**TITLE PAGE**

**Characterization of human clozapine-responsive T-cells**

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**Running title: Clozapine-induced agranulocytosis**

**Abstract**

Use of the atypical antipsychotic clozapine is associated with the development of life-threatening agranulocytosis. The delayed onset and the association with HLA variants are characteristic of an immunological mechanism. The aim of this study was to generate clozapine-specific T-cell clones (TCC) and characterise pathways of T-cell activation and cross reactivity with clozapine metabolites and olanzapine. T-cell lines and TCC were established by culturing healthy donor PBMC and PBMC from patients with agranulocytosis with clozapine. A total of 9 clozapine-responsive CD4+ TCC were generated from patients and healthy donors. Activation of TCC required antigen presenting cells, with clozapine interacting directly at therapeutic concentrations with several HLA-DR molecules. TCC were also activated with *N*-desmethyl clozapine and olanzapine at high concentrations. Marked changes in global protein expression profiles was observed when clozapine treatment was compared with olanzapine treatment and the medium control. *In silico* molecular docking of the study compounds into the HLA-DRB1\*15:01 peptide binding cleft revealed that clozapine and olanzapine bind in a similar confirmation to P4-P6 peptide binding pockets, while clozapine *N-*oxide, which does not activate the TCC, bound in a different confirmation. TCC secreted a combination of Th1, Th2, and Th22 cytokines and effector molecules, and expressed TCR Vβ 5.1, 16, 20 and 22 as well as chemokine receptors CXCR3, CCR6, CCR4 and CCR9. Collectively, this study demonstrates that a direct interaction of clozapine with HLA-DR molecules activates CD4+ TCC. Additional work is on-going to delineate their role in the neutrophil apoptosis seen in patients with agranulocytosis.

**Key words:** Drug hypersensitivity, agranulocytosis, clozapine, HLA, T-cells.

**Introduction**

Clozapine is an effective second generation antipsychotic drug used for the treatment of refractory schizophrenia. It was introduced into clinical practice in 1971 but severe and sometimes fatal agranulocytosis limited its use in approximately 1% of patients (1, 2). This resulted in its withdrawal in most countries; however, clozapine was reintroduced in the late 1980s because of its proven efficacy in the management of refractory schizophrenia and an absence of efficacious alternatives (3, 4). Patients on clozapine therapy must undergo extensive monitoring of leucocytes (especially neutrophils) weekly for the first 18 weeks of treatment, followed by fortnightly from week 18-52 and monthly afterwards (2, 3). Clozapine is discontinued in patients with neutrophil counts of less than 1.5 x 109/L (normal range; 2.0-7.0 x 109/L) in order to reduce the risk of infections and the development of agranulocytosis. Agranulocytosis associated with clozapine therapy is generally reversed 2-3 weeks after cessation of the drug (5) and cytokine therapy such as granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor has been used successfully to manage the adverse event (6, 7).

The delayed onset of agranulocytosis after initial clozapine exposure, followed by a rapid onset upon rechallenge are plausible reasons to support a role for the adaptive immune system in the disease pathogenesis. Multiple variants in a number of genes including HLA-DQA1, HLA-B, HLA-DRB1, SLOC1B3/SLOC1B7 and ACKR1 have been associated with the development of clozapine-induced agranulocytosis (8-12). The HLA allele associations are of particular importance as HLA proteins present antigenic determinants to T-lymphocytes. An early attempt to generate clozapine metabolite-responsive T-cells was unsuccessful (13), while a more recent study utilizing an “adapted immune assay” suggests that PBMC from patients with agranulocytosis might be selectively activated with the drug (14). This latter study reported a subtle increase in PBMC proliferation, but the nature of the responding cells were not defined. For this reason, the central theme of this project was to explore whether clozapine interacts with HLA proteins to activate T-cells.

Clozapine forms two main stable metabolites in humans: clozapine *N-*oxide and *N*-desmethyl clozapine (15, 16). Furthermore, cytochrome P450 and peroxidase metabolism of clozapine generates a reactive nitrenium ion that binds covalently to thiol-containing proteins (16-20). The formation of clozapine protein adducts results in a dose-dependent increase in the rate of spontaneous neutrophil apoptosis and this direct toxicity has been implicated in the pathogenesis of the agranulocytosis (21). Neo-epitopes formed through the adduction of protein might also activate patient T-cells when displayed as peptide fragments by HLA proteins on the surface of antigen presenting cells. If this clozapine does activate patient T-cells, it would go some way to explain the idiosyncratic nature of the adverse event (16, 21). Interestingly, the structurally-related drug olanzapine, which forms an analogous protein-reactive metabolite to clozapine, and modifies different neutrophil proteins and has a lower propensity to induce neutrophil toxicity (22, 23). These findings and the lower plasma concentrations of olanzapine (0.16µM, compared with 1.68µM for clozapine) may partly explain why olanzapine is not associated with agranulocytosis in humans. Olanzapine and clozapine metabolites were included in our analysis to explore the structural features of clozapine HLA binding and the subsequent T-cell response.

