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Killer cell Immunoglobulin-like Receptors in Immune Thrombocytopenia (ITP)

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disorder, characterised by an isolated low platelet count. Genes influencing activation of the immune system have been identified as influencing predisposition. Killer cell immunoglobulin-like receptors (KIR) control T-cell and Natural Killer cell function via inhibitory and activating signalling pathways. The inhibitory KIR2DL3, KIR3DL2 and KIR3DL1 are up-regulated in the T-cells of patients with ITP in remission relative to those with active disease, and an association of KIR2DS2/KIR2DL2 with ITP has also been reported. No comprehensive genotypic KIR analysis in ITP has been reported. We performed genotyping of all currently known KIR genes using sequence specific primer polymerase chain reaction (SSP-PCR) on a cohort of 83 adult patients with ITP (chronic/persistent or relapsed primary ITP identified by standard criteria) and 106 age matched healthy white volunteers. Non-white patients were not included in the analysis. There was an over-representation of KIR2DS3 (known to be in linkage disequilibrium with KIR2DS2/2DL2) and under-representation of KIR2DS5 (also protective against other immune mediated disorders) in adult ITP (odds ratio 0.16, C.I. 0.08-0.32, $P<0.001$). By multivariable binary logistic regression to adjust for age, sex and the effects of other KIR genes, the presence of KIR2DS2/2DL2 with KIR2DS5 abrogated the risk of KIR2DS2/2DL2 and the protective benefit of KIR2DS5. Further studies are required to establish the mechanistic basis for these observations and their potential impact on ITP therapy.

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1. **Seymour LA**, Nourse, JP, Crooks P, Wockner L, Bird R, Tran H and Gandhi MK. The presence of KIR2DS5 confers protection against adult immune thrombocytopenia. Tissue Antigens. 2014. 83(3)154-160.

Conference Abstracts:

1. **Seymour L,** Nourse JP, Crooks, P, Wockner L, Bird R, Tran H and Gandhi MK. The presence of KIR2DS5 confers protection against adult immune thrombocytopenia. Oral Presentation and Runner-up award. Haematological Society of Australia and New Zealand Scientific Meeting (Oct 2013)

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Book chapters:

1. Jones K, **Seymour L**, Gandhi MK. CD163 and TARC as Disease Response Biomarkers in Hodgkin Lymphoma. In: Preedy VR, Patel VB (eds). Cancer. Biomarkers in Disease: Methods, Discoveries and Applications, vol 2. Springer, Dordrecht. 2015.

Conference Abstracts:

1. Jones KL, Vari F, Keane C, Crooks P, Nourse JP, **Seymour LA**, et al. Serum CD163 and TARC as Disease Response Biomarkers in Classical Hodgkin Lymphoma. Oral Presentation, American Society of Hematology 54th Annual Meeting (Dec 2012). This work was also presented at the Highlights of ASH in North America (Feb 2013) and the Highlights of ASH in Asia (March 2013)

Publications included in this thesis

Seymour LA, Nourse, JP, Crooks P, Wockner L, Bird R, Tran H and Gandhi MK. The presence of KIR2DS5 confers protection against adult immune thrombocytopenia. Tissue Antigens. 2014. 83(3)154-160 – incorporated as Appendix 1.

Contributor	Statement of contribution
Author LAS(Candidate)	Designed experiments (70%) Performed experiments (70%) Analysed data (60%) Wrote the manuscript (70%)
Author JPN	Designed experiments (30%) Analysed data (15%)
Author PC	Performed experiments (30%)
Author RB	Wrote the manuscript (10%) Facilitated sample collection
Author MKG	Analysed data (15%) Wrote the manuscript (20%)
Author LW	Performed statistical analysis for Tables
Author HT	Facilitated sample collection

Contributions by others to the thesis

My supervisors, Professor Maher Gandhi and Associate Professor Robert Bird, contributed significantly to conception of the projects described in this thesis. No other significant contributions were made.

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Immune thrombocytopenia, killer cell immunoglobulin-like receptor, KIR2DS5, platelets, genotype, protection

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Abbreviations

ITP	Immune thrombocytopenia
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
ANA	Antinucleotide antibody
IVIg	Intravenous immunoglobulin
H.pylori	Helicobacter pylori
IgG	Immunoglobulin G
gp	Glycoprotein
AIHA	Autoimmune haemolytic anaemia
Rh	Rhesus
APC	Antigen presenting cell
TPO	Thrombopoietin
CD	Cluster differentiation
MAB	Monoclonal antibody
CTL	Cytotoxic T lymphocyte
IL	Interleukin
IFN	Interferon
Th	T helper
Treg	Regulatory T-cell
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
BAFF	B-cell activating factor
SLE	Systemic lupus erythematosus
NK	Natural killer
FCGR	FC gamma receptor
ITAM	Intracellular tyrosine-based activation motif
ITIM	Intracellular tyrosine-based inhibition motif
SNP	Single nucleotide polymorphism
ADCC	Antibody dependent cellular cytotoxicity
OR	Odds ratio
P	p-value
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
miR	Micro RNA
KIR	Killer cell immunoglobulin-like receptor

HLA	Human leukocyte antigen
ALL	Acute lymphoblastic leukaemia
CML	Chronic myeloid leukaemia
TKI	Tyrosine kinase inhibitor
IWG	International working group
PCR	Polymerase chain reaction
SSP-PCR	Sequence specific primer PCR
SSOP-PCR	Sequence specific oligonucleotide PCR
KCl	Potassium chloride
dNTP	deoxy nucleotide triphosphate
MgCl2	Magnesium chloride
C.I.	Confidence interval
LD	Linkage disequilibrium
NS	Not significant
TCR	T-cell receptor

1. Review of the Literature

1.1 Epidemiology, diagnosis and classification of ITP.

Immune thrombocytopenia (ITP), previously known as idiopathic thrombocytopenic purpura, is best defined as an acquired isolated thrombocytopenia without known underlying precipitating factors[1]. ITP occurs in both adults and children without any observable ethnic predilection, however there are age related differences in disease manifestation and management. Paediatric ITP usually follows a viral infection and although the thrombocytopenia can be marked, it is rarely associated with serious bleeding and often spontaneously resolves. Adult ITP has an incidence of 2.6-6.6 per 100 000 adults per year and a mortality rate of 1-4% per year[2]. ITP can be further subclassified into primary (occurring in isolation, ~80% of cases) or secondary (associated with a defined underlying disorder, ~20% of cases). Secondary ITP is often associated with autoimmune disorders, malignancy, infections[3] and certain drugs suggesting that ITP is a result of diverse mechanisms such as co-existing immune dysregulation and molecular mimicry after infection. The phases of ITP are currently classified as: newly diagnosed (up to 3 months from diagnosis), persistent (3-12 months from diagnosis) and chronic (>12 months since diagnosis)[4]. The clinical course of ITP is unpredictable, with no single therapy considered to be ‘gold standard’. Many patients with ITP are minimally symptomatic however there is a subset of patients who present with severe bleeding. The morbidity of ITP is based principally on the presence of bleeding, however studies have demonstrated that patients with ITP are also at increased risk of infection, thrombosis and haematologic malignancies[2].

In practice, a diagnosis of ITP is made in the setting of isolated thrombocytopenia following exclusion of other causes of thrombocytopenia. Appropriate first line investigations and other clinical considerations are outlined in table 1.

Table 1: First line investigations recommended when diagnosing ITP[5]

First line investigations:
Exclude exposure to heparin (e.g. recent surgery) and haematinic deficiencies.
Review of the peripheral blood count and film – exclude other abnormalities including other causes of thrombocytopenia, for example dysplastic features would implicate myelodysplasia[1].
Liver function testing and coagulation profile – exclude liver disease and consumptive coagulopathy as a cause of thrombocytopenia.
Test for HIV and HCV, ANA and Lupus anticoagulant[1]
Helicobacter Pylori*, particularly in patients in whom eradication would be used if testing was positive[1]. Note: serology may be falsely positive after intravenous immunoglobulin (IVIg).
No longer advised:
Bleeding Time (poor sensitivity and specificity).
Platelet autoantibody testing (poor sensitivity and specificity).

*H.pylori eradication has been shown to be successful in certain populations with a high incidence of H.pylori (e.g. Japanese) and less successful in western populations. For a review of H.pylori and ITP see the review by Stasi et al.[6]

1.2 Pathogenesis of ITP

The currently understood mechanisms involved in the pathophysiology of ITP are incompletely understood and multifactorial and considered primarily as a result of a dysregulated immune system. These are illustrated in figure 1.

