University of Oxford

Investigation Of Auto-Antibodies Targeting TNF-Related Peptides As Possible Biomarkers For Lethal Sepsis

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Background: Necroptosis is a programmed form of necrotic cell death. Necroptosis is thought to play an important role mediating the pathogenesis of inflammatory disease. Previously, a 30-mer peptide, P1213, within the most conserved region of TNF was found to be capable of promoting apoptosis. We discovered that autoantibodies targeting the P1213 region of TNF correlated with inflammatory disease, such as rheumatoid arthritis. It has been demonstrated that the autoantibodies convert TNF-related apoptosis into necroptosis when they bind to the apoptosis-inducing P1213 motif.

Objectives: Explore the link between autoantibodies against different regions of the TNF molecule and the clinical outcomes of severe sepsis, using serum samples of patients divided into those who died (lethal sepsis) and those who survived. In addition to explore cellular mechanisms whereby the autoantibodies may promote TNF-mediated necroptosis, and to assess whether the autoantibodies provide a potential biomarker for susceptibility to lethal sepsis.

Results: Through ELISAs and flow cytometry analysis, we found more than half of deceased sepsis patient sera (n=25) were positive for anti-P1213 autoantibodies. We discovered another conserved region, P21, which has the capacity to induce apoptosis similar to P1213. Part of P1213 and P21 are located physically adjacent to each other within the TNF conformational structure.

Conclusion: We have identified the autoantibodies as a potential biomarker to associate mortality in severe sepsis although not all deceased sepsis patients possess the autoantibodies. P1213 and/or P21 oligopeptides could be developed as neutralising ligands for the autoantibodies to protect sepsis patients from mortality.

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Chapter 1 – Introduction

1.1 TNF in inflammatory diseases

Tumour Necrosis Factor (TNFα) is a pleiotropic cytokine that is produced by various immune cells, but predominantly macrophages and T cells (Bradley, 2008). TNFα is an important inducer of a local inflammatory response which helps to contain infections. However, it has been found to play a role as a key mediator in acute and chronic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, and ankylosing spondylitis (Moudgil and Choubey, 2011) (Sticherling, 2016) (Ellis and Braley-Mullen, 2017). These conditions may ultimately be arisen by dysregulated TNFα signalling when regulation of immune cells is defective. It has been shown that TNFα as well as Tumour Necrosis Factor Receptor 1 and 2 (TNFR1 and TNFR2) are upregulated both locally and systemically in serum in the patients of these inflammatory diseases as TNFα levels correlate to the disease activity (Ślebioda and Kmieć, 2014). For example, synovial hyperplasia with inflammatory immune cells like T cells and macrophages is a common feature in rheumatoid arthritis, which consequently lead to over-production of inflammatory cytokines including TNFα. TNFα dictates the production of other proinflammatory cytokines such as interlekin-1 (IL-1) and interleukin-6 (IL-6), which in turn stimulate the production of matrix metalloproteinases (Kelley *et al.*, 2007), hence causing destruction against cartilage and bone at the inflamed joint.

1.2 The use of anti-TNFα as therapeutic

The crucial role of TNFα in inflammatory diseases can also be supported by the success of TNFα inhibitor as effective therapeutics on several chronic inflammatory diseases, including rheumatoid arthritis, psoriasis, some types of inflammatory bowel disease as well as ankylosing spondylitis. There are different forms of anti-TNFα biologic, including monoclonal antibodies (mAb) that target TNFα such as the chimeric mAb infliximab and the human mAb adalimumab, in addition to soluble protein which mimic TNFR such as etanercept (Kalliolias and Ivashkiv, 2015) (Li, Zheng and Chen, 2017) (Cohen and Sachar, 2017) (Garrouste *et al.*, 2016). However, one of the side effects of anti-TNFα treatments may reduce capacity of macrophages to kill intracellular microbes, which render patients become susceptible to mycobacterial infection (Ali *et al.*, 2013). Furthermore, although TNFα plays a key role in pathogenesis in animal models of acute inflammatory conditions such as sepsis, TNFα inhibitors are not effective in human sepsis, partly because other cytokines may also be responsible to the inflammatory condition. Interestingly, anti-TNFα biologic was initially designed to treat sepsis (Fisher *et al.*, 1996).

1.3 Biology of sepsis

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to bacterial infection (Singer *et al.*, 2016), where large quantities of bacterial products in patient's blood elevate cytokine productions, including TNFα and IL-1. These high levels of pro-inflammatory cytokines could eventually induce septic shock, a subset of sepsis with a great risk of mortality is associated with profound circulatory, cellular, and metabolic abnormalities, which may subsequently result in fall in blood pressure and disseminated intravascular coagulation (DIC)

(Semeraro *et al.*, 2010). In turn, DIC often lead to organ failure as blood perfusion is compromised. Hence, septic shock accounts very high mortality rate. In fact, sepsis is considered as a global health burden. It is responsible for 25.7% of admissions to 181 intensive care units (ICUs) over two years in England (McPherson *et al.*, 2013). In US, the mortality rates exceed 20% and is ranked first among causes of death in ICUs, where more than 200,000 patients die each year (Moore *et al.*, 2016). New reliable biomarkers both for diagnosis and as therapeutic targets in sepsis are much needed to reduce mortality.

1.4 TNFα signalling

TNFα initiates signalling by binding to TNFR1 or TNFR2. TNFR1 is expressed ubiquitously on most cell types whereas TNFR2 is only expressed on immune cells at low levels. The TNFα-TNFR2 signalling typically leads to immune modulatory functions and promotes tissue homeostasis and regeneration, but do not elicit cell death (Naudé *et al.*, 2011). On the other hand, TNFα-TNFR1 signalling results in the formation of complex I beneath the plasma membrane. TNFR1-associated dead domain protein (TRADD), an integral part of this receptor-coupled protein signalling complex, subsequently recruits receptor-interacting protein kinase 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2) upon activation, which in turn recruits cellular Inhibitor of apoptosis 1 and 2 (c-IAP1 and 2). The complex I plays a crucial role to downstream pro-survival NF-κB activation, which leads to inflammation, host defence, cell proliferation and survival. On the other hand, complex I can be altered by inhibition of NF-κB stimulation or due to TNFR1 signalling complex I destabilisation. As a result, TRADD and RIP1 detach membrane-associated complex I and migrate into cytoplasm, where they recruit Fas-associated dead domain protein (FADD) and caspase-8, caspase-10 and c-FLIP, thus forming complex IIa. In turn,

this stimulates the auto-catalytic capacity of caspase-8, eliciting apoptotic cell death with minimal immune activation. The exact mechanisms of how destabilisation of complex I and formation of complex IIa are unknown, but it has been shown that adaptor molecules, including c-IAPs and c-FLIP as well as A20 may be responsible for regulating the conformational change to the complex (Varfolomeev and Vucic, 2016).

1.5 TNFα-induced necroptosis

Recently, it has been discovered that TNFα-TNFR1 signalling can lead to the third outcome. This is demonstrated by using specific caspase inhibitor, z-VAD-fluoromethylketone (ZVAD), would result in necroptosis on L929 cells when combine with TNFα. Upon inhibition of caspase, RIP1 dissociated from complex IIa and form complex IIb with receptor-interacting protein kinase 3 (RIP3). The necrosome complex are subsequently auto-phosphorylated and allow phosphorylation of pseudokinase mixed lineage kinase domain-like (MLKL). This activates MLKL and induce its oligomerisation and migration to the plasma membrane to promote necroptosis by disrupting the membrane and triggering inflammation (Cai *et al.*, 2013). This regulated form of necrotic cell death has been implicated in inflammatory disease settings as high levels of RIP3 and MLKL are detected in ileum and colon of patients with inflammatory bowel disease and allergic colitis (Pierdomenico *et al.*, 2014). However, necroptosis is also being recognised as a key death pathway in physiological setting, such as mammalian bone growth plate. In addition, necroptosis is involved in host defence against certain apoptotic resistant viruses (e.g. cytomegalovirus) (Baillie, Sahlender and Sinclair, 2003).

The role of necroptosis in sepsis remains unclear, but recent mouse-model studies have revealed knock-out of RIP3 protect mice dying from lethal septic shock. Furthermore, the use of necrostatin-1, RIP1 inhibitor, also prolong mice survival significantly. These results indicate necrosis induced from RIP-1-RIP3 complex can lead to poor prognosis in sepsis and increase mortality (Duprez *et al.*, 2011).

Previous study from our group discovered that several conserved peptide regions of TNFα are related to cell death. For example, a 30-mer peptide (aa 110 – 140, P1213), was found to be capable of promoting apoptosis, whereas another region (aa 150 - 170, P16), can cause necrosis. They subsequently developed a mouse mAb, namely 244-12, which targets only the pro-apoptotic region (P1213) of TNFα (Lu *et al.*, 2016). They demonstrated that 244-12 binding and inhibiting P1213 convert low inflammatory apoptosis into high inflammatory necroptosis on mouse L929 cells, human chondrocytes and endothelial cells. It was postulated that 244-12-alike autoantibodies targeting pro-apoptotic motif of TNFα, P1213, can block TNFα-induced apoptosis, and these autoantibodies may contribute to the pathogenesis of inflammatory diseases, such as rheumatoid arthritis and sepsis, illustrated in Figure 1 below. Their latest study found these anti-P1213 autoantibodies within synovial fluid of some of the patients with rheumatoid arthritis before they received any TNFα-blocking treatments.