**Materials and methods**

**Human subjects**

PBMC from HLA-DQB1\*02:01 positive clozapine-naive donors from our HLA-typed biobank (24) and HLA-typed patients with agranulocytosis were used for the generation of drug-specific TCC. Basic demographics of the patients are summarized in Table 1. All the blood donors gave informed written consent to partake and the study was approved by the Liverpool local research ethics committee.

**Isolation of peripheral blood mononuclear cells**

PBMC were isolated from whole blood using density centrifugation. Briefly, blood was layered in a 1:1 volume ratio on top of lymphoprep from Axis-Shield PoC AA (Oslo, Norway) and centrifuged at 2000 rpm, for 25 min without brake. The PBMC buffy coat was collected using a Pasteur pipette and washed twice with HBSS buffer solution and then resuspend in T-cell culture media composed of RPMI 1640 containing 100 mg/ml penicillin, 100 U/ml streptomycin, 25 mg/ml transferrin, 10% human AB serum, 25 mM HEPES buffer and 2 mM L-glutamine (Sigma-Aldrich, UK).

**Activation of PBMC with test compounds**

PBMC (1.5 x 105) from patients and clozapine naïve healthy donors were cultured in triplicate with clozapine, *N*-desmethylclozapine, clozapine *N*-oxide or olanzapine in a 96 well U-bottomed plate. Cell culture media and phytohemagglutinin, PHA (10 µg/ml), were used as negative and positive controls, respectively. 3[H]-thymidine (0.5 µCi/well) was added to wells on day 5 for the final 16h of the culture period to assess drug-induced PBMC proliferation. Plates were harvested using TomTec Harvester 96 (Receptor Technologies) onto filter mats and sealed with scintillation wax and counted using a MicroBeta TriLux 1450 LSC β-counter (PerkinElmer). PBMC IFN-γ secretion was also quantified using ELISpot. ELISpot plates previously coated overnight with IFN‐γ capture antibody were washed to remove unbound antibody. Plates were blocked for 30min with cell culture media and PBMC (0.5 x 106) were cultured with graded concentrations of the test compounds. Cells were incubated at 37°C, 5% CO2 for 48 h and the ELIspot plates were developed according to manufacturer’s instruction (Mabtech, Nacka Strand, Sweden).

**Generation of test compound-responsive TCC**

PBMC were cultured with either clozapine (5 µM) or olanzapine (5 µM) in IL-2 (50 U/ml; Peprotech, NJ, USA) supplemented media for 14 days. TCC were generated by serial dilution and repeated PHA stimulation. Epstein–Barr virus (EBV)‐transformed B‐cell lines were generated from autologous PBMC with established methods (25) and used as antigen‐presenting cells. Drug-specificity was assessed by culturing irradiated autologous EBV-transformed B-cells (1×104/well) and TCC (5×104/well; 200μl) for 48h in duplicate wells. Proliferation was measured by the addition of [3H]-thymidine followed by scintillation counting. Drug-responsive TCC with stimulation index (SI) ≥ 2 were expanded for dose-response testing and functional assays (SI = mean cpm of drug-treated wells/mean cpm of medium-treated wells).

**Flow cytometry**

Flow cytometry was initially used to determine the CD4/CD8 phenotype of TCC. CD4-APC and CD8-PE fluorescence antibodies were purchased from BD Biosciences (Oxford, UK). T‐cell receptor (TCR) Vβ typing for 24 commonly expressed TCR was performed using the IOTest Beta Mark TCR Vβ Repertoire kit purchased from Beckman Coulter Ltd (High Wycombe, UK). Finally TCC were profiled for the expression of chemokine receptors including CXCR3, CLA, CCR1, CCR6, CD69, CCR4, CCR9, CXCR6, CCR2, CCR8 and E-cadherin using fluorescent antibodies purchased from BD Biosciences (Oxford, UK). Cells (10,000) were acquired using a FACSCanto II flow cytometer (BD Biosciences) and data was analysed by Cyflogic.

**Pathways of TCC activation with clozapine and structurally-related compounds**

The dose-dependent proliferative response of TCC (5×104/well; 200μl) was assessed by culturing irradiated EBV-transformed B-cells (1×104/well) with clozapine, *N*-desmethyl clozapine, clozapine *N-*oxide or olanzapine (all 0.5-100 µM) for 48h in triplicate wells at 37°C, 5% CO2. Proliferation was measured by the addition of [3H]-thymidine (0.5 µCi/well) followed by scintillation counting. IFN-γ secretion measured via ELISpot was used as a readout for TCC activation in certain experiments.

In initial experiments to assess pathways of TCC activation, the autologous EBV-transformed B-cells were omitted from the T-cell assays. Subsequently, EBV-transformed B-cells were pulsed for periods of 0.5 - 48 hours with clozapine (1 µM), prior to washing 3 times to remove the unbound drug. The EBV-transformed B-cells were then irradiated and co-incubated (1x104/50 µL) with TCC (5x104/50 µL) in the absence of soluble drug for a period of 48 hours, (37°C, 5% CO2). T-cell activation was quantified via proliferation measured using [3H] thymidine incorporation. TCC incubated with unpulsed autologous EBV-transformed B‑cells in the presence and absence of the free drug were used as controls. EBV transformed B-cells were also fixed with glutaraldehyde, prior to quenching with glycine and 3 washing steps to prevent antigen processing. TCC were incubated with fixed EBV-transformed B-cells in the presence and absence of clozapine for a period of 48 hours (37°C, 5% CO2). T-cell activation was again quantified via proliferation measured using [3H] thymidine incorporation.