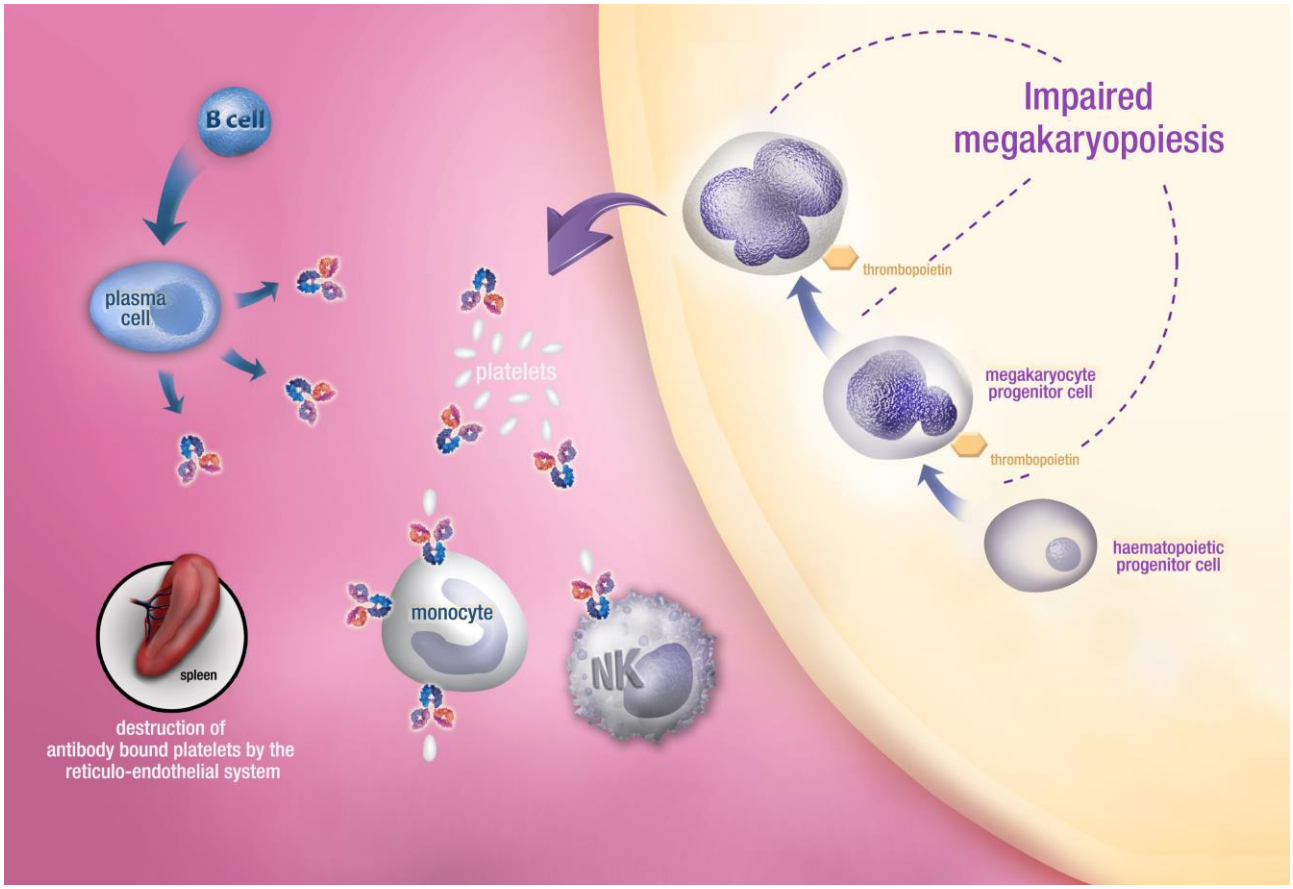

Figure 1. Graphical representation of currently accepted pathophysiological mechanisms in ITP. In antibody mediated destruction, T cells regulate the development of a limited number of B cell autoreactive clones which in turn develop into plasma cells. These secrete IgG autoantobodies directed against platelet surface glycoproteins, resulting in platelet destruction in the reticulo-endothelial system. Impaired megakaryopoiesis as an independent multifactorial mechanism as described on page 15, also contributes to the development of ITP.

Antibody mediated platelet destruction is the most widely accepted mechanism in ITP. This was first demonstrated in 1951 when James Harrington self-injected 500mL of blood from a patient with ITP. Within 3 hours his platelet count dropped below $10x10^9$/L and he developed petechiae and suffered a seizure. He later repeated the experiment on "all suitable staff from the Barnes Jewish Hospital" and together with Hollingsworth published the first report of a "thrombocytopenic factor" in the blood of patients with thrombocytopenic purpura[7]. Since then it has been shown that ITP occurs as a result of predominantly IgG antibody-mediated platelet destruction and variably impaired platelet production with the most common antigenic targets being platelet surface glycoproteins, gpIIb/IIIa[8], with other platelet surface antigens also implicated in a minority of cases[9]. The IgG antibodies directed against these glycoproteins appear to be derived from a limited number of B-cell clones[10]. Platelet destruction in ITP is generally considered to be primarily due to platelet opsonisation which not only results in accelerated clearance by macrophage mediated phagocytosis but may also affect platelet function and platelet

production[11]. Of note, anti-platelet antibodies are only detected in up to 60% of patients[11]. This is likely due to a combination of limited assay sensitivity and variability of epitopes. It has yet to be established why some individuals develop these anti-platelet auto antibodies to several structurally diverse platelet proteins. Unlike in warm autoimmune haemolytic anaemia (AIHA) where antibodies are often confined to epitopes within the Rh locus, in ITP, the proposed mechanism involves a 2-step process. Opsonisation of platelets by antibodies against surface proteins is followed by phagocytosis by antigen presenting cells (APC)s. Subsequent proteosomal degradation results in the formation and presentation of novel epitopes often derived from cytoplasmic proteins[12].

It has recently been demonstrated that reduced megakaryocytes and impaired megakaryopoiesis, is a significant factor in the development of thrombocytopenia. This was demonstrated by Louwes et al[12] by performing platelet kinetic studies using indium-111-labelled autologous platelets and clearly demonstrated a combination of both reduced platelet production and reduced platelet lifespan. Platelet production by megakaryocytes is regulated by thrombopoietin (TPO) which is constitutively synthesized in the liver and circulates to the bone marrow, where it binds TPO receptors (cMPL) on early megakaryocyte progenitors thereby stimulating their proliferation and differentiation. TPO also binds to circulating platelets, which reduces the TPO available to stimulate megakaryocytes, thus low platelet counts enable more TPO to reach the marrow to increase megakaryopoiesis. Paradoxically in ITP, serum TPO is not increased. The mechanistic basis for this observation remains to be elucidated but is postulated to be due to TPO clearance along with antibody-coated platelets. In ITP, there are no consistent morphological changes in megakaryocytes demonstrable morphologically however they may lack granularity, have reduced ploidy and may have fewer platelets budding from plasma membranes. Megakaryocyte apoptosis, due to increased caspase 3, has been reported, as well as fragments from degenerating megakaryocytes being phagocytosed by macrophages and neutrophils[13]. It has recently been appreciated that these megakaryocyte changes are likely due to autoantibody mediated mechanisms. Not only do megakaryocytes express platelet glycoproteins during their development making them targets of ITP antibodies, but some auto antibodies preferentially recognise megakaryocyte epitopes[14]. A study by McMillan *et al.*[15] showed that plasma (and IgG fractions) from patients with ITP suppressed *in vitro* megakaryocyte production from CD34+ progenitor cells and reduced megakaryocyte ploidy. Unfortunately little is known about the potential effects of ITP antibodies on downstream platelet development.

Mechanisms of platelet clearance in ITP involve splenic and liver clearance by Fcγ receptors, with a number of conventional treatment modalities (IVIG, anti-D, steroids and anti-CD20 monoclonal antibodies (MABs) inhibiting antibody-mediated platelet clearance at the Fcγ receptor. Other additional platelet clearance pathways may include complement mediated pathways (some ITP antibodies fix C3b to platelets)[16] and expression of P-selectin and phosphatidylserine associated with GP1b clustering platelet destruction signal[17].

The role of T-cell mediated cytotoxicity towards platelets and platelet precursors is also under investigation as a component of the pathogenesis of ITP. This has been postulated as a contributing mechanism based on the data of Roark *et al*[10] where it was shown that platelet specific antibodies derived from patients with ITP had restricted heavy chain rearrangement consistent with derivation from a single B-cell clone and somatic mutation in heavy and light-chain variable regions which is consistent with a classical T-cell dependent antigen-driven immune response. There is also a likely contribution from cytotoxic T-cell (CTL) direct lysis of platelets[18,19] and accumulation of CTLs in the bone marrow sufficient to disrupt thrombopoiesis[20].

Studies of cytokine profiles in chronic ITP also point towards a skewed T-cell response with increased IL-2 and IFN-γ, reduced IL-4 and IL-10, and decreased TGF-β consistent with a Th-0/Th-1 response[21,22]. This pattern of cytokines is associated with the differentiation and maturation of autoreactive B-cells into autoantibody producing plasma cells and the development of CTLs. Another study demonstrated that Th1/Th2 mRNA ratio was inversely correlated with the platelet count in adult patients with ITP which also infers a polarised Th1 response[23]. It has been postulated that this predominantly Th1 response in ITP is likely due to reduced numbers of circulating Th2 cells or the number and/or function of (CD4+/CD25(bright)/FOXP3+) regulatory T-cells (Tregs)[24].

Tregs are derived via two distinct pathways, thymus-derived Tregs and peripherally (or adaptive) derived Tregs which arise from naïve CD4+ T-cells encountering self-antigens[25]. The role of the Treg is to limit autoimmune responses and assist in the maintenance of self-tolerance[26]. Genetic deficiencies of Tregs are associated with diverse autoimmune type presentations such as IPEX syndrome in humans[27] and Scurfy in mice[28]. The contribution of reduced numbers of Tregs in the pathophysiology of ITP is still unclear.

Nishimoto *et al*. have shown that one third of nude mice, when reconstituted with Treg depleted T-cells developed antibody-mediated thrombocytopenia[29]. It has also been observed that there are reduced numbers and function of Tregs in the peripheral blood of patients with ITP (similarly with other autoimmune disorders)[30] and it is thought that this may contribute towards reduced tolerance and the expansion and differentiation of auto reactive B-cells.

Th17 cells are a subset of T-helper cells which produce IL-17 and like Tregs, are also derived from undifferentiated naïve CD4+ T-cells[31]. They have an established role in mediating host response to bacterial and fungal infection at mucosal surfaces by triggering the release of antimicrobial peptides and recruiting neutrophils. Increased Th-17 cells and elevated plasma IL-17 have been demonstrated in adults and children with severe autoimmune disease[32], including ITP[33]. The mechanism of IL-17 and Th17 cells in autoimmunity still remains to be elucidated.