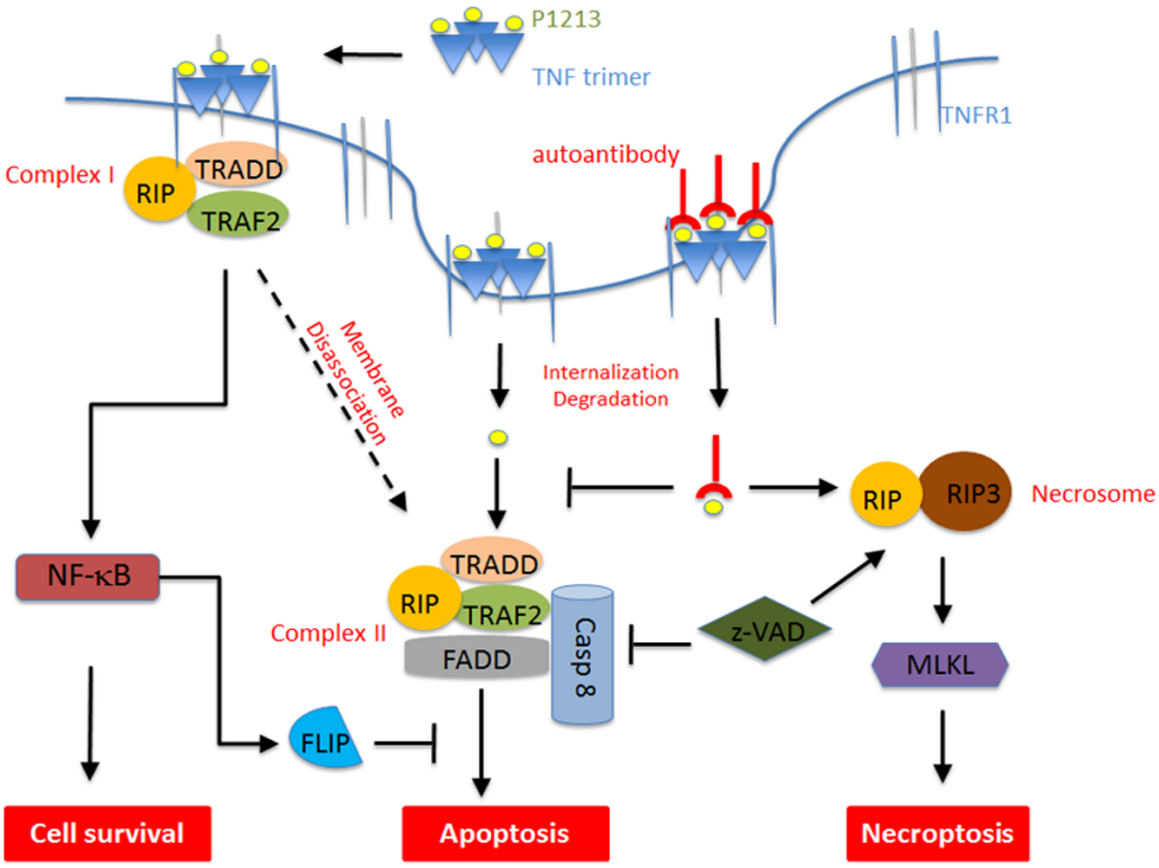


Figure 1. Proposed mechanism of action of P1213 autoantibody on TNF-induced cell death.

Hypothesis

We propose that autoantibodies targeting P1213 play a role in necroptosis, and in patients with severe sepsis and we hypothesise that presence of autoantibodies will correlate with lethality.

To address this hypothesis, experimental work in this thesis is divided into two chapter. Chapter 3 explores whether there is a link between autoantibodies against different regions of the TNFα molecule and the clinical outcomes of severe sepsis, using serum samples of patients divided into those who died (lethal sepsis) and those who survived.

Building on this, Chapter 4 investigates possible cellular mechanisms whereby the autoantibodies may promote TNFα-mediated necroptosis, and to assess whether the autoantibodies provide a potential biomarker for susceptibility to lethal sepsis.

Chapter 2 - Materials and methods

Human Specimens

Sera samples were obtained from 40 consecutive ICU patients with sepsis from the Shanghai Tenth People's Hospital, China. They were all studied with informed consent in full compliance with national and institutional ethical requirements (COREC number COREC06/ Q1606/139). Sepsis and septic shock was diagnosed according to the guidelines laid out at the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (1992). Sera were collected within 14 days of admission to hospital. Sera samples were collected from 25 healthy adult donors from Shanghai, China and Oxford, UK. 23 sera/plasma samples from myasthenia gravis patients were kindly provided by Prof. Nick Willcox. All samples were stored at −80°C until analysis.

Patients clinical characteristic

Seven out of 40 sepsis patients were excluded because of incomplete clinical information. 25 sepsis patients deceased shortly after diagnosis and admission to hospital, whereas eight patients recovered from severe sepsis (Table 1).

Table 1. Demographics and infection site of sepsis patients.

	Deceased	Recovered
Number of patients	25	8
Male	11	6
Female	14	2
Age range (years)	55-87	39-88
Median Age (years)	80	63
Infection Site:		
Severe Pneumonia	16	2
cholecystitis	2	2
peritonitis	2	2
Urinary tract infection	1	2
pancreatitis	1	
pelvic infection	1	
Unknown	2	

Cell lines and maintenance

Mouse fibrosarcoma L929 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco #10500064). Cells were grown at 37°C, 5% CO2 and 95% humidity. Medium containing all supplements is hereafter referred to as complete medium.

To avoid high confluency, immortalised cells were sub-cultured every 2-4 days by enzymatic dissociation with Trypsin/EDTA (0.25% Trypsin, 0.02% EDTA; Sigma-Aldrich #T4049). Following medium aspiration, cells were washed in

Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich #14190250) and trypsinised for 2-5 min at 37°C. Cells were resuspended in complete medium and according to their growth rates, split at a ratio of 1:3 to 1:10.

Cryopreservation and recovery of cells

Cells to be cryopreserved were resuspended in FBS containing 10% (v/v) DMSO, aliquoted and cooled slowly in freezing containers (Cryo 1°C Mr. Frosty, Nalgene) at -80°C. For long-term storage, cells were transferred to a liquid nitrogen tank (-196°C). Cryopreserved cells were recovered by thawing at 37°C, resuspension drop-wise into pre-warmed complete medium, centrifugation (400 x g, 5 min) and final resuspension in fresh complete medium.

ELISA

ELISA plates were coated with protein or peptide at 2-10 µg/ml, overnight at 4ºC or 2 hours at 37ºC, followed by washing and blocking with 5% BSA or 5% milk (#70166, Sigma-Aldrich, UK) (diluted in PBST, 0.01 % Tween). Plates were then washed, and then incubated with patient sera (1:100 diluted in PBS and 5% BSA or 5% milk) or mAb at room temperature for 1 hour unless otherwise specified, and washed. Anti-human IgG γ heavy chain-specific (catalogue number H10007, Invitrogen), anti-mouse Ig secondary antibody conjugated with HRP, monoclonal anti-human IgG1-Biotin antibody (catalogue number B6775, Sigma-Aldrich, Mo, USA) were used to detect the reactions. Plates were read at $OD._{450}$ nm.

Cytokine Arrays

Cytokines and chemokine were detected in serum samples (diluted 1:100) from 4 recovered and 4 deceased sepsis patients and 4 healthy controls using a

proteome profiler human cytokine array kit (R and D Systems, MN, USA) as per the manufacturer's instructions.

Dot blot assay

Serum samples from anti-P1213 autoantibody positive sepsis patients (n=2), anti-P1213 autoantibody negative sepsis patients (n=2) and healthy controls (n=2) were selected to compare the IgG concentration by dot blot assay. 2µl of serum (diluted 1:200 with PBS) was spotted onto nitrocellulose membrane and dried at room temperature, followed by blocking with 5% skim milk in PBST for 60 minutes. The membrane was washed with PBST and incubated with a FITC conjugated rabbit anti-human IgG H&L (Abcam, UK) (diluted 1:25000 in PBST) for 60 minutes at room temperature, followed by 4 5-minute PBST washes and development with the enhanced chemiluminescence (ECL) system.