**Cytokine secretion from TCC activated with clozapine and structurally-related compounds**

The cytokine secretion from TCC was assessed using ELIspot plates coated for the following cytokines and effector molecules; IFN-γ, granzyme B, perforin, Fas L, IL-5, IL-10, IL-13, IL-17 and IL-22. Following overnight incubation, the ELIspot plate was blocked and TCC were co-incubated with autologous EBV transformed B-cells in the presence and absence of clozapine, olanzapine, *N-*desmethyl clozapine and clozapine *N-*oxide (37°C, 5% CO2). Following 48-hour incubation, the ELIspot plate was developed according to the manufacturer’s instructions and spots were counted using an AID ELIspot reader.

**HLA restriction and HLA mismatch assays**

To delineate the HLA molecules involved in the activation of TCC, the TCC (5×104/well; 200μl) were cultured with autologous EBV-transformed B-cells (1×104/well) and clozapine in the presence or absence of anti‐human HLA class I, HLA class II and anti‐HLA‐DR antibodies or isotype control antibodies (BD Pharmingen, San Jose). Proliferation was measured using [3H] thymidine. An HLA mismatch assay using heterologous EBV-transformed B-cells as antigen presenting cells (from HLA-typed donors) was used to identify HLA allele restriction of the TCC. Clozapine-responsive TCC were cultured as described above with either autologous EBV‐transformed B-cells or a panel of 13 heterologous EBV‐transformed B-cells expressing similar or dissimilar HLA alleles of interest (Table 2). The initial selection of heterologous antigen presenting cells for the HLA mismatch assay was based on the expression (or not) of HLA‐DQB1\*02:01 allele reported in GWAS studies (11, 26) and a variety of HLA-DRB1 alleles.

***In silico* modelling of molecular interaction between HLA-DRB1\*15:01 binding peptide and drugs**

As the activation of certain TCC with clozapine was restricted to HLA-DRB1\*15:01, the established crystal structure (PDB code 1bx2) (27) was used to generate drug binding models with Pymol (2.0, Schrodinger). After removal of the HLA-DRB1\*15:01 binding peptide, GOLD 5.2 (CCDC software) (28) was used to dock clozapine, clozapine *N*-oxide, and olanzapine to the binding groove, with the binding site defined as 15 Å around the binding point. A generic algorithm with ChemPLP as the fitness function was used to generate 10 binding modes per ligand. Default settings were retained for the “ligand flexibility”, “fitness and search options”, and GA settings.

**Characterisation of clozapine- and olanzapine-induced changes in TCC protein expression**

To investigate clozapine- and olanzapine-induced changes in protein expression, TCC (1 x 106) were cultured with clozapine (1.68 µM; plasma concentration or 5 µM; *in vitro* optimum stimulatory concentration) or olanzapine (0.16 µM; plasma concentration or 5 µM; optimum *in vitro* stimulatory concentration) in the presence of autologous EBV-transformed B-cells (0.2 x 106) for 8, 24 and 48 h at 37°C, 5% CO2. Untreated cells were also set up as negative controls. After incubation, CD19-conjugated beads (Miltenyi Biotec, Gadbach, Germany) were used for the negative selection of TCC and changes in protein expression determined by iTRAQ. In preliminary experiments, protein lysates for pure TCC were compared with CD19-conjugated magnetic bead selected TCC using gel electrophoresis and coomassie blue staining. Furthermore, TCC protein expression post drug-stimulation was examined with coomassie blue staining before iTRAQ was performed on samples.

**Statistics**

T cell proliferation and ELISpot assays were performed in triplicate wells and statistical significance (\*p ≤ 0.05) was determined using Mann Whitney test (GraphPad Prism software).ANOVA was used to determine significant changes in drug-induced protein expression (statistical significance; \*p ≤ 0.05).

**Results**

**Detection of clozapine-responsive TCC from drug-naïve individuals and patients with agranulocytosis**

Significant clozapine- or olanzapine-induced activation of PBMC from patients with agranulocytosis and healthy donors was not detected, using [3H] thymidine and ELISpot to measure proliferation and IFN-γ release, respectively (results not shown). Thus, greater than 800 and 1200 TCC were generated from clozapine/olanzapine-treated PBMC from healthy donors expressing HLA-DQB1\*02:01 (n=3) and patients with agranulocytosis (n=3), respectively.

Four clozapine-responsive TCC were identified from donor HLA-997 after initial testing using proliferation as a readout (medium control or clozapine [5 µM] in duplicate; figure 1). Following mitogen-driven expansion, the TCC were found to be CD4+ 9 (n=3) or CD8+ (n=1), they expressed varied T-cell Vβ receptors (16, 20 and 22; the final TCC did not express any of the Vβs that cover 80% of the known receptors) and proliferated in the presence of clozapine (1-100 µM) in a dose-dependent manner. Figure 1C shows the proliferative response of the 3 CD4+ TCC. Clozapine-responsive TCC were not detected from donors HLA-998 and HLA-999. Initial specificity testing using olanzapine suggested the presence of 2 olanzapine-responsive TCC from donor HLA-999; however, both TCC did not proliferate after expansion to graded concentrations of olanzapine and were therefore unsuitable for further assays.