Tolerance check point defects are also likely to be involved in the immune dysregulation associated with ITP. Immune tolerance mechanisms involve the monitoring of the lymphocyte repertoire and the induction of anergy in auto reactive lymphocytes. At present, it is unclear how immune tolerance is disturbed in primary and secondary ITP. It has been postulated by Cines *et al*.[34] that immune tolerance defects in ITP may arise during early B-cell development in the bone marrow (central tolerance defects), during differentiation with skewed B-cell subsets emerging or in the setting of immune stimulation (peripheral tolerance). One immune tolerance homeostasis checkpoint regulator is the cytokine B-cell activating factor BAFF which is a member of the tumour necrosis factor family and involved in regulating B-cell maturation, survival and immunoglobulin production. Thien *et al*.[35] has shown that increased BAFF expression leads to the rescue of auto reactive B-cells from anergy and as such, likely has an important role in autoimmunity[35]. In keeping with this theory, Emmerich *et al*. has shown that there is an increased prevalence of the (-871) TT genotype of the BAFF promoter as well as increased serum BAFF in ITP[36]. Elevated BAFF has also been demonstrated in patients with other autoimmune diseases such as SLE, rheumatoid arthritis and Sjogren syndrome[37,38]. However, the functional effect of the promoter region polymorphism is yet to be established.

The role of NK cells in the pathogenesis of ITP is unclear. There are several reports of reduced and functionally defective NK cells in ITP[39–41] but no definitive mechanism has been established. Given the variability in response to rituximab in ITP, further studies are warranted to enhance understanding of the role of NK cell function in ITP pathogenesis and their impact on rituximab mediated antibody dependent cellular cytotoxicity (ADCC).

1.3 Genetics of ITP

As mentioned above, the primary mechanism of platelet clearance in ITP is due to FCγ receptor (FCGR) mediated clearance of opsonised platelets by macrophages. The human FCGR family is comprised of three major classes of receptors: FCGR1, FCGR2 and FCGR3. Each of these arise from multiple transcripts derived from different genes on chromosome 1 (FCGR1a and 1b, FCGR2a, 2b and 2c, and FCGR3a and 3b)[42]. The majority of these receptors are involved in signal transduction via an intracellular tyrosine-based activation motif (ITAM). FCGR2a however expresses an intracellular tyrosine-based inhibition motif (ITIM). The FCGR1 subtypes are both high affinity receptors for monomeric IgG with the other families being lower-affinity receptors interacting only with complex or multimeric IgG[43]. There is increased heterogeneity within the FCGR subtypes FCGR2a, 3a and 3b by functional single nucleotide polymorphisms (SNPs) which appear randomly distributed among populations with variable distribution between ethnic groups[44,45]. It appears as though FCGR SNPs may have crucial roles in the phagocytosis of IgG opsonised platelets and thus a role in the pathogenesis of ITP. Ghesquieres *et al*, Bredius *et al*, Sanders *et al* and Salmon *et al* have demonstrated that inheritance of higher or lower affinity FCGR alleles may be associated with the predisposition towards FCRG related immune and infectious diseases[46–49].

It has been shown that the FCGR2a-131H (histidine) allele has a higher binding affinity for IgG than FCGR2a-131R (arginine)[43,50]. Other SNPs identified that may be relevant in ITP include the FCGR3a-V158 (valine) as opposed to phenylalanine (F) polymorphism. IgG1-FCGR3a co-crystallization studies show that FCGR3a interacts with the lower hinge region of IgG1[51] with FCGR3a-158V having a higher IgG1 binding affinity than FCGR3a-158F[52]. It has been hypothesized that the FCGR3a-158V polymorphism is associated with enhanced clearing of IgG-platelet complexes by ADCC by both macrophages and NK cells. FCGR3a transcripts have been shown to be higher in NK cells

from individuals with FCGR3a-158V/V compared with FCGR3a-158V/F or FF genotypes with V/V homozygotes having enhanced *in vitro* ADCC activity[53].

Wang *et a.*[54] performed a meta analysis in order to clarify the association between FCGR2a H131R polymorphism and ITP[54]. They found no significant predisposition towards ITP in the adult data reviewed and speculated that the conflicting results between individual studies were likely a result of ethnic diversity, clinical heterogeneity and small sample size. They did, however, find an association in Caucasian children. This was originally demonstrated by Carcao *et al.* in a cohort of 98 predominantly Caucasian children in Canada, compared with controls[55]. This study not only demonstrated an association between FCGR2a-131H and ITP but also demonstrated an association with FCGR3a-158V alone and in combination with FCGR2a-131H. They did not see any association with either of these polymorphisms and the development of chronic ITP. Our laboratory has also demonstrated an increased frequency of the FCGR3a-158V/V genotype in chronic or relapsed ITP compared with age , sex and ethnicity matched controls (OR = 3.05, P = 0.007)[56]. Other studies[57,58], however, did not demonstrate any significant difference in FCGR3a-158 polymorphisms which may reflect differences in ethnic composition and categorisation of ITP (our study only looked at relapsed and chronic ITP, while other studies did not define ITP categories).

The use of the anti-CD20 monoclonal antibody, Rituximab, is widespread in ITP, however only a subset of patients will respond and the exact mechanism of action is uncertain. Cooper *et al.*[59] demonstrated that a cohort of patients with FCGR3a-158V/V polymorphisms were more likely to respond to rituximab. They also demonstrated that non responders to rituximab had increased CD8 positive CTLs compared with rituximab responders, thus supporting a role for T cell mediated cytotoxicity[59].

Other genetic lesions which may be associated with ITP include those in genes associated with T-cell activation such as cytokines associated with activation and survival, CD72 gene polymorphisms, and DNA hypermethylation of FOXP3 promoter regions affecting Treg activity[60–66]. Micro RNAs have also been investigated and it has been shown that increased miR-155 is associated with decreased SOCS1 in ITP. This is thought to be associated with regulation of cytokines given that they also demonstrated the negative correlation of miR-155 and the cytokines IL-4, IL10 and TGF-β1 levels and the positive correlation with IL-17A levels[67]. Interestingly, aberrant miR-155 expression has also been

seen in the autoimmune disorders rheumatoid arthritis, systemic lupus erythematous, multiple sclerosis and type 2 diabetes mellitus[68–71]. Zhao[72] showed that miR-130A (targets TGFB1 and IL18 genes) was reduced in chronic active ITP compared with normal controls.

1.4 Killer cell immunoglobulin-like receptors (KIR)

KIRs are a family of 14 polymorphic and highly homologous genes and two pseudogenes, encoded on chromosome 19[73] which regulate effector functions of NK cells and a subset of $CD4^+$ and $CD8^+$ T lymphocytes (that lack CD28 expression). They were first identified serologically by Moretta *et al.*[74], with functional specificity preceding molecular characterisation. The receptors are subdivided based on the number of extracellular Immunoglobulin domains (2D or 3D) and can be inhibitory or activating. Activating KIRs lack the immuno tyrosine inhibitory motif (ITIM) present in the intracellular domain of inhibitory KIRs due to a carboxy-terminal truncation of the protein, and have thus been named as short forms of the receptors. For example KIR3DS1 (short form) is an activating receptor highly related to the KIR3DL1 (long form) inhibitory receptor. Humans possess between four and 14 KIR receptor genes in their genome with variable numbers of genes inherited as a haplotype by non-homologous recombination.

There are two major classes of KIR haplotypes identified as shown in figure 2. The A haplotype contains four genes (KIR2DL1, KIR2DL3, KIR3DL1, KIR2DS4) in addition to the framework genes (KIR3DL3, KIR3DP1, KIR2DL4, KIR3DL2), representing a predominately inhibitory haplotype. There are many B haplotypes, containing various combinations of the activating KIR genes in combination with framework genes. KIR genotype expression varies between different immune effector cells, and determines whether there is phenotypic dominance of KIR-mediated inhibition over activation. The phenotypic expression of KIRs on NK cells is variable with regulating factors poorly understood. It is, however, accepted that HLA class I antigens and self-tolerance plays a fundamental role in NK cell “education” in the bone marrow[75].

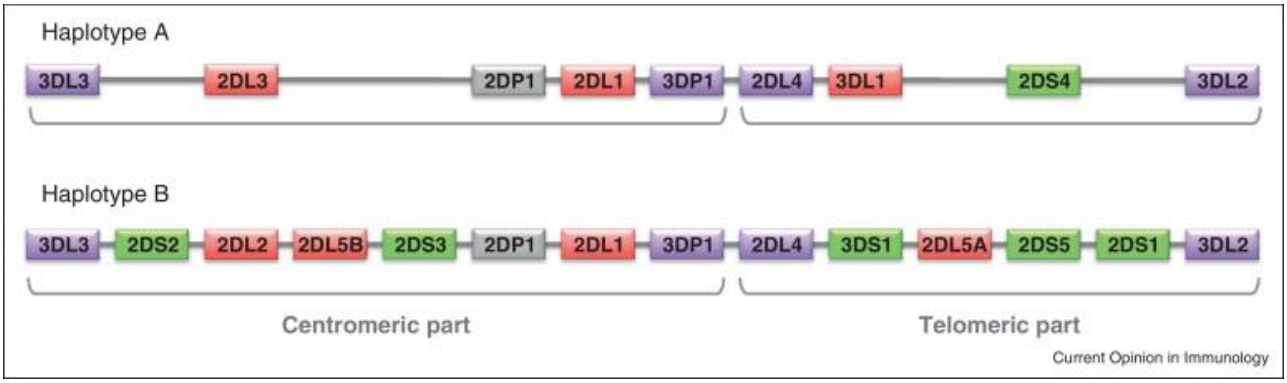

Figure 2. Schematic representation of KIR haplotypes on the KIR gene locus. Reproduced with permission (licence number: 3746860651258)[76]**.**

In those instances in which a KIR ligand has been identified, they are typically HLA-class I molecules (generally HLA-Cw but also HLA-Bw and HLA-A loci), with the same ligand binding both the activating KIR and its inhibitory counterpart. Both receptor and ligand pairs need to expressed within the individual in order of a functional interaction to occur. This in itself provides a challenge as HLA and KIR genes segregate differently on different chromosomes[77]. The specific HLA ligands are known for most but not all the KIRs (Table 2)[76]. Unlike HLA-class I / T-cell receptor interactions, it is believed that peptides are not required for the HLA-class I / KIR interaction[78].

Table 2: KIR molecules and HLA ligands.