Flow cytometry

Adherent cells were first detached from the culture surface using Trypsin/EDTA (0.25% Trypsin, 0.02% EDTA; Sigma-Aldrich #T4049). After cells were detached, 400ul of Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich #14190250) was added into each well to stop trypsinisation. Centrifuged the cells at 300g for 10 min. Prepared 2% FBS in PBS with CellEvent™ Caspase 3/7 Green Detection Reagent (#C10427, Invitrogen) (1 ul dye in 1 ml 2% FBS in PBS) and added 300ul into cells to resuspend with the pellet. Incubated cells at 37°C in the dark for 90 min. Added 0.5 µl SYTOX® AADvanced dead cell stain (#C10427, Invitrogen). Fluorescence was detected using a FACSCalibar flow cytometer (BD Biosciences) and quantified with FlowJo analysis software (FlowJo LLC).

Chapter 3 – The screening of autoantibodies against TNFα related peptide in patients with severe sepsis explore whether it is correlated to patient's prognosis

Introduction:

Our group previously confirmed autoantibodies against the P1213 motif of TNFα may contribute in affecting the chronic pathogenesis of rheumatoid arthritis (Lu et.al. unpublished), we then postulated that the autoantibodies may also play a role in acute inflammatory disease such as sepsis. We were given sera samples of sepsis patients with various prognosis. Our aim is to identify biomarker that correlate to the clinical outcome of sepsis, especially to detect autoantibodies against P1213 of TNFα and assess whether the autoantibodies are correlated to lethal sepsis. Therefore, this chapter mainly focuses on screening of autoantibodies from sera samples of sepsis patients using ELISA.

Results:

3.1 Total serum IgG and anti-TNFα antibody levels do not correlate with indication of sepsis

Upon receival of sera from sepsis patients and healthy donors from China, we examined cytokine and chemokine profiles of four sera samples among 25 deceased and eight recovered sepsis patients respectively, and four healthy controls using Proteome Profiler Human Cytokine Array Panel A. All eight patients had higher cytokine and chemokine levels than in any of the controls (Table 2). Strangely, TNFα as well as other major proinflammatory cytokines were not detected within all the sera samples we tested despite sera from those patients were diagnosed with

severe sepsis. In addition, we compared the total serum IgG levels from two sera samples of each group using a dot blot assay, and none of them had shown the correlation with the clinical outcomes as high level of serum of IgG may occur in recovered patients as well as healthy donors (Fig. 3.1).

Table 2. Cytokine/Chemokine profiles of deceased and recovered sepsis patients. Cytokine and chemokines levels detected within four sera samples from deceased sepsis patients, recovered sepsis patients and healthy donor respectively, were assessed using Proteome Profiler Human Cytokine Array Panel A.

Cytokine/Chemokine	Deceased (n=4)	Recovered (n=4)	Control (n=4)
Serpin E1	4/4	1/4	0/4
MIF	2/4	1/4	0/4
CD40 Ligand	2/4	1/4	0/4
ICAM-1	4/4	4/4	0/4
IL-1ra	1/4	0/4	0/4
CCL5	2/4	2/4	0/4
CXCL11	1/4	1/4	0/4
CXCL12	0/4	2/4	0/4

***number in the table represents number of cytokine positive patients/total number of patients tested**

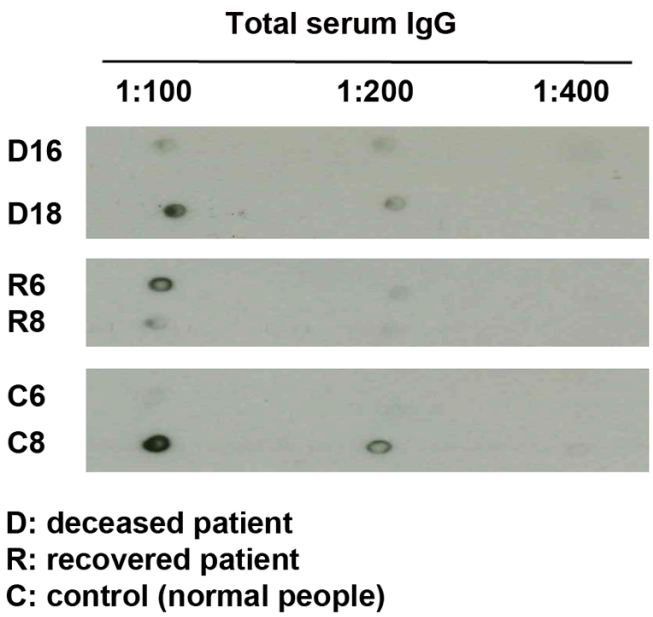
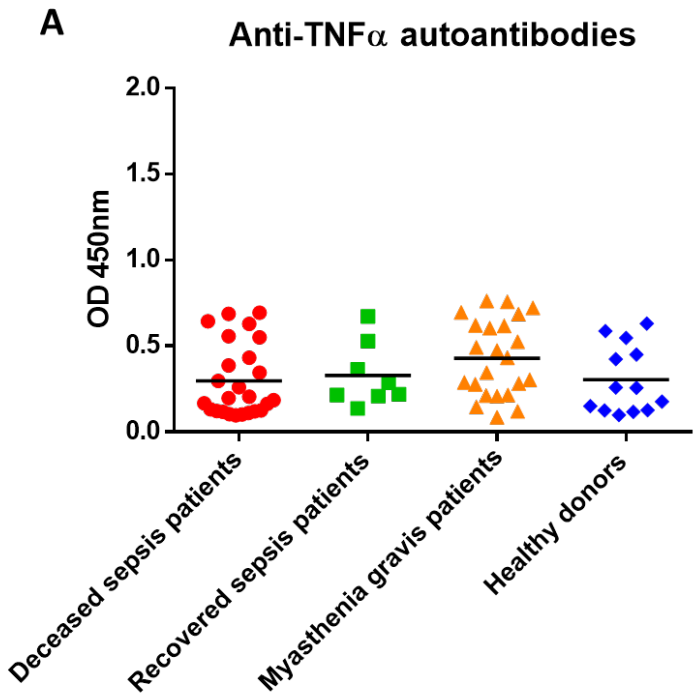


Figure 3.1. Dot blot assay for comparing the total serum IgG levels association with sepsis prognosis. Two sera from deceased, recovered or normal control respectively were dot blotted to a nitrocellulose membrane at the titres shown above. After drying, the membrane was probed with anti-human IgG conjugated to horseradish peroxidase (HRP).

To assess whether anti- whole TNFα autoantibodies are related to mortality of sepsis, direct ELISA were performed on sera samples from all deceased sepsis patients (n=25), recovered sepsis patients (n=8), healthy donors (n=13), as well as myasthenia gravis patients (n=23) to detect levels of anti-TNFα autoantibodies. The results indicated anti- whole TNFα autoantibodies were present at roughly equal quantities in serum samples of both sepsis patients and healthy donors, hence the presence of TNFα antibodies is not restricted to sepsis patients (Figure 3.2A).


A
Anti-TNFα autoantibodies
OD 450nm
2.0
1.5
1.0
0.5
0.0
Deceased sepsis patients
Recovered sepsis patients
Myasthenia gravis patients
Healthy donors