Initially, 6 clozapine-responsive TCC were identified from agranulocytosis patient 2; however, only 2 proliferated in a clozapine dose-dependent-manner after further expansion (figure 1B and C). Both TCC were CD4+, one expressed Vβ 5.1, while the Vβ expressed by the other TCC was not detected with our testing kit. Clozapine-responsive TCC were not detected from patients 1 and 3 PBMC. Furthermore, TCC from all 3 patients were not stimulated to proliferate with olanzapine.

**Characterisation of chemokine receptor expression and clozapine-induced TCC cytokine secretion**

CD4+ TCC from patients with agranulocytosis and healthy donors expressed high levels of CXCR3, and CCR4, while CCR6 and CCR9 were expressed on 2 of the TCC (figure 2A). When activated with clozapine, the CD4+ TCC secreted a combination of Th1 (IFN‐γ) and Th2 (IL-5, IL-10, IL-13) cytokines. Several TCC also secreted IL-17 and/or IL-22 alongside the cytolytic molecules granzyme-B, perforin and Fas-ligand. The single CD8+ TCC secreted similar cytokines, albeit to a lesser extent (figure 2B and C). IFN-γ, granzyme B and Fas L ELISpot were used to assess the kinetics of cytokine/effector molecule release from the TCC. All three mediators were secreted in a time-dependent manner, with maximum levels observed after 24-48 h (figure 2D).

**Pathway of activation of clozapine-responsive TCC**

The proliferative response of TCC with clozapine was dependent on the presence of antigen presenting cells (autologous EBV-transformed B-cells) (figure 3 A, figure 4 A and B). Antigen presenting cells pulsed with clozapine for 0.5-48 h and washed extensively before co-culture with TCC failed to induce a proliferate response (figure 3A). In contrast, proliferation was detectable when TCC were incubated with clozapine and glutaraldehyde-fixed antigen presenting cells (figure 3A). Peroxidase-catalysed metabolism of clozapine generates a nitrenium ion that covalently modifies cysteine residues protein; glutathione binds to the nitrenium ion and when in excess blocks the protein binding interaction (20, 29). The addition of glutathione (1mM) to cell culture medium did not reduce clozapine-induced proliferation of TCC (figure 3B). Collectively, these data indicate that clozapine activates TCC via a direct and processing-independent binding interaction with HLA molecules.

HLA mismatching experiments were conducted to investigate whether HLA-DQB1\*02:01 was involved in the presentation of clozapine to the CD4+ TCC. These experiments involved culturing TCC with clozapine and a panel of heterologous antigen presenting cells before measuring proliferation of the TCC. The proliferative response of TCC from both healthy donors and patients with agranulocytosis was not associated with antigen presenting cell expression of HLA-DQB1\*02:01 (figure 4A and B). Instead, the activation of the TCC mapped to HLA-DR molecules. TCC from the healthy donors were activated when clozapine was cultured with antigen presenting cells expressing HLA-DRB1\*07:01 (expressed on the autologous antigen presenting cells) and HLA-DRB1\*15:01. In contrast, the activation of TCC from patients with agranulocytosis was associated with HLA-DRB1\*11 molecules. Proliferative responses were detected with antigen presenting cells expressing HLA-DRB1\*11:01 (expressed on the autologous antigen presenting cells) and HLA-DRB1\*11:04 (figure 4B). HLA-DR restriction of the response of TCC with clozapine was confirmed through the use of an HLA-DR blocking antibody. TCC were not stimulated to proliferate with clozapine when autologous antigen presenting cells were pre-treated with the blocking antibody (figure 4C).

**Cross-reactivity of clozapine-responsive TCC with clozapine metabolites and olanzapine**

TCC proliferation and cytokine release, measured using [3H] thymidine incorporation and ELISpot, respectively, were used to assess cross-reactivity of clozapine with its two major stable metabolites (clozapine *N*-oxide and *N*-desmethyl clozapine) and olanzapine (figure 5A). Three patterns of cross reactivity were observed with the clozapine-responsive TCC (figure 5B). One TCC was activated with *N*-desmethyl clozapine and olanzapine, but the minimum stimulatory concentration for proliferation and cytokine release with both compounds was approximately 10-fold higher than clozapine (figure 5B and C). The second TCC was activated with olanzapine, while the third TCC was activated with *N*-desmethyl clozapine. For these TCC, the minimum stimulatory concentration of olanzapine and *N*-desmethyl clozapine was the same as clozapine. None of the TCC were activated with clozapine *N*-oxide (figure 5B).

***In-silico* molecular docking of clozapine, olanzapine and clozapine *N*-oxide with HLA-DRB1\*15:01**

Availability of the X-ray crystal structure of HLA-DRB1\*15:01 enabled modelling of the potential binding interaction between clozapine, clozapine *N*-oxide and olanzapine and the peptide binding groove. All 3 compounds to occupied the P4-P6 binding pockets of HLA-DRB1\*15:01 (figure 6A). The P1 pocket is small and hydrophobic and cannot accommodate the large aromatic groups of the test compounds. In contrast, the P4 binding pocket with Ala100 is able to accommodate aromatic substituents. Clozapine and olanzapine bound to P4/P6 pockets with a similar conformation due to structural rigidity. Clozapine closely interacts with the key amino acid residues within the binding groove, forming multiple H-bonds with Arg191 and Asp206 (figure 6B and C). In contrast, clozapine *N*-oxide occupies the P6 pocket with a different conformation, with the *N*-oxide moiety pointing inside toward the binding groove to interact with Trp187 and Asn68 (figure 6B and D).