KIR	**HLA Ligand**
KIR2DL1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DL2	HLA-C1: C*01, C*03, C*07, C*08 HLA-C2: C*0501, C*0202, C*0401 HLA-B: B*4601, B*7301
KIR2DL3	HLA-C1: C*01, C*03, C*07, C*08 HLA-C2: C*0501, C*0202 HLA-B: B*4601, B*7301
KIR3DL1	HLA-A and HLA-B expressing Bw4 epitope HLA B*08, B*27, B*57, B*58 HLA-A: A*24, A*23, A*32
KIR3DL2	HLA-A: A*03, A*11
KIR3DL3	Unknown
KIR2DL5A and B	Unknown

KIR2DL4	HLA-G
KIR2DS1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DS2	Unknown
KIR2DS3	Unknown
KIR2DS4	HLA-C: C*0501, C*1601,C*0202 HLA-A: A*1102
KIR2DS5	Unknown
KIR3DS1	Unknown

Genetic studies strongly implicate the presence of certain KIR genotypes, KIR haplotypes or KIR/HLA compound genotypes in susceptibility and outcome to diverse disease processes such as psoriasis/psoriatic arthritis[79], rheumatoid arthritis/vasculitis, type I diabetes[80,81], scleroderma[82], multiple sclerosis[83,84], pre-eclampsia[85], resistance to HIV[86,87] and HCV viral infection[88], coronary artery disease, and melanoma[89]. In haematological conditions, KIR genotype associations have been seen in allogeneic and autologous stem cell transplantation[90,91], risk for relapse in paediatric acute lymphoblastic leukaemia (ALL)[92], chronic myeloid leukaemia (CML) and response to tyrosine kinase inhibitors (TKI)[93,94] and incidence of plasma cell myeloma[95]. It is likely that there is disease modification by specific KIR-ligand interactions rather than by global T-cell or NK cell responsiveness[87]. There is increasing data supporting the important role of KIR/KIR ligand interactions and NK cell alloreactivity in both adult and paediatric allogeneic bone marrow transplant influencing both graft versus disease and graft versus host effects[96,97].

1.5 KIRs and ITP

There are very few previous studies exploring the role of KIRs in ITP. Olsson *et al*[18] first identified a potential role for KIRs in ITP. They investigated the role of T cells in the pathogenesis of ITP by performing a DNA microarray with CD3+ T-cells derived from patients with active ITP, ITP patients in remission and healthy controls. They demonstrated that genes involved in a Th1 response (interferon-γ and IL-2 receptor-β) as well as several cytotoxic genes (Apo-1/Fas, granzyme A, granzyme B and perforin) had increased expression in ITP patients compared with controls. They also showed that several KIR genes had increased expression in patients in remission compared with patients with active ITP and normal control subjects. Using flow cytometry, they demonstrated that increased CD3+ lymphocytes expressing KIR3DL1 and KIR3DL2 were

present in patients in remission compared with active ITP. These findings, in combination implicate CTLs in the pathogenesis of ITP. Functional studies were then performed using both autologous platelets and HLA-mismatched donor platelets as target cells and patient autologous T-cells and NK cells. They found platelet lysis occurred only when using T-cells as effector cells with no effect seen using NK cells. Based on this, they proposed that T-cell mediated cytotoxicity contributes to the destruction of platelets in ITP, which is inhibited by up regulation of inhibitory KIRs resulting in disease remission. They did not, however perform ADCC based functional studies, and as such could not conclude anything about the role of antibody mediated CTL or NK cell responses.

Our laboratory has previously performed genotyping on a cohort of patients with ITP and matched controls and found that the KIR2DS2/KIR2DL2 compound genotype was associated with adult persistent/chronic and relapsed ITP. In a similar study, reported as an abstract, performed in the USA, Boulad *et al*.[98] performed KIR genotyping on a cohort of patients with active ITP (receiving treatment at the time) and 213 controls taken from a database. The ethnicity, age and genders of patients and controls was not reported. They found that expression of two inhibitory KIRs (KIR2DL1 and KIR2DL2) was lower in patients with ITP compared with controls ($P<0.002$) and that response to rituximab was strongly related to KIR expression with KIR2DL1 expression higher and KIR2DL3 expression lower in patients who did not respond to rituximab.

In the only other study to date investigating KIRs and ITP, El-Beblawy et al.[99] performed comprehensive KIR genotyping in Egyptian children with childhood ITP. This study demonstrated that the KIR2DL2-/KIR2DS2- genotype was overrepresented in children with ITP compared with controls.

2. Hypothesis and Aims

Hypothesis:

That KIR genes and/or haplotypes influence the protection against and/or susceptibility to adult ITP.

Aim:

To assess the influence of all currently known KIR genes (with the exception of KIR3DL3 which is present in all haplotypes) on protection/susceptibility, to adult ITP in

a well-defined cohort. Results will be compared with age matched healthy volunteers in order to explore any disease associations with KIR genes and haplotypes.

3. Methodology

3.1 Patients/Healthy Volunteers

One hundred and twenty-four patients with chronic and relapsed ITP were accrued from multiple centres in Australia as part of a sponsored clinical trial (ML20948; NCT00475423). DNA was available for 95 patients consented for participation in this laboratory sub-analysis. Diagnosis was made according to the then current American Society of Hematology guidelines[100]. Patients were only eligible if they met stringent criteria. To ensure that only primary ITP was included, patients with lymphoproliferative disorders, multi-system autoimmune disorders, drug-induced thrombocytopenia or seropositivity for Human Immunodeficiency virus, Hepatitis B or C were excluded. Newly diagnosed ITP patients were not included. Patient characteristics including ethnicity were collected prospectively as part of the trial protocol. The study was approved by the relevant Hospital/Research Institute Ethics Committees and was performed in accordance with the Declaration of Helsinki. Signed informed consent was obtained from all patients in the study.

Patients were divided into two groups. The first denoted as ‘persistent/chronic ITP’ was defined as ITP for more than 3 months requiring ongoing therapy to maintain a platelet count of ≥ 30 x 10^9/L. This definition is similar to the phases of disease as laid out by the International Working Group (IWG)[5] . The second group was termed ‘relapsed ITP’. These were patients with an initial response as defined per IWG: namely a platelet count ≥ 30x 10^9/L and at least 2-fold increase in the baseline count with absence of bleeding, without the requirement for ongoing therapy. Relapse in these patients was defined as a platelet count falling to below 30 x 10^9/L. It included patients with one or multiple relapses; for the former, relapse had to occur within 12 months of diagnosis. One hundred and six healthy adult volunteers, with no history of haematological or autoimmune disorders of any kind, served as a control cohort. Gene and polymorphism frequencies differ with ethnicity [101], therefore for accurate interpretation of results, we ensured that our control population were all white. Study of healthy controls was approved by the Queensland Institute of

Medical Research Ethics Committee and signed informed consent was obtained from all participants.

3.2 SSP-PCR

DNA was previously extracted from cryopreserved peripheral blood cell pellets (patients) or buccal scrapes (healthy volunteers) using a commercially available DNA extraction kit. Analysis was performed in batches. All samples were analysed in the same laboratory. Of the 16 currently known KIR genes, we analysed 13 of these. KIR3DL3 was omitted from the analysis as it is present in 100% of the population. KIR2DL2 and KIR2DS2 were previously analysed by this laboratory[56] so were not repeated. The 13 remaining KIR genes were analysed by a modified version of published SSP-PCR in a set of 7 multiplex PCR reactions using the primers included in table 3[102]. Each PCR was performed in a reaction volume of 15 µL with a final concentration of 1.5x PCR buffer II (10 mM Tris–HCl and 50 mM KCl), 200 µM of each dNTP, 0.6 µM of each forward and reverse primer (with exception of 3DP1F82 and 3DP1F83 were used at 0.3 µM final concentration), 2 mM of MgCl2, 0.5 unit of AmpliTaq DNA polymerase and 10 ng genomic DNA. PCR amplification was performed using an ABI 9700 Gene-Amp PCR System using the set-specific thermal cycling conditions as previously published[102] and shown in table 4. Following amplification, 5 µL of the products (for sets 1,2,4,5,6,7) and 10 µL for set 3 were electrophoresed in 3% agarose gel containing ethidium bromide at low voltage for 40 minutes. A low range ladder was also included. An example of an agarose gel of KIR genotyping is shown in figure 2.

Four of the seven duplex PCR sets performed include primers targeting a framework KIR gene (sets 4 and 6) and serve as an internal positive control to confirm the successful PCR amplification in each of these reactions. The remaining four PCR sets (sets 1, 3, 5 and 7) target at least one frequently occurring KIR gene, and therefore, most individuals are likely positive for one of the two genes tested in each of these sets. There was at least one of these KIR genes positive in all our reactions in both ITP patients and controls.

Table 3: SSP-PCR Primer combinations.

Set	KIR	Forward primer sequence	Reverse primer sequence	Amplicon (bp)
1	3DS1	AGCCTGCAGGGAACAGAAG	GCCTGACTGTGGTGCTCG	320
	2DL1	GTTGGTCAGATGTCATGTTTGAA	TCCCTGCCAGGTCTTGCG	144
2*	3DL3	GCAATGTTGGTCAGATGTCAG	GGGAGC(C/T)GACAACTCATAGGGTA	202
	2DL2	AGGGGGAGGCCCATGAAT	AGTGGGTCACTCGAGTTTGACC	160
3	2DL4	TCAGGACAAGCCCTTCTGC	CACTCAGTGGGGGAGTGC	243
	2DS3	TCCTATGACATGTACCATCTATCCAC	GAAGCATCTGTAGGTTCCTCCT	138
4	2DP1	GGGGGCCTGGCCACATGA	CACCGAAGAATCACATGTCCTG	693
	2DS5	ACAGAGAGGGGACGTTTAACC	ACAACTGATAGGGGGAGTGAGT	153
5	3DP1	GTGTGGTAGGAGCCTTAGAAAGTG	TGAAAACGGTGTTTCGGAATAC	280/399
		GTACGTCACCCTCCCATGATGTA		
	2DS1	TTCTCCATCAGTCGCATGAA	GAGGGTCACTGGGAGCTGAC	100
6	2DL3	CCTTCATCGCTGGTGCTG	GCAGGAGACAACTTTGGATCA	814
	2DL5	CATCTATCCAGGGAGGGGAG	ACTCATAGGGTGAGTCATGGAG	151
7	3DL2	CCCATGAACGTAGGCTCCG	GACCACACGCAGGGCAG	133
8	3DL1	GCAGGGAACAGAACAGCC	CCGTGTACAAGATGGTATCTGTA	275
	2DS4	CGGTTCAGGCAGGAGAGAAT	GTTTGACCACTCGTAGGGAGC	199/221

* Set 2 PCR results not included as 3DL3 was present in 100% of patients and controls and 2DS2 in this cohort was reported in a previous publication and not repeated.