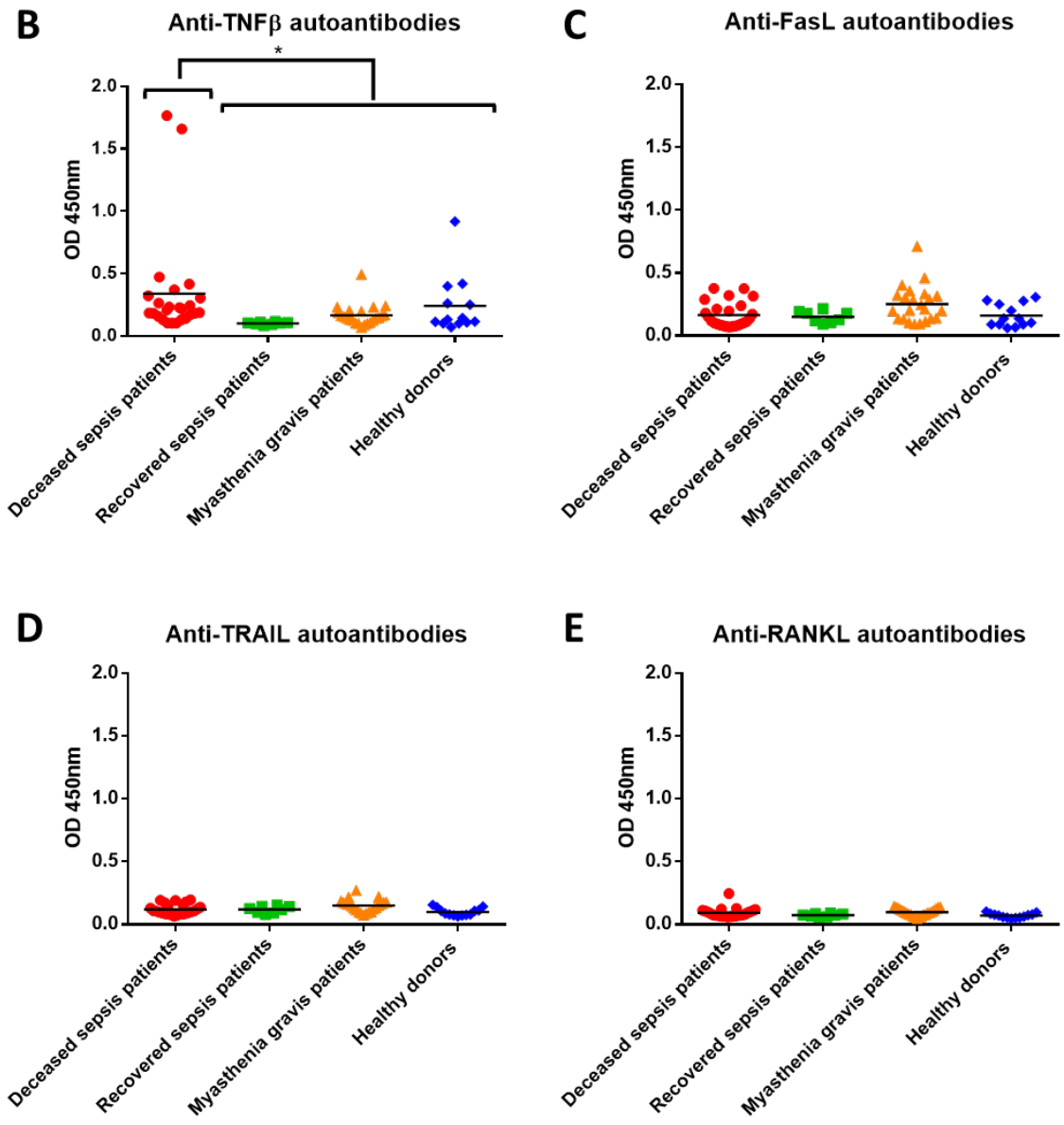


Figure 3.2. Direct ELISA detection of serum autoantibody levels against five different TNF superfamily ligands, including TNFα (A), TNF-β (B), FasL (C), TRAIL (D) and RANKL (E), in sepsis patients and myasthenia gravis patients. Using ELISA plates coated with 2 μg/ml TNF superfamily members respectively. After blocking with milk, each plate was probed by sera, at dilutions of 1:100, from 25 deceased sepsis patients, 8 recovered sepsis patients, 23 myasthenia gravis patients and 13 healthy donors. And followed by incubation with HRP-conjugated goat anti-human IgG secondary antibody. Absorbance following addition of TMB was measured at 450 nm. Each data point represents average of n = 3 replicates. Significance was evaluated by Mann-Whitney U test for comparison to deceased sepsis patients group (*, $P<0.05$).

3.2 High level of anti-TNF-β autoantibodies are presented in lethal sepsis patients

Same experiments were performed on the same group of sera samples to measure the concentrations of autoantibodies targeting various TNF superfamily proteins, including TNF-β, FAS ligand, TRAIL and RANKL. These TNF family members are known to associate cell death, are mostly type II transmembrane proteins with an extracellular, homotrimeric C-terminal TNF homology domain (Bossen *et al.*, 2006).

Interestingly, very high levels of autoantibodies targeting TNF-β are detected specifically within sera from some deceased sepsis patients ($P \leq 0.05$) whereas autoantibodies against the rest of TNF superfamily proteins were detected in relatively low quantities with no significant difference across different patient groups (Figure 3.2).

3.3 Higher level of anti-P1213 and anti-P21 autoantibodies are detected in severe sepsis

Our next interest focused on investigating autoantibodies targeting TNFα peptide, especially P1213, in sepsis patients. We identified a noticeable despite statistically non-significant association that high level of anti-P1213 autoantibodies were detected within sera samples of more than a third of deceased sepsis patients (n = 25) when compared to only one serum with elevated level anti-P1213 autoantibodies of recovered patients (n = 8) and healthy donors (n = 13) combined in this ELISA result (Fig. 3.3). Apart from P1213, we also explored autoantibodies targeting other peptide regions of soluble form of TNFα, 20-mers overlapping TNFα peptides P9 to P22, in relation to severe sepsis. There were significant increases ($P \leq 0.05$) of quantities of autoantibodies against P9, P13 to P15 region and P17 to P22 region within sera of deceased patients when compared to recovered patients and healthy

donors combined. In particular, the levels of anti-P13 and anti-P21 autoantibodies were presented much significantly higher ($P \leq 0.01$) within sera of deceased patients than the other group (Fig. 3.4).

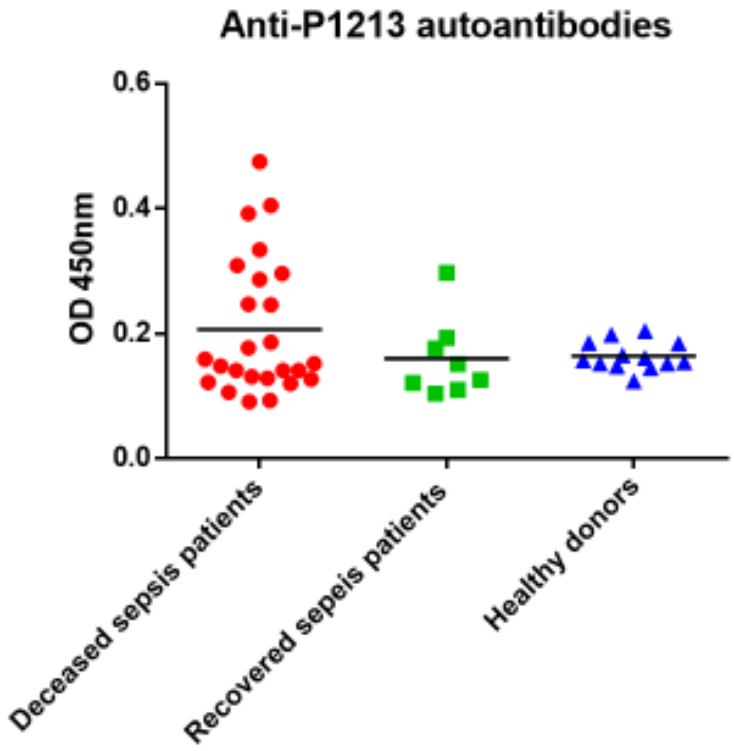


Figure 3.3. Sepsis patients sera ELISA for the detection of autoantibody targeting TNFα 30-mers overlapping peptide P1213. 10 µg/ml of peptide were coated in ELISA plates, followed by blocking with BSA. As for Figure 3.2, sera from 25 deceased sepsis patients, 8 recovered sepsis patients and 13 healthy donors, were diluted at 1:100 with BSA, to be used as probe against P1213 on the plate. And followed by incubation with HRP-conjugated goat anti-human IgG secondary antibody. Absorbance following addition of TMB was measured at 450 nm. Each data point represents average of n = 3 replicates.

We then performed another ELISA to measure autoantibodies against P1213 and P21 to confirm our previous finding with sera samples from all deceased sepsis patients (n=25), recovered sepsis patients (n=8), healthy donors (n=25), in addition to myasthenia gravis patients (n=23) (Fig. 3.5). Cut-off values, determined by mean + 2 S.D. of recovered sepsis patients group, were utilised to quantify the number of patients or healthy individuals who are positive for the autoantibodies (Table 3). The results indicated more than half of deceased sepsis patients were positive for anti-P1213 autoantibodies. Therefore, anti-P1213 autoantibodies significantly correlated to mortality of sepsis ($P \leq 0.01$) (Table 4). However, it was shown that anti-P1213 autoantibodies are found in some healthy individuals. Interestingly, nearly half of myasthenia gravis patient sera also had anti-P1213 autoantibodies. Furthermore, lethal sepsis and myasthenia gravis patient sera reacted with P21 in a similar way to P1213 (Fig. 3.5). Thus, similar to anti-P1213 autoantibodies, this suggested anti-P21 autoantibodies also related to mortality of sepsis ($P \leq 0.01$) (Table 4).