**Drug- and dose-dependent changes in T-cell protein expression**

Changes in protein expression following TCC stimulation with either clozapine (1.68 µM [therapeutic] or 5 µM [stimulatory for all TCC]) or olanzapine (0.16 µM [therapeutic] and 5 µM [stimulatory for all TCC]) for 8-48 h was investigated using iTRAQ. Approximately 2300 proteins were quantified across all the samples. Preliminary experiments showed a similar pattern of protein migration from pure TCC or CD19 antibody-conjugated bead sorted TCC (figure S1A and B), suggesting that the purification step did not interfere with protein expression. All the TCC stimulated with clozapine showed similar patterns of protein expression that clustered together as demonstrated by the principal component analysis (PCA) and hierarchical cluster heat map (figure 7A and B).

TCC cultured with the plasma concentration of clozapine showed significant changes in protein expression when compared with the same TCC stimulated with the olanzapine plasma concentration, figure 7C. Each red dot on the volcano plot represents a protein with a significant change in expression post drug treatment. Red dot plots to the left of the graph are down regulated proteins while dot plots to the right hand side represent upregulated proteins. Changes in protein expression for clozapine at 5 µM was greater than treatment with 1.68 µM clozapine, suggesting a dose-dependent effect (figure 7C). TCC stimulated with 5 µM olanzapine displayed more changes in protein expression compared with 0.16 µM of olanzapine but significantly less change in protein expression compared with clozapine (5 µM) (figure 7C). On the whole, clozapine treatment resulted in significantly more changes in protein expression compared with olanzapine (figure S2).

**Analysis of proteins involved in immunological processes**

Panther gene ontology software was used to cluster the observed protein after stimulation with either clozapine (1.68 µM) or olanzapine (0.16 µM) for 48 h. Most of the proteins were involved in cellular and metabolic processes. Interestingly, only the TCC activated with the plasma concentration of clozapine (1.68 µM) showed upregulation in proteins involved in immunological process (figure 8A). The 7 proteins upregulated post clozapine stimulation included immunoglobulin heavy constant mu, tyrosine-protein kinase JAK3, signal transducer and activator of transcription 5A, long-chain-fatty-acid-CoA ligase 1, activator of 90 kDa heat shock protein ATPase homolog 1, T-cell surface antigen CD2 and guanylate-binding protein 2 (fold changes 0.3-8.5; figure 8b). Intriguingly, stimulation of TCC with 30x the olanzapine plasma concentration (5 µM) resulted in the upregulation of 5 proteins (tyrosine-protein kinase JAK3, signal transducer and activator of transcription 5A, guanylate-binding protein 2, T-cell surface antigen CD2 and activator of 90 kDa heat shock protein ATPase homolog 1) that were also observed with clozapine treatment (figure 8B). Further analysis of clozapine-induced changes in protein expression using ingenuity pathways analysis revealed that upregulation of syntaxin 11 (STX11) indirectly activates the IL-1β pro-inflammatory signalling pathway via platelet factor 4 (PF4) and HLA-DR (figure 8C).

**Discussion**

Clozapine is distinctive in that it has been reinstated to clinical practice after withdrawal due to serious and sometimes fatal agranulocytosis. This is because clozapine is the most efficacious drug available for the management of treatment-resistant schizophrenia. Its use is accompanied by monitoring of haematological parameters, especially white blood cell counts, to detect the development of neutropenia at the earliest possible time-point and prevent the neutropenia from progressing to agranulocytosis. The delayed onset of neutropenia (1-2 weeks) and agranulocytosis (3-4 weeks) after initial clozapine exposure, the rapid onset upon rechallenge and multiple HLA associations are plausible reasons to support an immune-mediated pathogenesis (8). Hence, there is a need to examine whether clozapine stimulates human T-cells to proliferate and secrete effector molecules.