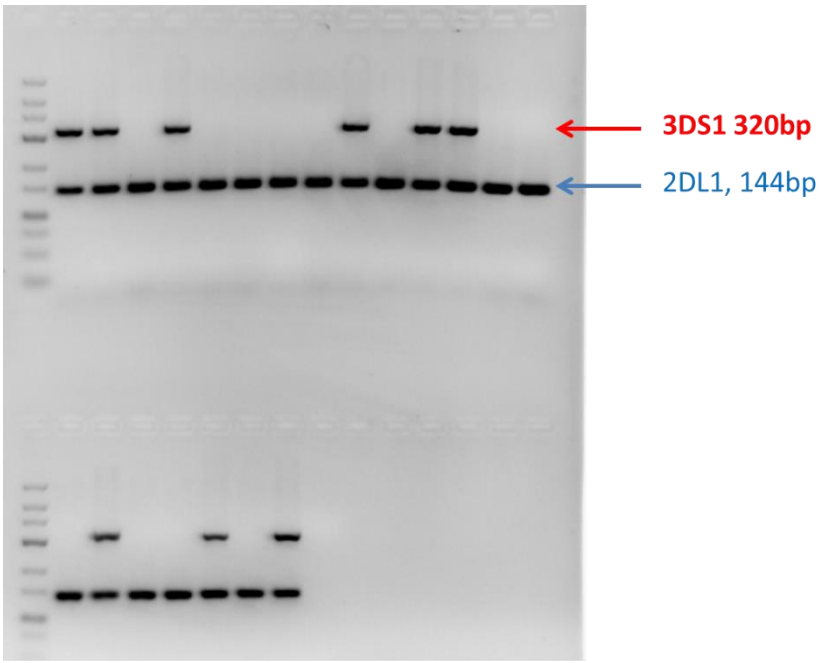

Figure 3. An example of an agarose gel demonstrating the distinct bands of KIR3DS1 and KIR2DL1 in the set 1 PCR.

Table 4: Set specific thermocycling conditions.

PCR	Set	Segments	Cycling conditions
1	3,6,8	Denaturation	95^0C 3 min
		Cycle 1	7 cycles of 94^0C for 15s, 66^0C for 20s, 72^0C for 45s
		Cycle 2	40 cycles of 94^0C for 15s, 64^0C for 20s, 72^0C for 45s
		Final extension	72^0C for 7 min
		Hold	4^0C infinity
2	4,7	Denaturation	95^0C 3 min
		Cycle 1	7 cycles of 94^0C for 15s, 68^0C for 20s, 72^0C for 45s
		Cycle 2	40 cycles of 94^0C for 15s, 66^0C for 20s, 72^0C for 45s
		Final extension	72^0C for 7 min
		Hold	4^0C infinity
3	2	Denaturation	95^0C 3 min
		Cycle 1	7 cycles of 94^0C for 15s, 68^0C for 20s, 72^0C for 30s
		Cycle 2	35 cycles of 94^0C for 15s, 66^0C for 20s, 72^0C for 30s
		Final extension	72^0C for 7 min
		Hold	4^0C infinity
4	1,5	Denaturation	95^0C 3 min
		Cycle 1	7 cycles of 94^0C for 15s, 63^0C for 20s, 72^0C for 45s
		Cycle 2	40 cycles of 94^0C for 15s, 61^0C for 20s, 72^0C for 45s
		Final extension	72^0C for 7 min
		Hold	4^0C infinity

3.3 HLA-allotyping

Tier 1 HLA-C allotyping was performed by ProImmune Limited (Oxford, United Kingdom) on 71 patient samples using a PCR-SSOP method. HLA typing was not performed on the control samples and 12 of the patient samples due to insufficient quantities of DNA available.

3.4 Statistics

As a first exploratory analysis, potential associations between disease status and KIR genotypes were assessed via Pearson's chi-squared test or when expected counts were small, Fisher's exact test. KIR genotypes with unadjusted p-values less than 0.05 were considered as potential candidates to include in multivariable modelling. However, due to known patterns of association (i.e. genotypes in linkage disequilibrium) it was important to assess the relationship between genotypes in order to ensure that genotypes that were co-linear (i.e. highly associated) weren't included in the same model given that including variables that are co-linear in a statistical model tends to produce spurious results. Co-linearity was assessed by Pearson's chi-squared test statistic, where p-values less than 0.05 indicated highly associated genes that were not suitable for consideration in the same model. This was also confirmed by LD analysis which again is designed to assess the level of association between genotypes. Once the candidate genes had been identified they were included in a binary logistic regression analysis in order to assess the effect the genes had in combination with each other on the binary outcome of disease status. As age and gender were both considered potential confounders, models were adjusted for the effects of age and gender regardless of their significance. As unadjusted and adjusted odds ratios were similar, adjusted odds ratios are reported. All analysis was performed in SPSS v.19. An alpha level of 0.05 was considered statistically significant.

4. Results

4.1 Study population

Ethnicity was available for all 95 patients, with 83 (84%) of patients identifying themselves as white. Due to gene and gene polymorphism frequencies differing with ethnicity, only the white cases were analysed. The mean age of patients was 50.5 years (range 18–85) with 47 (57%) females, 37 (45%) with chronic/persistent ITP and 44 (53%) with relapsed ITP, two people (2.4%) had primary/acute refractory ITP. In the 106 healthy white controls, 72 (68%) were women and mean age was 40.5 years (range 23–68 years).

4.2 Genotype and allele frequencies

We have previously shown that the distributions observed in the healthy controls for the KIR2DS2/2DL2 genes were in agreement with those previously reported and did not

deviate from the Hardy Weinberg Equilibrium (P>0.1)[56,103]. The distribution of KIR genes in both cases and healthy participants are shown in Table 5. The KIR gene frequencies did not differ between adult ITP patients and controls with the exception of KIR2DS3 (odds ratio [OR] =2.28, C.I. 1.19-4.35, P=0.012), KIR2DS5 and KIR2DL5 (OR=0.16, C.I. 0.08-0.32, P<0.001 and OR=0.47, C.I. 0.25-0.88, P=0.017 respectively). From this we deduce that KIR2DS3 confers an increased risk and KIR2DS5 and KIR2DL5 confer protection (Figure 4). There were no significant differences noted between KIRs and the subtypes of ITP (chronic/persistent and relapsed ITP) so for the rest of the analysis these subtypes have been combined and referred to as ITP.

A Linkage Disequilibrium (LD) plot demonstrating the LD present between the genes in our analysis is shown in Figure 5. From this it can be seen that our results are consistent with, and validated the LD data of Gourraud[104]. Based on our LD findings and the test of association in Table 6, we can further explain the significant associations demonstrated in Table 5. The genes that show a conferral of risk (KIR2DS2/KIR2DL2 and KIR2DS3) are highly positively associated. The genes that confer protection are weakly negatively associated with KIR2DS2 (KIR2DL5; p=0.05) or not associated at all (KIR2DS5; p=0.24). KIR2DL5 and KIR2DS5 are both positively associated (Chi-squared test; p-value<0.001; data not shown), however as KIR2DL5 has a weak negative association with KIR2DS2/KIR2DL2 and KIR2DS3, we would expect it to correlate with another gene that has a protective effect regardless of any biological relationship. As KIR2DL5 confers a more moderate degree of protection to adult ITP and as it is both associated with genes on the centromeric and telomeric region it is not an appropriate candidate for further modelling. However, KIR2DS5 is independently protective of ITP when adjusted for age, sex and KIR2DS2/2DL2 genotype (Table 7).

Table 5: Distribution of genes amongst the cases and controls with accompanying unadjusted odds ratios.

	Case n=83	**Control n=106**			
gene	**present (% of case)**	**present (% of controls)**	**odds ratio**	**95% CI**	**P value**
KIR2DL2 (susceptibility)	50 (60.2%)	47 (44.3%)	1.90	(1.06 - 3.41)	**0.030**
KIR2DS2	50	45	2.05	(1.14 -	**0.015**

				3.68)		
(susceptibility)	(60.2%)	(42.5%)				
KIR3DS1	34	39	1.19	(0.66 - 2.15)	0.559	
	(41.0%)	(36.8%)				
KIR2DL1	81	106	NA		0.192	*
	(97.6%)	(100.0%)				
KIR2DS5	37	88	0.16	(0.08 - 0.32)	**<0.001**	
(protective)	(44.6%)	(83.0%)				
KIR2DP1	82	103	2.39	(0.24 - 23.39)	0.632	*
	(98.8%)	(97.2%)				
KIR2DL4 F/W	82 (98.8%)	106 (100.0%)	NA		0.439	*
KIR2DS3	31	22	2.28	(1.19 - 4.35)	**0.012**	
(susceptibility)	(37.3%)	(20.8%)				
KIR3DP1 (del) F/W	80	104	0.51	(0.08 - 3.14)	0.655	*
	(96.4%)	(98.1%)				
KIR3DP1 (undel)	30	27	1.66	(0.89 - 3.10)	0.113	
	(36.1%)	(25.5%)				
KIR2DS1	31	50	0.67	(0.37 - 1.20)	0.176	
	(37.3%)	(47.2%)				
KIR2DL3	41	61	0.72	(0.40 - 1.28)	0.265	
	(49.4%)	(57.5%)				
KIR2DL5	51	82	0.47	(0.25 - 0.88)	**0.017**	
(protective)	(61.4%)	(77.4%)				
KIR3DL1	78	102	0.61	(0.16 - 2.35)	0.509	*
	(94.0%)	(96.2%)				
KIR2DS4 (undel)	32	40	1.04	(0.57 - 1.87)	0.908	
	(38.6%)	(37.7%)				
KIR2DS4 (del)	69	96	0.51	(0.22 - 1.22)	0.128	
	(83.1%)	(90.6%)				
KIR3DL2 F/W	83 (100.0%)	105 (99.1%)	NA		1.000	*

* indicates situations where the Fisher's Exact Test was used to calculate the P value. In other cases, Pearson's Chi-squared test was used. F/W = framework genes which are present in all haplotypes.

