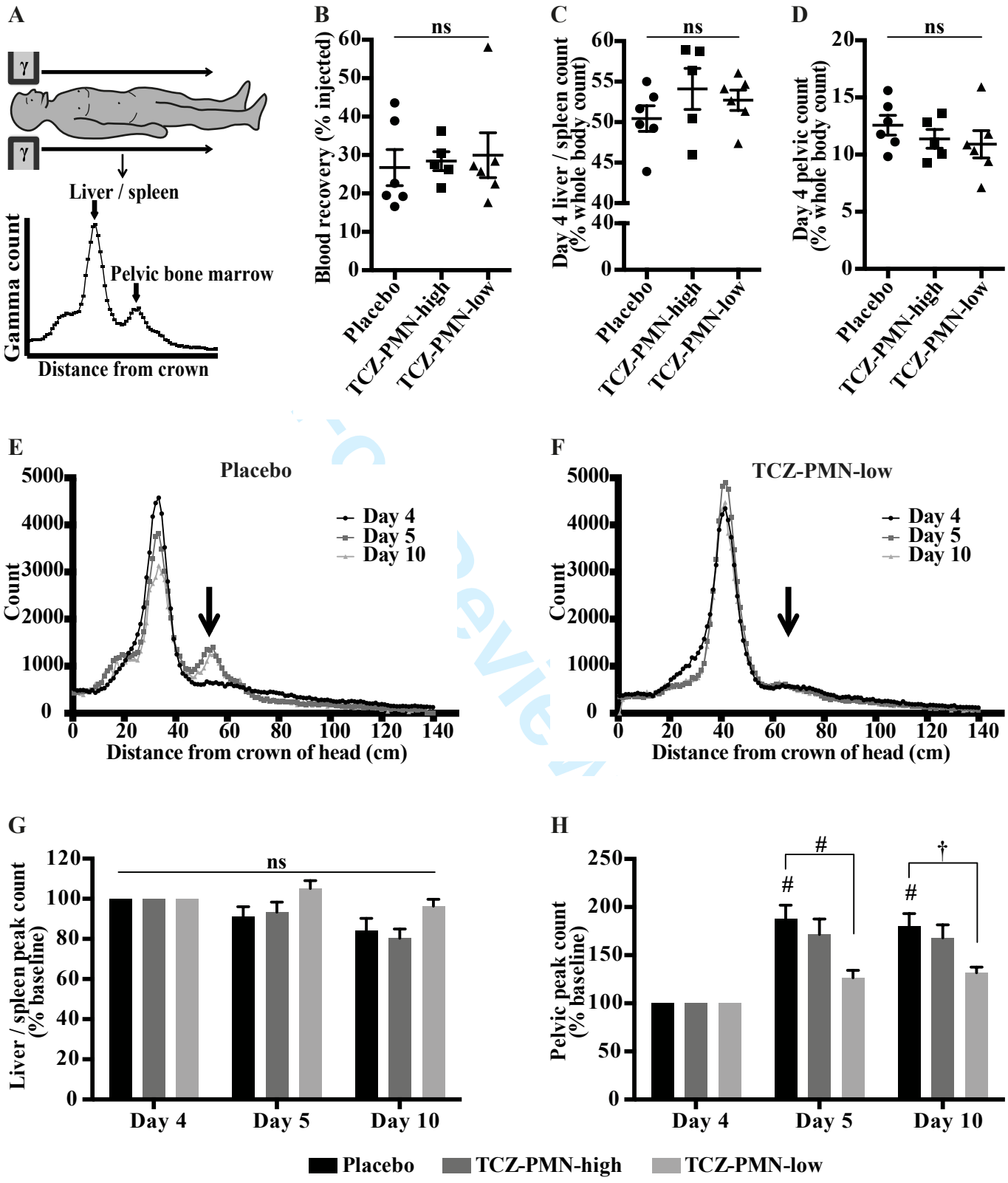

A
Isotype
0 min control
30 min control
30 min fMLP
% Maximum
CD11b
CD16
CD62L
CD162
FSC
B
CD11b MFI
C
CD16 MFI
D
CD62L MFI
E
CD162 MFI
F
Forward scatter
G
% Shape changed cells
ns
Day 0
Day 4
Placebo
TCZ-PMN-high
TCZ-PMN-low

Figure 5.


A
γ
γ
Liver / spleen
Pelvic bone marrow
Gamma count
Distance from crown
B
ns
Blood recovery (% injected)
Placebo
TCZ-PMN-high
TCZ-PMN-low
C
ns
Day 4 liver / spleen count (% whole body count)
Placebo
TCZ-PMN-high
TCZ-PMN-low
D
ns
Day 4 pelvic count (% whole body count)
Placebo
TCZ-PMN-high
TCZ-PMN-low
E
Placebo
Day 4
Day 5
Day 10
Count
Distance from crown of head (cm)
F
TCZ-PMN-low
Day 4
Day 5
Day 10
Count
Distance from crown of head (cm)
G
ns
Liver / spleen peak count (% baseline)
Day 4
Day 5
Day 10
H
#
#
#
†
Pelvic peak count (% baseline)
Day 4
Day 5
Day 10
Placebo
TCZ-PMN-high
TCZ-PMN-low