Initial experiments aimed to detect the presence of clozapine-responsive T-cells in blood of patients with agranulocytosis using the lymphocyte transformation test and IFN‐γ ELISpot assay, which measure proliferation and cytokine release, respectively. Patients were selected primarily according to clinical diagnosis of a clozapine-associated adverse event; however, given the growing evidence for the role of HLA-DQB1 variants and in particular HLA-DQB1\*02:01 in neutropenia and agranulocytosis (8-12), 2 out of the 3 patients expressed this allele. Negative results were obtained with both assays using PBMC from all 3 patients. Similar findings have been reported previously with PBMC from patients with flucloxacillin-induced liver injury in patients expressing HLA-B\*57:01+ (30). Furthermore, PBMC diagnostic assays such as the lymphocyte transformation test are known to have a low sensitivity in patients with Stevens Johnson syndrome and toxic epidermal necrolysis the most severe forms of cutaneous drug-induced event (31). In both examples, the negative results were associated with a low frequency of drug-responsive T-cells circulating in the periphery when the assays were conducted, often years after the adverse event. Consequently, almost 600 TCC were generated from clozapine-exposed patient T-cell lines and assessed for clozapine-specific T-cell responses. As a comparator, in excess of 900 TCC were expanded from PBMC of 3 healthy donors expressing HLA-DQB1\*02:01. Low numbers of clozapine-responsive TCC were generated from one patient with agranulocytosis and one healthy donor and these clones were then characterized in terms of phenotype and function. At this point, it is interesting to speculate why the TCC were not detected in all patients and healthy donors. TCC may exist in all individuals expressing the relevant HLA molecules for antigen presentation; however, their low frequency in certain individuals thwart detection. Alternatively, immune regulatory pathways (e.g., immune checkpoints, Tregs) might restrict the expansion of clozapine-responsive T-cells in certain individuals. Finally, it is feasible that only a small percentage of the population express clozapine T-cell receptor clonotypes. In this respect, a preferential T-cell receptor usage has recently been described in HLA-B\*15:02+ patients that develop carbamazepine-induced severe cutaneous adverse reactions (32).

All but one of the clozapine-responsive TCC were CD4+. Clozapine exposure resulted in a dose-dependent proliferative response and the secretion of Th1 and Th2 cytokines, IL22 and cytolytic molecules. The clones expressed chemokine receptors CCR4 and CXCR3, which are associated with Th2 and Th1 cytokine secretion, respectively (33). It is plausible that activation of clozapine-specific TCC alters the cytokine microenvironment and enhances the rate of spontaneous neutrophil apoptosis or the apoptosis induced by the nitrenium ion of clozapine when formed locally within neutrophils and neutrophils precursors (21). A previous study suggesting that Fas ligand expression regulated by Foxo3a on neutrophils is implicated in neutrophil apoptosis highlights the potential importance of the cytolytic molecules secreted by clozapine-responsive TCC in the adverse event (34). Thus, in-going experiments we are developing an autologous neutrophil TCC culture system to study neutrophil antigen presentation and the killing of neutrophils by the clozapine-responsive TCC.

The association of clozapine-induced neutropenia/agranulocytosis with the expression of HLA class II variants prompted us to explore pathways of clozapine-specific T-cell activation. Clozapine was found to interact directly with HLA molecules expressed on the surface of the antigen presenting cells to activate the CD4+ TCC. Evidence for this was several-fold. First, TCC proliferation and cytokine release with clozapine was dependent on the presence of antigen presenting cells (EBV-transformed B-cells). Second, fixation of antigen presenting cells with glutaraldehyde, which blocks protein processing (35), did not prevent the activation of TCC. Third, TCC were not activated with clozapine-pulsed antigen presenting cells. Fourth, glutathione, which blocks clozapine metabolite modification of proteins (20, 29), did not inhibit the activation of the clones. Finally, anti-HLA blocking antibodies inhibited T-cell activation. A panel of antigen presenting cells generated from 14 healthy donors expressing different HLA-DRB1 and HLA-DQB1 alleles were used to explore the HLA molecules involved in the presentation of clozapine to TCC. These studies revealed that activation of TCC mapped to HLA-DR molecules: HLA-DRB1\*11:01 and 11:04 for the patient TCC and HLA-DRB1\*07:01 and 15:01 for the healthy donor TCC. Interestingly, all 4 HLA-DR molecules have a sequence homology of 90% or more (<https://www.ebi.ac.uk/ipd/imgt/hla/align.html>). Thus, activation of the TCC occurs as a consequence of a clozapine HLA-DR binding interaction, with HLA-DQ molecules playing no apparent role.

Major metabolites of clozapine (*N-*desmethyl clozapine and clozapine *N*-oxide) and the anti-psychotic medication olanzapine, which is not associated with a high incidence of agranulocytosis, have similar core structures and thus were assessed for their ability to activate the TCC. TCC were stimulated to proliferate and secreted cytokines and cytolytic molecules with *N-*desmethyl clozapine and/or olanzapine, but not clozapine *N-*oxide. The high resolution X-ray crystallographic structure of HLA-DRB1\*15:01 is available and was used to model and compare the theoretical docking of the clozapine, olanzapine and clozapine *N-*oxide within the HLA peptide binding cleft. Clozapine, and olanzapine bound to P4/P6 pockets with a similar conformation due to their structural rigidity, whereas clozapine *N-*oxide occupied the P6 pocket with a different conformation. Although these modelling data should be interpreted with a degree of caution as they do not incorporate the peptide sequence that would complete the structure of the T-cell binding epitope, they do provide a possible explanation for the observed cross-reactivity profile of the TCC.