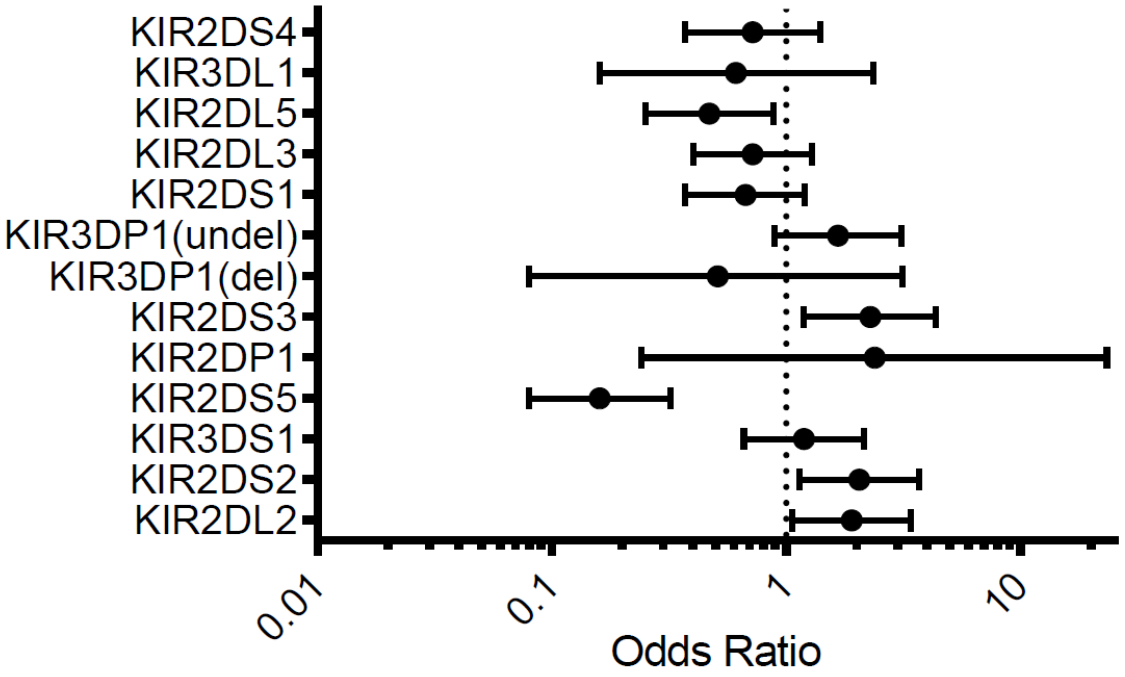

Figure 4. Odds ratio of KIR genotypes in patients versus controls

Genetic associations of KIRs are shown as odds ratios with 95% CI. Those to the left of the central line confer protection over ITP and those to the right confer increased risk. Those conferring protection are 2DS5 (P<0.001) and 2DL5 (P=0.017) and those conferring risk include 2DS3 (P=0.012), 2DS2 (P=0.015) and 2DL2 (P=0.03).

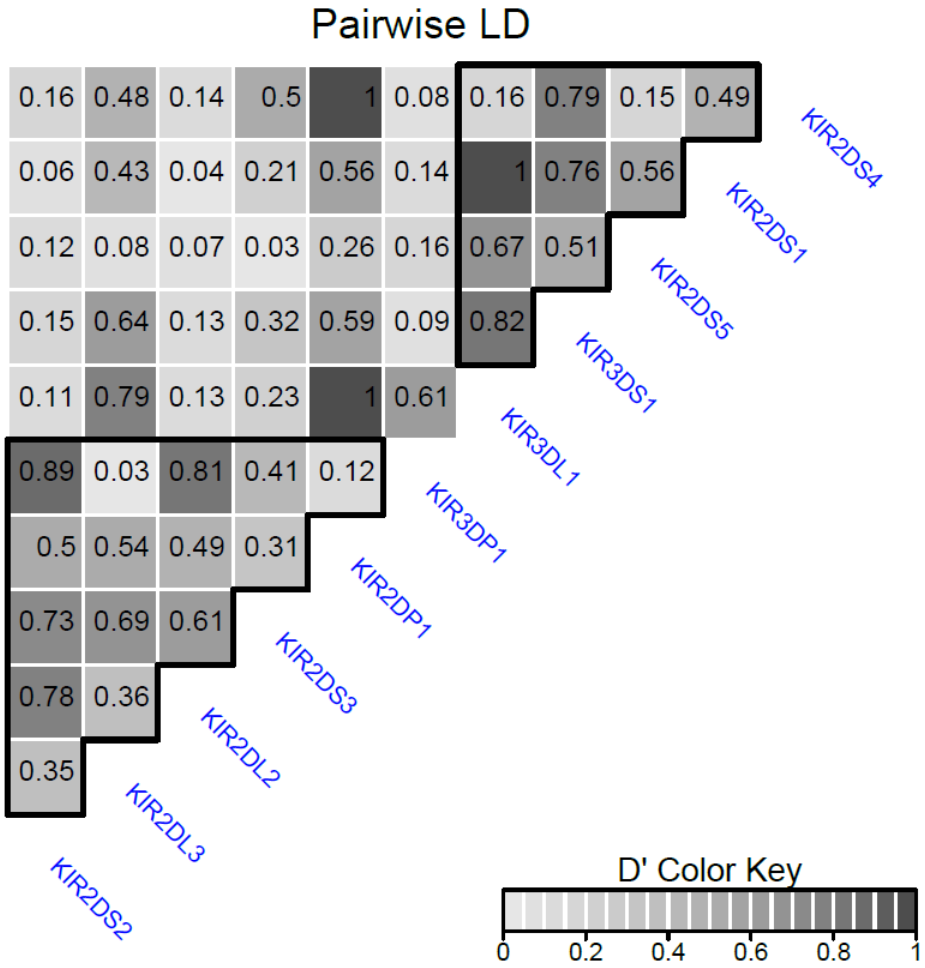

Figure 5. Linkage disequilibrium plot demonstrating LD between KIR subtypes.

The grey scale highlights the strength of the LD present between KIRs with the darkest grey demonstrating strong LD and the lighter grey, weak LD. LD estimates within the outlined regions are a result of pairwise LD comparisons to other KIR subtypes located in the same region. Those in the telomeric LD region (KIR2DS4, KIR2DS1, KIR2DS5, KIR3DS1, KIR3DL1) tend to be more highly associated with each other than to those on the centromeric LD region (KIR3DP1, KIR2DP1, KIR2DS3, KIR2DL2, KIR2DL3, KIR2DS2) and vice versa. KIR2DL4, KIR2DL1, and KIR3DL2 are not plotted, as they are present in at least 99% of subjects. Furthermore KIR2DL5 is not plotted as KIR2DL5A is on the telomeric region and KIR2DL5B is on the centromeric LD region.

Table 6: Test of association between presence and absence of KIR2DS2 in both cases and controls. KIR2DL2 and KIR2DS3 are significantly positively associated while KIR2DL5 is borderline significantly but negatively associated.

	KIR2DS2 absent (total = 94)		KIR2DS2 present (total = 95)		
KIR Gene	**absent**	**present**	**absent**	**present**	**p-value**
KIR2DL2	82 (87.2%)	12 (12.8%)	10 (10.5%)	85 (89.5%)	**<0.001**
KIR2DS3	87 (92.6%)	7 (7.4%)	49 (51.6%)	46 (48.4%)	**<0.001**
KIR2DL5	34 (36.2%)	60 (63.8%)	22 (23.2%)	73 (76.8%)	**0.050**
KIR2DS5	28 (29.8%)	66 (70.2%)	36 (37.9%)	59 (61.2%)	0.24

Table 7: Unadjusted and adjusted (for age and sex) odds ratios for a multivariable binary logistic regression containing KIR2DL2/KIR2DS2 and KIR2DS5.

	Unadjusted			Adjusted for Sex and Age		
	OR	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
KIR2DL2+/KIR2DS2+	2.37	(1.21 - 4.62)	0.01	2.24	(1.11 - 4.54)	0.025
KIR2DS5+	0.15	(0.07 - 0.31)	<0.01	0.16	(0.07 - 0.34)	<0.001

4.3 KIR haplotypes

Using the known defined KIR haplotypes[105], study participants were allocated haplotypes based on whether or not they only carried the 9 genes present on the haplotype A (*KIR3DL3–2DL3–2DP1–2DL1–3DP1–2DL4–3DL1–2DS4–3DL2)*. If they only carried these genes they were considered homozygous for the haplotype A and allocated the genotype A/A. Since haplotype B can contain the same genes as haplotype A, it is only possible to say that an individual contains at least one B haplotype. Hence, all other

participants were considered to carry at least one B haplotype (either BB or AB) and were allocated the genotype B/-.