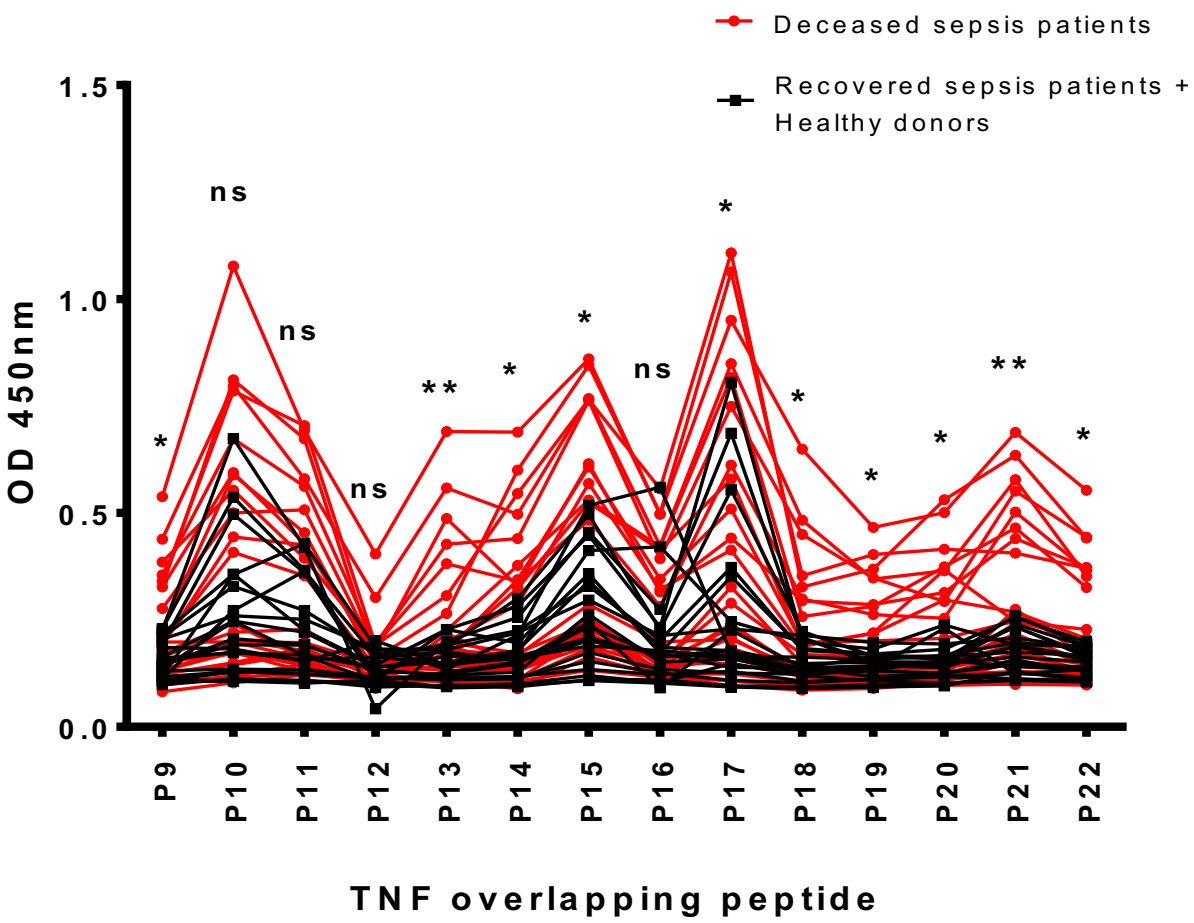


Figure 3.4. Sepsis patients sera ELISA for the detection of autoantibody targeting soluble TNFα 20-mers overlapping peptide from P9 to P22. 10 µg/ml of peptide were coated in ELISA plates, followed by blocking with BSA. As for Figure 3.3, sera from 25 deceased sepsis patients, 8 recovered sepsis patients and 13 healthy donors, were diluted at 1:100 with BSA, to be used as probe against P9 to P22 respectively on the plate. And followed by incubation with HRP-conjugated goat anti-human IgG secondary antibody. Absorbance following addition of TMB was measured at 450 nm. Each data point represents average of n = 3 replicates. Statistical significance was determined by unpaired t-test for comparison between autoantibodies level within sera of deceased sepsis patients and combination of recovered sepsis patients and healthy donors (ns, non-significant; *, $p \leq 0.05$; **, $p \leq 0.01$).

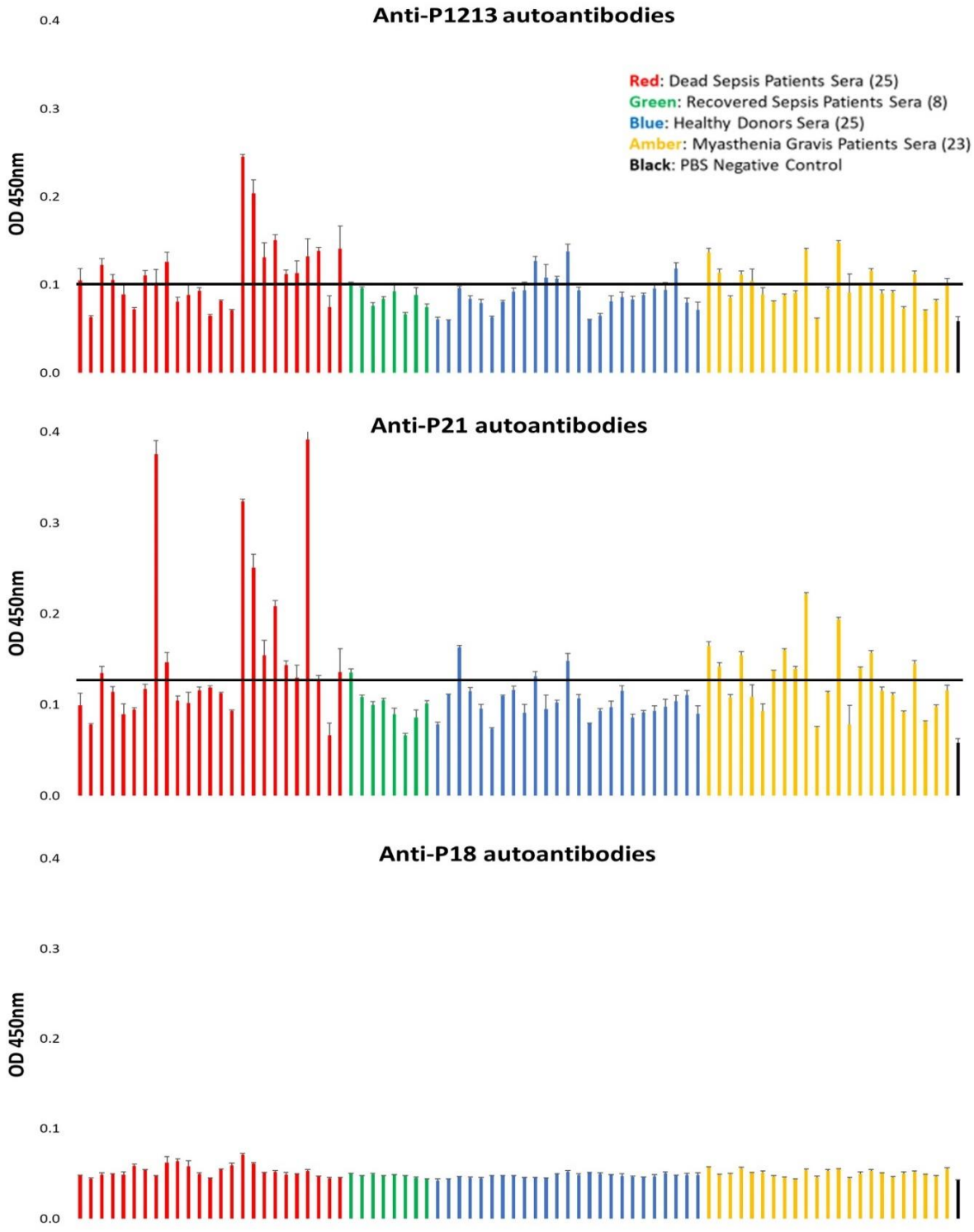


Figure 3.5: Serum autoantibody levels against TNFα peptides P1213 (top), P21 (middle) and negative control P18 (bottom) (tested at 1:100 by ELISA and blocked with milk). Cut-off values (Mean + 2 S.D. of recovered sepsis patients group) are used to determine whether autoantibodies are detected in each serum. Error bars represent mean ± S.E.M. of n = 3 replicates.

Table 3: Number and percentage of human serum samples with anti-p1213 and anti-p21 autoantibodies detected according to Figure 1.

Human Serum Samples		Anti-P1213 Antibodies Detected (%)	Anti-P21 Antibodies Detected (%)
Sepsis Patients (33)		15 (45%)	12 (36%)
	Dead (25)	15 (60%)	11 (44%)
	Recovered (8)	0 (0%)	1 (13%)
Healthy Donors (25)		5 (20%)	3 (12%)
Myasthenia Gravis Patients (23)		9 (39%)	11 (48%)

Table 4: Statistical comparison of autoantibody levels in groups of human serum samples using independent (unpaired) T-test. Average and standard deviation of each group's OD value are shown.

	Sepsis Patients (N=33)		**Healthy Donors (N=25)**			**Dead Sepsis Patients (N=25)**		**Recovered Sepsis Patients (N=8)**			**Recovered Sepsis Patients (N=8)**		**Healthy Donors (N=25)**		
	Mean	SD	Mean	SD	*P*-value	Mean	SD	Mean	SD	*P*-value	Mean	SD	Mean	SD	*P*-value
P1213	0.106	0.038	0.088	0.019	0.030 *	0.113	0.041	0.085	0.010	0.006 **	0.085	0.010	0.088	0.019	0.541
P21	0.140	0.079	0.104	0.020	0.019 *	0.153	0.086	0.099	0.018	0.008 **	0.099	0.018	0.104	0.020	0.561

SD = Standard deviation

* *P*-value statistically significant (<0.05)

** *P*-value highly statistically significant (<0.01)

Chapter 4 – Assessment of possible mechanisms of autoantibodies within sera from sepsis patients lead to necroptotic cell death

Introduction:

Our results in Chapter 3 proved the presence of anti-P1213 autoantibodies within sera samples of deceased sepsis patients. Our next interest is focus on mechanism of how these autoantibodies convert TNFα-related apoptosis into necroptosis. To that end, we used sera which contain anti-P1213 autoantibodies form deceased sepsis patients together with TNFα *in vitro* to induce necroptosis on TNFα-sensitive cell-line, mouse fibroblast L929 cells.