The TCC were activated with clozapine at therapeutic plasma concentrations. In contrast, the concentration of olanzapine required to activate the same clones exceeded the plasma concentration of 0.16 µM by 30-50 fold. This suggests that the lower daily dose of olanzapine might be in part responsible for the lack of T-cell activation in patients and the significantly lower incidence of blood dyscrasias, when compared with clozapine (36, 37). To further investigate the nature of the T-cell response induced with clozapine and olanzapine, TCC were stimulated with both compounds at therapeutic and T-cell stimulatory concentrations (clozapine [1.68 µM, 5 µM], olanzapine [0.16 µM, 5 µM]) and drug-induced changes in protein expression were analyzed at 8, 24 and 48 h using iTRAQ. PCA and hierarchal cluster revealed that clozapine stimulation resulted in greater number of drug-associated changes in protein expression, irrespective of dose, treatment duration or the TCC. A greater number of significant TCC protein changes (both up and down regulated) were also observed in the volcano plots presented in figure 7c, when clozapine treatment was compared with olanzapine at both therapeutic and T-cell stimulatory concentrations. Panther gene ontology assessment showed increased expression of immune system processes with clozapine, but not olanzapine at therapeutic concentrations. However, a similar pattern of upregulated immune-related proteins was observed when TCC treated with therapeutic clozapine an optimal T-cell stimulatory olanzapine concentrations were compared. Expression of tyrosine-protein kinase JAK 3, signal transducer and activator of transcription 5A, guanylate-binding protein 2, T-cell surface antigen CD2 and activator of 90kDa heat shock protein ATPase homolog 1 was increased with both conditions. Missense mutations in JAK3 has previously been shown to cause pathway hyperactivation resulting in cytokine hyper-responsiveness (38, 39). In addition JAK3-STAT5A signalling induced by IL-2 is critical for T-cell proliferation (38, 40). Activator of 90kDa heat shock protein ATPase homolog 1 activates HSP90AA1 resulting in an increase in chaperone activity and plays a critical role in the regulation of phenotype and function of human T-cells (41). Finally, T-cell surface antigen CD2 is a costimulatory receptor found on the surface of T-cells and NK cells. CD2 interacts with CD58 on antigen presenting cells to trigger T-cell activation (42).

Although, clozapine is the drug of choice for the management of patients with refractory schizophrenia, idiosyncratic agranulocytosis remains the major issue. Data presented herein demonstrates that clozapine activates human CD4+ T-cells via an HLA-DR restricted pathway. Future research is needed to delineate the precise role the T-cells plays in clozapine-induced adverse events in patients and indeed whether the adaptive immune system acts to exaggerate the direct toxic and stress pathways induced by myeloperoxidase generated clozapine metabolites.

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**List of abbreviations**

Human leukocyte antigen, HLA; peripheral blood mononuclear cells, PBMC; phycoerythrin, PE; allophycocyanin, APC; fluorescein isothiocyanate, FITC; T-cell clone, TCC; principal component analysis, PCA.

**Figure legends**

**Figure 1. Generation of clozapine-responsive TCC from HLA-DQB1\*02:01+ healthy donors and patients with agranulocytosis.** PBMC from (A) clozapine-naïve healthy donors and (B) patients with agranulocytosis were cultured with either clozapine (5 µM) or olanzapine (5 µM) for 14 days. TCC were generated from the T-cell lines by serial dilution and repeated mitogen stimulation. Fast growing TCC were selected, expanded and tested for clozapine/olanzapine responsiveness. TCC (0.5 x 105) were cultured with autologous EBV-transformed B-cells (0.1 x 105) and clozapine (5 µM) or olanzapine (5 µM) in duplicate cultures for 48 h at 37°C, 5% CO2. [3H]-thymidine (0.5µCi/well) was added for the last 16 h of the culture period. TCC with SI >2 (mean cpm in drug-treated wells / mean proliferation in control wells with medium) were expanded for further phenotypic and functional characterisation. C. Dose-dependent proliferation of clozapine-specific TCC (0.5 x 105) cultured with autologous EBV-transformed B-cells (0.1 x 105) and drug in triplicate cultures for 48 h at 37°C, 5% CO2. Proliferation was measured through the addition of [3H]-thymidine (0.5µCi/well).

**Figure 2. TCC express CXCR3 and CCR4, and secrete cytokines and cytolytic molecules markers following clozapine exposure.** (A)Phenotyping of clozapine-responsive CD4+ TCC was performed using fluorescent-conjugated antibodies. Fluorescence intensity was measured using a FACS canto II flow cytometer, counting a minimum of 10,000 events. TCC were stained with antibodies for CXCR3-APC, CLA-FITC, CCR1-PE, CCR6-APC, CD69-FITC, CCR4-PE, CCR9-APC, CXCR6-PE, CCR2-APC, CCR8-FITC and E-Cad-PE. (B, C, D) ELISpot was used for the detection of IFN‐γ, IL‐5, IL-10, IL‐13, IL‐17, IL‐22, granzyme B, perforin, and Fas‐ligand from clozapine exposed TCC. TCC (0.5 x 105) were cultured with autologous EBV-transformed B-cells (0.1 x 105) and clozapine (5-10 µM) in ELISpot plates pre-coated with the respective capture antibodies for 48 h. Plates were then developed using specific secondary antibodies and the secretion of proteins was visualized as spot counts. (B) ELISpot images showing cytokine/cytolytic molecule secretion from a representative CD4+ TCC. (C) Quantification of cytokine/cytolytic molecule secretion from 3 CD4+ and 1 CD8+ TCC using spot counts. (D) Time-dependent secretion of IFN‐γ, granzyme-B and Fas ligand from 2 representative CD4+ TCC.