79% of patients with adult ITP were genotype B/- and 92% of healthy participants had this genotype. Logistic regression demonstrates that genotype B/- confers protection against ITP (Table 8). Given that KIR2DS5 is part of haplotype B, we then investigated whether the protective effect of haplotype B is due to the presence of KIR2DS5. When both KIR2DS5 and genotype B/- are considered in conjunction, the effect of KIR2DS5 dominates the effect of genotype B/- rendering it non-significant (OR= 1.3, C.I. 0.4-4.27, P=0.66). This demonstrates that KIR2DS5 is driving the protective effect of genotype B/- more so than the other genes in genotype B/-.

Table 8: Unadjusted and adjusted (for age and sex) odds ratios for a binary logistic regression containing haplotype B/- .

	Unadjusted			**Adjusted for Sex and Age**		
	OR	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Haplotype B/-	0.37	(0.15 - 0.92)	0.033	0.31	(0.12 - 0.84)	0.021

4.4 Compound Genotypes

As previously shown, KIR2DL2/2DS2 is significantly related to increased risk of developing ITP; more so than just KIR2DL2 and KIR2DS2 alone[56]. Including KIR2DL2/2DS2 in our analysis, omits the need to include KIR2DS3 (as it exhibits strong LD with KIR2DL2 and KIR2DS2). Table 9 shows that there is increased risk of ITP in the KIR2DS2/2DL2 positive population that are KIR2DS5 negative (adjusted for age/gender, OR=4.48, C.I. 1.17–17.15, P=0.029), and also that there is a decreased risk of ITP (protective) in those that are KIR2DS5 positive and KIR2DS2/2DL2 negative. Interestingly, in those positive for KIR2DS2/2DL2 and KIR2DS5 both the independent risk and protective effects appear to be ameliorated (P=NS).

Table 9: Confidence intervals for the combinations of 2DS2/2DL2 compound genotype and 2DS5 demonstrates that the combination of 2DS2/2DL2 and 2DS5 largely negates the risk of 2DS2/2DL2 and the protective benefit of 2DS5 (adjusted for age and sex).

	Unadjusted			**Adjusted for Sex and Age**		
	OR	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
2DS2/DL2-/2DS5-	1			1		
2DS2/2DL2+ / 2DS5-	5.12	(1.42 - 18.41)	0.012	4.48	(1.17 - 17.15)	0.029
2DS2/2DL2-/ 2DS5+	0.24	(0.09 - 0.61)	0.003	0.24	(0.09 - 0.64)	0.004
2DS2/2DL2+ / 2DS5+	0.41	(0.16 - 1.03)	0.058	0.40	(0.15 - 1.08)	0.069

4.5 HLA-C allotypes

The ligand for KIR2DS5 is unknown. However, as the HLA-C2 allotype has been associated with protection against ankylosing spondylitis and acute rejection of renal allograft [106], we investigated the distribution of HLA-C allotypes in our ITP cohort. HLA-C allotypes were allocated into relevant KIR ligand groups, C1 and C2, based on the table published by Khakoo *et al.*[107]. In the patient samples tested, we found that 44% had the genotype C1C1, 8% C2C2 and 48% C1C2. This distribution of allotypes was not significantly different (p=0.35) to the “healthy” population (i.e. those with a resolved hepatitis C infection) reported in Khakoo *et al.* [107]. As such there is no evidence to suggest there is an interaction between ITP and HLA-C allotype. Furthermore there was no association found between HLA-C type and KIR2DS5 positivity in patients with ITP (P=0.77).

5. **Discussion**

In this study, I have performed the first prospective case-control genetic association study comparing the comprehensive KIR repertoire with the presence of adult ITP. Analysis was restricted to a cohort of well-defined chronic/persistent and relapsed ITP patients. KIR genotype was compared with healthy control participants. Data were adjusted for the effects of age and sex. I identified a number of significant associations, including notably the novel finding of the protective influence of the presence of KIR2DS5. Logistic regression analysis showed the protective effect conferred by KIR2DS5 dominates

the effect of the B-haplotype. I also demonstrated certain KIR genes associated with increased risk of the development of ITP. Previously we found an association of the compound genotype KIR2DS2/2DL2 with ITP and extended to this in the current study by showing that KIR2DS3 also confers increased risk[56]. It has previously been described by Middleton[108] that KIR2DS3 can map to both centromeric and telomeric regions of the KIR gene cluster which could in theory affect the interpretation of its LD relationship with KIR2DS2 and KIR2DL2. Although I did not determine the allelic position of the KIR2DS3 gene in our patient and control groups, I did determine through LD analysis that KIR2DS3 is in strong LD with KIR2DS2 and KIR2DS3 and the increased risk seen with this was not independent.

For comparison of KIR frequencies I tested 106 white volunteers. Those with a history of autoimmunity or cancer were specifically excluded. The frequency of haplotype B in our white control population (92%) was higher than that in the published literature of white populations (mean 75%)[107]. The majority of published KIR frequency studies are from European populations, and there is no frequency data of KIRs in white Australians available. This highlights the importance of ethnic variability when studying KIR associations.

Although KIR disease association studies have been carried out in a variety of autoimmune, infectious and malignant diseases, the protective effect of KIR2DS5 has been shown in only a few other diseases (ankylosing spondylitis, acute rejection of renal allograft)[106]. Howe and colleagues also showed a non-significant trend with KIR2DS5 being underrepresented in patients with the bone marrow failure syndromes Aplastic Anaemia and Paroxysmal Nocturnal Haemoglobinuria[109]. KIR2DS5 is also associated with leukemia free survival after HLA identical hematopoietic stem cell transplantation[110]. The mechanism for the protective effect of the activating KIR2DS5 seen in ITP is not known, but may involve altering the balance of inhibitory and activating KIRs to favour induction of immune tolerance and the subsequent reduced risk of developing autoimmunity[106]. MHC class I (particularly HLA-C) molecules are known to be ligands for specific KIRs [111]. However, no ligand has been identified for KIR2DS5. It has been suggested that there is an association between HLA-C2 and protection against ankylosing spondylitis[106], but no evidence of an interaction between KIR2DS5 and any HLA class I molecule has be demonstrated. It may be that associations of KIR2DS5 and ITP are due to LD and actually

reflect other KIR/HLA interactions[106]. I have, however demonstrated that in adult ITP, the protective effect of KIR2DS5 is independent and not associated with LD or HLA-C allotype.

This study adds to the accumulating evidence that KIRS contributes to the pathogenesis of adult ITP. A pathogenetic model for the association of KIR genotype with ITP is unclear. KIR proteins are expressed in both NK and effector T-cells. The activation of these cells is controlled by a balance between a genetically determined repertoire of activating and inhibitory KIRs[112]. It has been demonstrated that in both cell types, activating KIRs are involved in direct cell-mediated cytotoxicity although through different mechanisms. Activating KIRs in NK cells results in the direct activation of the cell, however in cytotoxic $CD8^+$ T-cells, activating KIRS act as co-stimulatory receptors that boost the response to T-cell receptor (TCR) cross linking and ultimate cell activation[113].

In the ground-breaking study by Olsson *et al*, (discussed above), autologous T-cells from patients with active chronic ITP (but not from those with ITP in remission) were shown to mediate platelet destruction[18]. By contrast, autologous NK cells were incapable of inducing direct platelet cytolysis. Moreover, the inhibitory KIR2DL3, KIR3DL2 and KIR3DL1 were up-regulated in the T-cells of patients with ITP in remission relative to those with active disease[18]. Olsson selected the KIRs to be investigated by using $CD3^+$ T-cell microarray to investigate for preferential gene expression in 8 patients with active ITP, 6 patients with ITP in remission and 6 healthy controls. Although this technique did not identify KIR2DS5 as a potential protective candidate, the numbers were small and comprehensive KIR genotyping not performed. Similarly, my study was not designed to measure levels of KIR gene or protein expression within T- or NK-cells. Along these lines, it remains to be definitively established whether KIR2DL2/KIR2DS2 and KIR2DS5 genes influence T-cell-mediated platelet cytotoxicity. Alternatively, they may influence indirect destruction of autoantibody bound platelets by NK-cells. Notably, NK-cell mediated destruction of platelets by ADCC was not tested in the study of Olsson *et al.* Although it is known that *in-vitro* KIR manipulation can modulate ADCC[112], the role of NK-cell-mediated ADCC in ITP has yet to be determined.

My thesis provides a rational platform for future studies to confirm and further elucidate the functional effect of KIRS, in particular KIR2DS5 in ITP. These should include comprehensive KIR genotyping in other ITP cohorts, in particular paediatric ITP, secondary ITP and ITP in different ethnicities. Given the highly variable responses to

rituximab seen in ITP, functional studies looking at the impact of KIRs on conventional rituximab dosing and the effect of other independent influencing factors such as FCGR receptor polymorphisms are warranted. Functional assays that test the ability to enhance rituximab anti-ITP responses by blocking inhibitory KIRs would also be of interest. Lirilumab is a first-in-class mAb that prevents inhibitory Killer-cell immunoglobulin-like receptors (KIRs) on NK-cells engaging with their ligand. Specifically, it blocks HLA-C1/C2 engagement to the 3 main inhibitory KIRs 2DL-1 (universally expressed) and KIR2DL-2/-3. My data indicates that these are present in 100% (-1), -44.3% (-2), 57.5% (-3) of healthy Australian Caucasians, values which are consistent with non-Australian data. Along these lines, identification of the HLA ligand to KIR2DS5 will not only assist in establishing the mechanistic basis for my observations but may provide a potential target for therapeutic manipulation.

In summary, in a comparison of relevant healthy control volunteers and a tightly defined cohort of adult ITP patients, the presence of KIR2DS5 was independently associated with protection against ITP. By multivariable binary logistic regression to adjust for age, sex and the effects of other KIR genes, the compound genotype of KIR2DS2/2DL2 with KIR2DS5 abrogated the risk of KIR2DS2/2DL2 and the protective benefit of 2DS5. These findings shed new light on the immunobiology of adult persistent/chronic and relapsed ITP.

6. References

1. Neunert, C. *et al.* The American Society of Hematology 2011 Evidence-Based Practice Guideline for Immune Thrombocytopenia. *Blood* **117,** 4190–4207 (2011).