Results:

4.1 P21 is physically adjacent to P1213 within conformational structure of TNFα

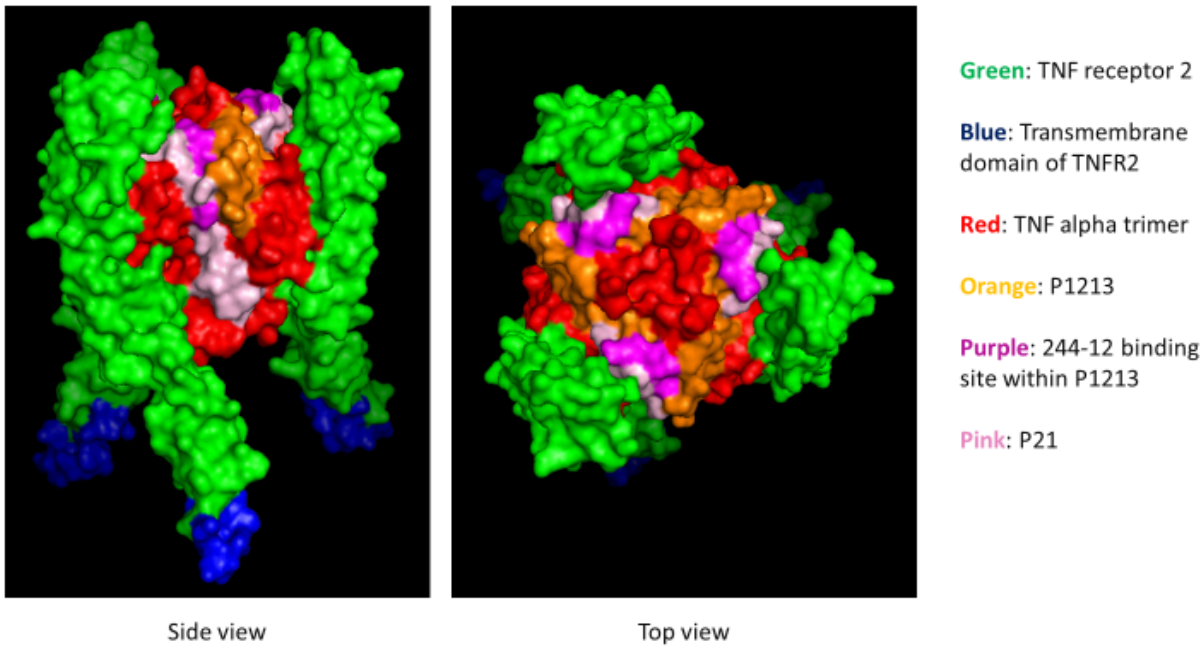
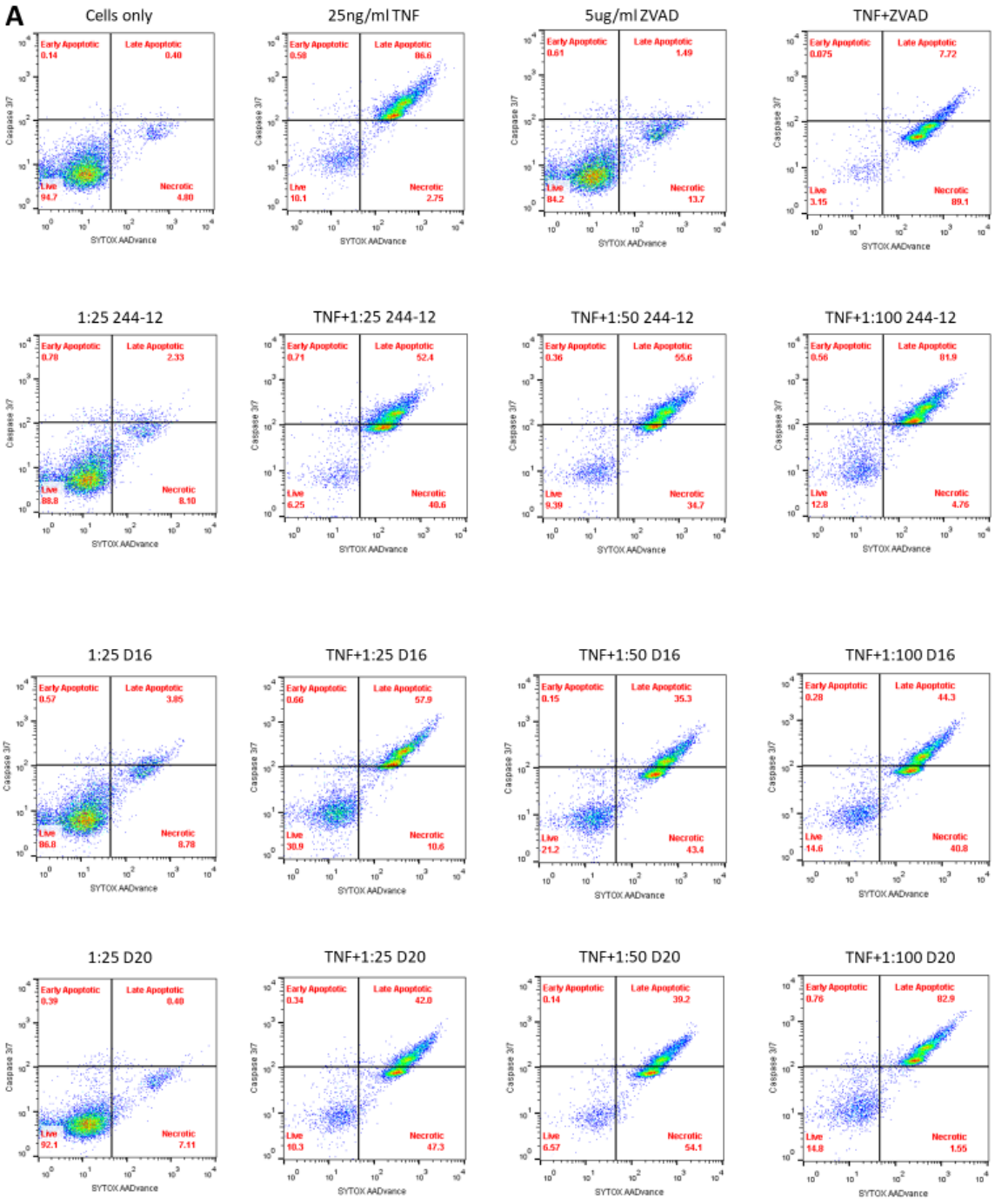


Figure 4.1. 3D structural representation of TNFα binding TNFR2. TNFR2 (Green), TNFα (Red), P1213 region of TNFα (Orange), mAb 244-12 binding region of P1213 (Purple) and P21 region of TNFα (Pink). Left panel: side view. Right panel: top view. Molecular figures were made with PYMOL.

In order to perceive the mechanism of how anti-P1213 autoantibodies can convert TNFα-related apoptosis into necroptosis when they bind to the apoptosis-inducing P1213 motif of TNFα, we used PyMOL, a molecular visualisation software, to construct a 3D crystal structure of a TNFα-TNFR2 complex (Fig 4.1). We discovered the precise positions of TNFα motifs that are believed to be associated with necroptotic-inducing autoantibodies, including P1213, mAb 244-12 binding site and P21. Interestingly, these autoantibodies-related motifs are located close together at the apical site of the TNFα structure, exposing to the top of the complex. In fact, part of P1213 and P21 are located adjacent to each other within the conformational structure of TNFα. It is postulated that the autoantibodies specific to P1213 may also target P21 as both P1213 and P21 belong to the same pro-apoptotic motif of TNFα.