**Figure 3. Clozapine activates TCC through a direct binding interaction with HLA molecules.** (A) TCC (0.5 x 105) were cultured clozapine in the presence or absence of autologous EBV-transformed B-cells (APC; 0.1 x 105), or with autologous EBV-transformed B-cells (0.1 x 105) pulsed with clozapine for 0.5-48 h. EBV-transformed B-cells were washed repeatedly to remove unbound clozapine prior to culturing with the TCC. EBV-transformed B-cells were also fixed with glutheraldehyde to prevent protein processing before addition to the T-cell assay. (B) TCC (0.5 x 105) were cultured clozapine and autologous EBV-transformed B-cells (0.1 x 105) in the presence and absence of glutathinoe (1 mM) for 48 h. [3H]thymidine (0.5µCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured using a β-counter.

**Figure 4. Clozapine binds to HLA-DR molecules activate CD4+ TCC.** (A, B) TCC (0.5 x 105) were cultured clozapine and either autologous or heterologous antigen presenting cells (APC; 0.1 x 105) expressing various HLA-DRB1 and DQB1 alleles for 48 h at 37°C, 5% CO2. (see table 2 for the full HLA type of the antigen presenting cells). (C) TCC (0.5 x 105) were cultured clozapine and autologous EBV-transformed B-cells (0.1 x 105) in the presence or absence of an HLA-DR blocking antibody and the isotype control for 48 h. [3H]thymidine (0.5µCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured using a β-counter.

**Figure 5. Clozapine-responsive TCC cross-react with *N*-desmethyl clozapine and olanzapine.** (A) Structure of clozapine, its major stable metabolites (clozapine *N*-oxide and *N*-desmethyl clozapine) and olanzapine. (B, C) Patterns of cross-reactivity of clozapine-responsive TCC with clozapine *N*-oxide, *N*-desmethyl clozapine and olanzapine. TCC and antigen presenting cells were cultured with either clozapine, stable metabolites or olanzapine using cell numbers described above. Cross reactivity was determined by [3H] thymidine and beta counting.

**Figure 6. *In-silico* molecular docking of clozapine, clozapine *N*-oxide and olanzapine into the HLA-DRB1\*15:01 peptide binding pocket.** (A) Docking of clozapine, clozapine *N*-oxide, and olanzapine with HLA-DRB1\* 15:01 (PDB code 1bx2) shows all compounds bind to the P4-P6 pocket. (B) Clozapine and olanzapine bind to HLA-DRB1\*15:01 with similar conformation whereas clozapine *N*-oxide interacts differently. (C) Close-by view of the interaction between clozapine and HLA-DRB1\*15:01. H-bonding interactions between clozapine and residues Arg191 and Asp206 are highlighted. (D) Close-by view of the interaction between clozapine *N*-oxide and HLA-DRB1\*15:01. H-bonding interactions between clozapine and residues Trp187 and Asp68 are highlighted. Docking images were created using PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.)

**Figure 7. Clozapine and olanzapine concentration-dependent changes in TCC protein expression.** TCC were cultured clozapine in the presence of autologous EBV-transformed B-cells for 24-48 h. B-cells were depleted prior to protein expression profiling using ITRAQ. (A) PCA plot of clozapine or olanzapine-induced changes in T-cell protein expression after 8-48 h. (B) Hierarchical cluster analysis showing clozapine-and olanzapine-dependent changes in T-cell protein expression, 24-48 h post drug stimulation. Clustering of the clozapine samples is clearly illustrated. (C) Volcano plot illustrating the fold change in protein expression for TCC treated with plasma concentrations or optimum T-cell stimulatory concentrations of clozapine and olanzapine when compared with control cells exposed to culture medium. Red dots represent proteins whose expression has changed by a significant amount. Statistical analysis was performed using ANOVA.

**Figure 8. Analysis of changes in expression of TCC proteins involved in immunological processes following clozapine or olanzapine treatment.**

(A) Classification of clozapine- and olanzapine-induced changes in protein expression on the basis of biological process using pathway using PANTHER gene list analysis software. (B) Bar charts showing upregulated TCC proteins involved in immunological processes upon treatment with therapeutic clozapine (1.68 µM) and optimal T-cell stimulatory olanzapine (5 µM) concentrations. (C) Ingenuity pathway analysis of TCC protein expression changes involved in cell to cell signalling interactions and the inflammatory response after clozapine treatment.

**Figure S1. Protein expression in TCC before and after depletion of antigen presenting cells.** (A) Coomassie blue stain comparing pure T-cell protein lysates and T-cell lysates from CD19 antibody conjugated bead sorting from T-cell/antigen presenting cell co-culture. (B) Coomassie blue stain of protein lysates from TCC stimulated with clozapine (1.68 µM, 5 µM) and olanzapine (0.16 µM, 5 µM) for 8-48 h.

**Figure S2. Dose- and drug-dependent changes in protein expression.** Volcano plot of fold change in protein expression for T-cells treated with plasma concentrations of clozapine (1.68 µM) relative to treatment with olanzapine (0.16 µM) and clozapine compared with olanzapine at T-cell stimulatory concentrations. Red dots represent proteins whose expression has changed by a significant amount. Analysis was performed using ANOVA.

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