4.2 Anti-P1213 positive sera from deceased sepsis induce necroptosis



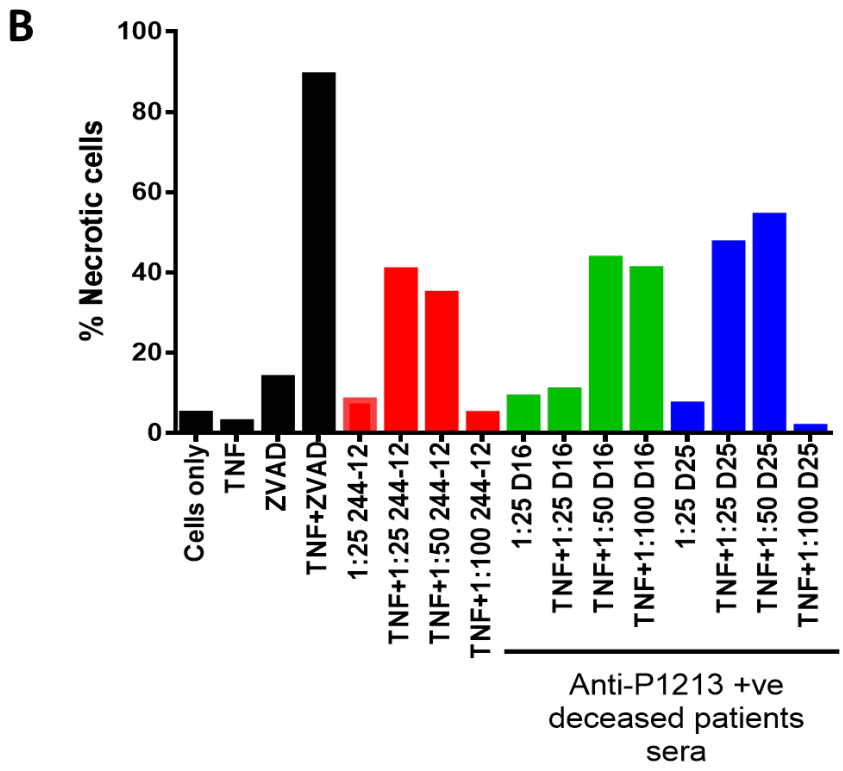


Figure 4.2. Flow cytometric analysis of different dilutions of anti-P1213 positive sera from deceased sepsis patients and mAb 244-12 in combination of TNFα on L929 cells. (A) L929 cells were seeded in 12-well plates at a density of 150,000 cells/well. 6 h later, 25 ng/ml TNFα were added in combination of various dilutions (1:25, 1:50 & 1:100) of anti-P1213 positive sera, D16 and D25, from deceased sepsis patients and mAb 244-12. 5ng/ml ZVAD were added as positive control which render necroptotic cell death. Sera or 244-12 alone as control did not cause cell death. Cells were then harvested 12 h later and processed for flow cytometry as described in the Materials and Methods section, using CellEvent Caspase-3/7 Green Detection Reagent in combination with SYTOX Green Dead Cell Stain. (B) Graphical representation of comparison of frequency of necrotic cell in % subsequent to different treatments.

After demonstrating the presence of anti-P1213 autoantibodies within sera samples of severe sepsis patients according to our results in Chapter 3, we proceeded to perform *in vitro* cell-based assays to examine the necroptotic effect of sera from deceased sepsis patients. Anti-P1213 autoantibodies-positive sera were selected in different dilutions and treated with TNFα on TNFα-sensitive cell-line, mouse fibroblast L929 cells. Notable necroptotic cell death was observed when cells were incubated in TNFα and autoantibodies-positive sera from deceased sepsis patients, D16 and D25, at 1:50 dilution (Fig 4.2). The combination of mAb 244-12 at both 1:25 and 1:50 dilutions with TNFα also induce necroptosis on L929 cells. Interestingly, when cells were treated TNFα alone or TNFα with anti-P1213 autoantibodies-negative sera from deceased sepsis patients, recovered patients and healthy donors, the outcome of L929 cells tended to be apoptotic cell death where caspase are activated and cleaved (Fig 4.3).

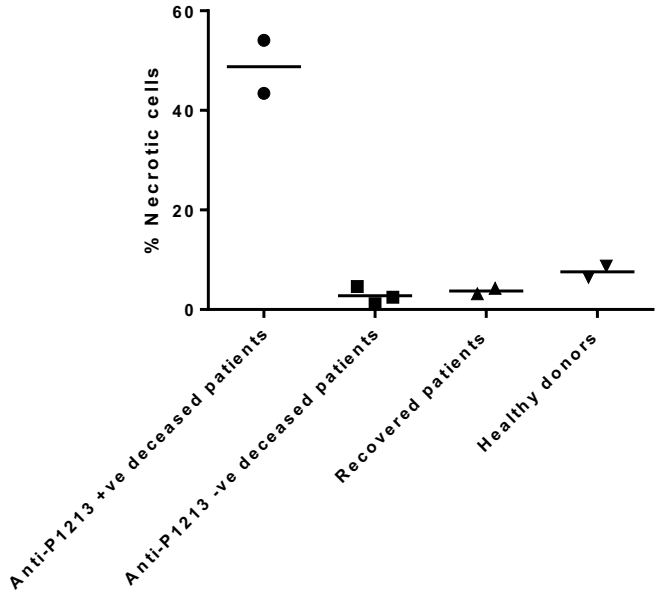


Figure 4.3. Flow cytometric analysis of different human sera from sepsis patients and healthy donors in combination of TNFα on L929 cells. L929 cells were seeded in 12-well plates at a density of 150,000 cells/well. 6 h later, 25 ng/ml TNFα were added in combination of human sera, diluted at 1:50, of anti-P1213 positive and negative sera from deceased sepsis patients, recovered patients and healthy donors. Cells were then harvested 12 h later and processed for flow cytometry as described in the Materials and Methods section, using CellEvent Caspase-3/7 Green Detection Reagent in combination with SYTOX Green Dead Cell Stain to compare frequency of necrotic cell in % depends to different sera samples added.

4.3 P1213 rescues necroptotic cell death caused by sera from sepsis patients

To explore the possibility of using P1213 overlapping peptide as an agonist for autoantibodies within the human serum, thus inhibiting their effect to convert apoptosis to highly inflammatory necroptosis, we mixed large dose of P1213 (up to 50 μM) with mAb 244-12 or anti-P1213 autoantibodies positive sera samples prior combining with TNFα to treat the cells. The results indicated P1213 reduced 244-12-induced necroptotic cell death in a dose-dependent manner (Fig 4.4A). This suggested P1213 is a competitive inhibitor for the autoantibodies. 50 μM P1213 also rescued necroptotic cell death moderately (Fig 4.4B). However, because both P1213 and P21 are apoptotic inducer, they have relatively high cytotoxicity than other peptides (Fig 4.5), as it had been shown that P1213 and P21 started to elicit apoptosis in L929 cells on their own with doses higher than 100 μM.

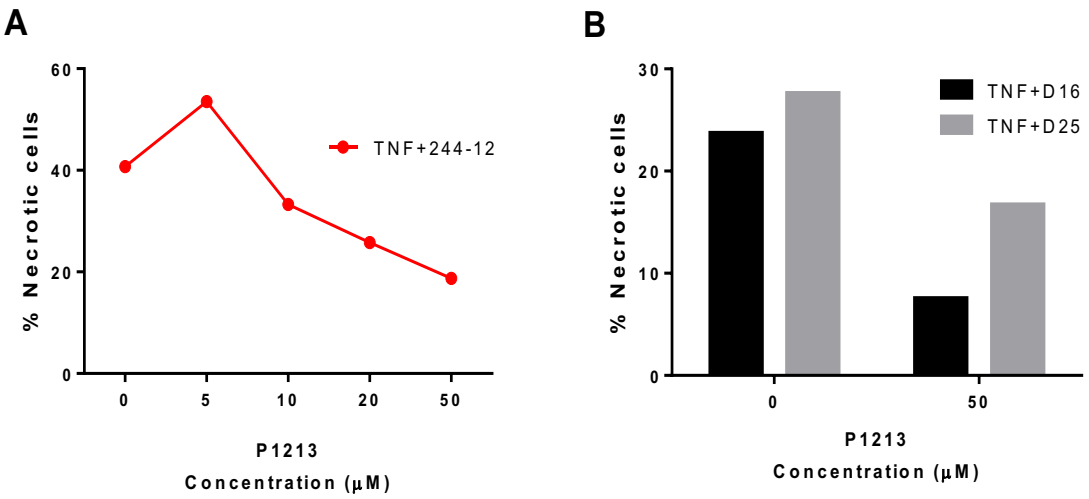


Figure 4.4. Flow cytometric analysis to determine whether P1213 can reduce necroptosis caused by anti-P1213 positive sera from deceased sepsis patients and mAb 244-12 in combination of TNFα on L929 cells. (A) L929 cells were treated with 25 ng/ml TNFα with mAb 244-12 (diluted at 1:50) in addition to different doses of P1213 (0, 5, 10, 20, 50 μM) to compare the resultant frequency of necrotic cells in %. (B) L929 cells were treated with 25 ng/ml TNFα with anti-P1213 positive sera from deceased sepsis patients (diluted at 1:50), D16 and D25, with or without 50 μM P1213 to assess whether P1213 can effectively rescue necroptosis by comparing frequency of necrotic cells in %.

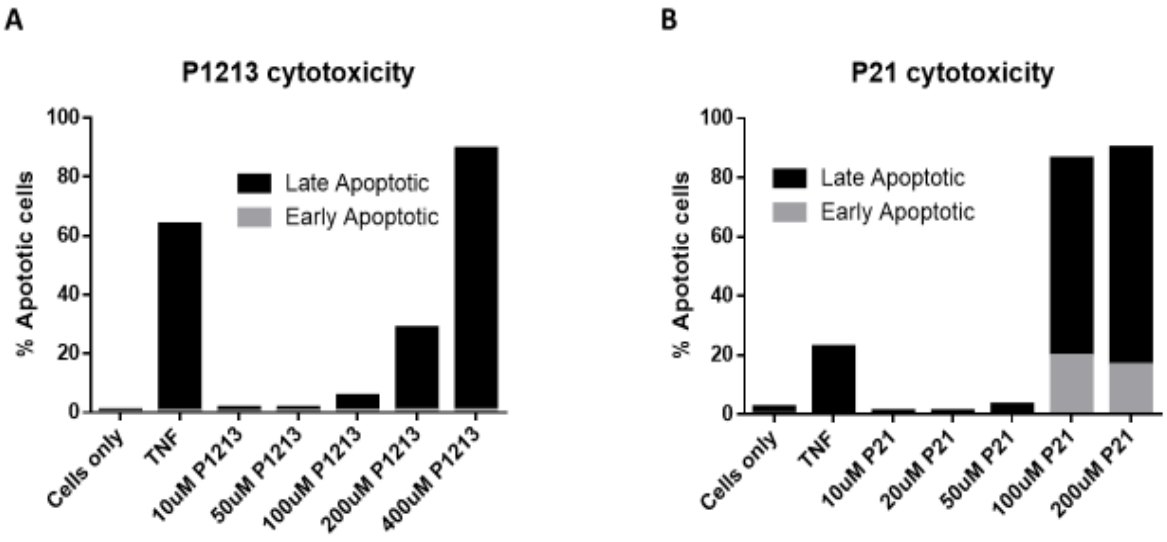


Figure 4.5. Assessing the cytotoxicity of TNFα overlapping peptide P1213 and P21 on L929 cells using flow cytometric analysis. (A) L929 cells were seeded in 12-well plates at a density of 150,000 cells/well. 6 h later, various doses (from 10 to 400 μM) of P1213 (A) and P21 (B) as well as 25 ng/ml TNFα were added respectively with the cells. Cells were then harvested 12 h later and processed for flow cytometry as described in the Materials and Methods section, using CellEvent Caspase-3/7 Green Detection Reagent in combination with SYTOX Green Dead Cell Stain to measure frequency of apoptotic cells based on corresponding treatments.

Chapter 5 - Discussion

Sepsis, regarded as a global healthy concern, is the deadliest condition in ICUs and has mortality rates over 20% in both developed and developing countries (Moore *et al.*, 2016). There is currently no direct cure for severe sepsis, with TNFα inhibitor has not proved beneficial in human sepsis despite TNFα being considered as the major mediator of acute inflammatory conditions. Until recently, high inflammatory TNFα-induced necroptosis has been discovered and found correlated to the prognosis of sepsis in animal models (Declercq, Vanden Berghe and Vandenabeele, 2009). However, the exact mechanism of how necroptosis contribute to septic shock remains unclear. There is a desperate need to discover new reliable biomarkers both for diagnosis and as therapeutic target in order to drastically reduce mortalities that are caused by lethal sepsis.

In this study, we detected and measured the necroptotic effect of anti-P1213 autoantibodies within sera samples of sepsis patients with different prognosis, as well as healthy donors. Through mostly ELISAs and flow cytometry analysis, we have identified the autoantibodies as a potential biomarker to correlate mortality in severe sepsis although not all deceased sepsis patients possess the autoantibodies. This illustrates the complexity of sepsis, whereby various factors or cytokines could be involved in the pathogenesis, thus therapy targeting only a single factor like anti-TNFα therapy does not prove beneficial toward sepsis in most cases (Jarrett *et al.*, 2003). It is also understood that the anti-P1213 autoantibodies can appear within small part of healthy populations. This suggests that the autoantibodies are not suitable for indicating sepsis morbidity. However, anti-P1213 autoantibodies should

be considered as a good prognosis indicator as a significant portion of deceased sepsis patients group possess the autoantibodies.

We also detected relatively high levels of anti-P21 autoantibodies within large proportion of deceased sepsis patients. Interestingly, the properties of P21 and P1213 are very similar, as they are both negative charged, capable to induce apoptosis, and more importantly, they are located very closely together in a TNFα conformational structure. This raised possibility that autoantibodies targeting P1213 motif are the same autoantibodies which target P21. The 3D crystal structure of TNFα-TNFR2 complex showed that the autoantibodies binding site and P1213/P21 motif are located at the top of the complex, where it is conveniently exposed allowing antibody-protein interaction. This suggest that the binding between of apoptosis-inducing motif of TNFα and autoantibodies is highly feasible.

Serendipitously, we found anti-TNF-β autoantibodies specifically within deceased sepsis patients. The links between TNF-β and severe sepsis remains elusive. However, one article revealed that individuals who produce TNF-β with a specific intron polymorphism, LTA (+252), are predisposed to severe sepsis(Watanabe *et al.*, 2010). It is postulated that severe sepsis patients with LTA (+252) render production of autoantibodies specifically targeting TNF-β. Anti-TNF-β autoantibodies could be regarded as a possible biomarker for severe sepsis and thus improving clinical management of sepsis.

Interestingly, our data demonstrated considerable amount myasthenia gravis patients also possess the anti-P1213 autoantibodies. Myasthenia gravis arises from autoantibodies that target specifically against neuromuscular junction in patients (Uzawa *et al.*, 2016). This raises speculation that the autoantibodies are prevalent in

various inflammatory and autoimmune disorders as they have been detected in rheumatoid arthritis and sepsis. In that case, Anti-P1213 could be a significant biomarker for many TNFα-mediated inflammatory conditions. This also proposes that necroptosis plays a crucial role in the pathogenesis of across several inflammatory diseases.

There were a few limitations in our experiments especially with the use of human serum samples. In ELISA experiments, we do not have a positive control for comparison with human sera when we were measuring levels of autoantibodies against P1213 within the serum samples. Our only positive control, mAb 244-12, are mouse derived antibodies. We were not able to quantify the amount of autoantibodies detected as standard curve could not be generated since we used different secondary antibodies due to the species difference. Hence, we would only be able to measure and compare autoantibodies levels between human sera samples in OD values. This can be resolved if we use chimeric mAb with human Fc receptors.

Furthermore, there was only a limited amount of sera samples we could experiment with, particularly with sera of deceased sepsis patients. Experiments using sera could only be done in higher dilution factor (1:100) to enough sera samples for future experiments. Also, there were only eight sera samples of recovered sepsis patients, which is insufficient to perform statistical comparison between patient groups. Moreover, all sera samples of sepsis patients are Chinese, thus we could not rule out ethnicity may influence results. Additionally, most results of flow cytometry analysis are treated as preliminary data since they lack replicates. This is due to time limitation of my 1-year research project. Lastly, our *in vitro* studies on anti-P1213 autoantibodies are limited on a mouse cell line, L929. It would be more suitable to

apply human serum containing the autoantibodies on human cell line, to examine more representative effect of the human autoantibodies.

The exact mechanism of how anti-P1213 autoantibodies induce necroptosis remains elusive. However, we speculate it involves the binding affinity of TNFα-TNFR interactions. We hypothesise that when the anit-P1213 autoantibodies bind to P1213 motif of TNFα, the interaction may cause 3D conformational change which differ to TNFα without autoantibodies. This slight change of shape may alter TNFα's hydrophobicity hence the net charge of the entire soluble molecule may change. This in turn may affect its subsequent TNFα-TNFR interactions, which could increase or decrease its binding affinity. It has been shown that TNFα-TNFR1 interactions are pro-cell death when comparing to TNFα-TNFR2 interactions, which do not stimulate apoptotic cell death as TNFR2 tend not associate with death domain (Varfolomeev and Vucic, 2016). Interestingly, TNFα-TNFR1 interactions take only seconds to initiate downstream signalling (Grell *et al.*, 1998). We postulate that the change of conformational shape on TNFα by anit-P1213 autoantibodies may ultimately results in lower binding affinity of TNFα-TNFR1 interactions thus longer binding. As a result, apoptotic signalling to be altered in to necroptotic signalling. This theory can be proved by measuring changes of binding affinity of TNFα-TNFR1 interactions after anti-P1213 autoantibodies interact with TNFα.

As shown in Figure 4.4, P1213 can be used as a therapeutic as an anti-P1213 autoantibodies agonist, which would block the autoantibodies affecting TNFα signalling, thus minimising necroptosis and inflammation. However, its efficacy is relatively low and higher dose of P1213 could result in apoptotic cell death on its own. Another potential therapeutic agent to inhibit necroptosis caused by anti-P1213

autoantibodies would be necrostatin-1, RIP1 inhibitor. Animal models using necrostatin-1 has shown effective protection against septic shock and prolonged the survival (Duprez *et al.*, 2011). However, inhibition on RIP1 with necrostatin-1 also lower TNFα-induced apoptotic cell death as RIP1 is key for formation of complex IIa in TNFα signalling.

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