2. Cohen, Y. C., Djulbegovic, B., Shamai-Lubovitz, O. & Mozes, B. The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts. *Arch. Intern. Med.* **160,** 1630–1638 (2000).

3. Stasi, R., Willis, F., Shannon, M. S. & Gordon-Smith, E. C. Infectious causes of chronic immune thrombocytopenia. *Hematol. Oncol. Clin. North Am.* **23,** 1275–1297 (2009).

4. Provan, D. *et al.* International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood* **115,** 168–186 (2010).

5. Rodeghiero, F. *et al.* Standardization of Terminology, Definitions and Outcome Criteria in Immune Thrombocytopenic Purpura of Adults and Children: Report from an International Working Group. *Blood* **113,** 2386–2393 (2009).

6. Stasi, R., Willis, F., Shannon, M. S. & Gordon-Smith, E. C. Infectious Causes of Chronic Immune Thrombocytopenia. *Hematol. Oncol. Clin. North Am.* **23,** 1275–1297 (2009).

7. HARRINGTON, W. J., MINNICH, V., HOLLINGSWORTH, J. W. & MOORE, C. V. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J. Lab. Clin. Med.* **38,** 1–10 (1951).

8. Chan, H., Moore, J. C., Finch, C. N., Warkentin, T. E. & Kelton, J. G. The IgG subclasses of platelet-associated autoantibodies directed against platelet glycoproteins IIb/IIIa in patients with idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **122,** 818–824 (2003).

9. Van Leeuwen, E. F., Van Der Ven, J. T., Engelfriet, C. P. & Von Dem Borne, A. E. Specificity of Autoantibodies in Autoimmune Thrombocytopenia. *Blood* **59,** 23–26 (1982).

10. Roark, J. H., Bussel, J. B., Cines, D. B. & Siegel, D. L. Genetic analysis of autoantibodies in idiopathic thrombocytopenic purpura reveals evidence of clonal expansion and somatic mutation. *Blood* **100,** 1388–1398 (2002).

11. Stasi, R. *et al.* Idiopathic thrombocytopenic purpura: Current concepts in pathophysiology and management. *Thromb. Haemost.* (2007). doi:10.1160/TH07-08-0513

12. Louwes, H., Zeinali Lathori, O. A., Vellenga, E. & de Wolf, J. T. Platelet kinetic studies in patients with idiopathic thrombocytopenic purpura. *Am. J. Med.* **106,** 430–434 (1999).

13. Houwerzijl, E. J. *et al.* Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. *Blood* **103,** 500–506 (2004).

14. Stahl, C. P., Zucker-Franklin, D. & McDonald, T. P. Incomplete antigenic cross-reactivity between platelets and megakaryocytes: relevance to ITP. *Blood* **67,** 421–428 (1986).

15. McMillan, R., Wang, L., Tomer, A., Nichol, J. & Pistillo, J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. *Blood* **103,** 1364–1369 (2004).

16. Cines, D. B. & Schreiber, A. D. Immune Thrombocytopenia. *N. Engl. J. Med.* **300,** 106–111 (1979).

17. Urbanus, R. T. *et al.* Patient autoantibodies induce platelet destruction signals via raft-associated glycoprotein Ibα and Fc RIIa in immune thrombocytopenia. *Haematologica* **98,** e70–e72 (2013).

18. Olsson, B. *et al.* T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat. Med.* **9,** 1123–1124 (2003).

19. Zhang, F. *et al.* Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura, Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur. J. Haematol. Eur. J. Haematol.* **76,** 427, 427–431, 431 (2006).

20. Olsson, B., Ridell, B., Carlsson, L., Jacobsson, S. & Wadenvik, H. Recruitment of T cells into bone marrow of ITP patients possibly due to elevated expression of VLA-4 and CX3CR1. *Blood* **112,** 1078–1084 (2008).

21. Semple, J. W. *et al.* Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity. *Blood* **87,** 4245–4254 (1996).

22. Ogawara, H. *et al.* High Th1/Th2 ratio in patients with chronic idiopathic thrombocytopenic purpura. *Eur. J. Haematol.* **71,** 283–288 (2003).

23. Panitsas, F. P. *et al.* Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response. *Blood* **103,** 2645–2647 (2004).

24. Ling, Y., Cao, X., Yu, Z. & Ruan, C. Circulating dendritic cells subsets and CD4+Foxp3+ regulatory T cells in adult patients with chronic ITP before and after treatment with high-dose dexamethasome. *Eur. J. Haematol.* **79,** 310–316 (2007).

25. Bluestone, J. A. & Abbas, A. K. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* **3,** 253–257 (2003).

26. Gratz, I. K., Rosenblum, M. D. & Abbas, A. K. The life of regulatory T cells. *Ann. N. Y. Acad. Sci.* **1283,** 8–12 (2013).

27. Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **27,** 20–21 (2001).

28. Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27,** 68–73 (2001).

29. Nishimoto, T., Satoh, T., Simpson, E. K., Ni, H. & Kuwana, M. Predominant autoantibody response to GPIb/IX in a regulatory T-cell-deficient mouse model for immune thrombocytopenia. *J. Thromb. Haemost.* **11,** 369–372 (2013).

30. McKenzie, C. G. J., Guo, L., Freedman, J. & Semple, J. W. Cellular immune dysfunction in immune thrombocytopenia (ITP). *Br. J. Haematol.* **163,** 10–23 (2013).

31. Harrington, L. E. *et al.* Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6,** 1123–1132 (2005).

32. Murphy, C. A. *et al.* Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* **198,** 1951–1957 (2003).

33. Guo, Z. X. *et al.* The role of Th17 cells in adult patients with chronic idiopathic thrombocytopenic purpura. *Eur. J. Haematol.* **82,** 488–489 (2009).

34. Cines, D. B., Bussel, J. B., Liebman, H. A. & Luning Prak, E. T. The ITP syndrome: pathogenic and clinical diversity. *Blood* **113,** 6511 –6521 (2009).

35. Thien, M. *et al.* Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity* **20,** 785–798 (2004).

36. Emmerich, F. *et al.* High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **136,** 309–314 (2007).

37. Cheema, G. S., Roschke, V., Hilbert, D. M. & Stohl, W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune–based rheumatic diseases. *Arthritis Rheum.* **44,** 1313–1319 (2001).

38. Tan, S.-M. *et al.* Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. *Arthritis Rheum.* **48,** 982–992 (2003).

39. Semple, J. W., Bruce, S. & Freedman, J. Suppressed natural killer cell activity in patients with chronic autoimmune thrombocytopenic purpura. *Am. J. Hematol.* **37,** 258–262 (1991).

40. XU, R. *et al.* Elevated NKT cell levels in adults with severe chronic immune thrombocytopenia. *Exp. Ther. Med.* **7,** 149–154 (2014).

41. Talaat, R. M., Elmaghraby, A. M., Barakat, S. S. & EL-Shahat, M. Alterations in immune cell subsets and their cytokine secretion profile in childhood idiopathic thrombocytopenic purpura (ITP). *Clin. Exp. Immunol.* **176,** 291–300 (2014).

42. van de Winkel, J. G. J. & Capel, P. J. A. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol. Today* **14,** 215–221 (1993).

43. Parren, P. W. *et al.* On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. *J. Clin. Invest.* **90,** 1537–1546 (1992).

44. Satoh, T. *et al.* Fcγ receptor IIB gene polymorphism in adult Japanese patients with primary immune thrombocytopenia. *Blood* **122,** 1991–1992 (2013).

45. Joutsi, L., Javela, K., Partanen, J. & Kekomäki, R. Genetic polymorphism H131R of Fcγ receptor type IIA (FcγRIIA) in a healthy Finnish population and in patients with or without platelet-associated IgG. *Eur. J. Haematol.* **61,** 183–189 (1998).

46. Ghesquières, H. *et al.* FCGR2A and FCGR3A polymorphisms in classical Hodgkin lymphoma by EBV status. *Leuk. Lymphoma* 1–7 (2013). doi:10.3109/10428194.2013.796048

47. Bredius, R. G. M. *et al.* Fcã Receptor Iia (Cd32) Polymorphism In Fulminant Meningococcal Septic. *J. Infect. Dis.* **170,** 848–853 (1994).

48. Sanders, L. A. M. *et al.* Fcã Receptor Iia (Cd32) Heterogeneity In Patients With Recurrent Bacterial Respiratory Tract Infections. *J. Infect. Dis.* **170,** 854–861 (1994).

49. Salmon, J. E. *et al.* Altered distribution of Fcγ receptor IIIA alleles in a cohort of Korean patients with lupus nephritis. *Arthritis Rheum.* **42,** 818–823 (1999).

50. Denomme, G. A. *et al.* Activation of platelets by sera containing igg1 heparin-dependent antibodies: an explanation for the predominance of the Fcγrlla 'low responder' (his131) gene in patients with heparin-induced thrombocytopenia. *J. Lab. Clin. Med.* **130,** 278–284 (1997).

51. Sondermann, P., Huber, R., Oosthuizen, V. & Jacob, U. The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. *Nature* **406,** 267–273 (2000).

52. Koene, H. R. *et al.* Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* **90,** 1109–1114 (1997).

53. Hatjiharissi, E. *et al.* Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the FcγRIIIa-158 V/V and V/F polymorphism. *Blood* **110,** 2561–2564 (2007).

54. Wang, D., Hu, S.-L., Cheng, X.-L. & Yang, J.-Y. FCGR2A rs1801274 polymorphism is associated with risk of childhood-onset idiopathic (immune) thrombocytopenic purpura: evidence from a meta-analysis. *Thromb. Res.* **134,** 1323–1327 (2014).

55. Carcao, M. D. *et al.* Fcgamma receptor IIa and IIIa polymorphisms in childhood immune thrombocytopenic purpura. *Br. J. Haematol.* **120,** 135–141 (2003).

56. Nourse, J. P. *et al.* The KIR2DS2/DL2 genotype is associated with adult persistent/chronic and relapsed immune thrombocytopenia independently of FCGR3a-158 polymorphisms. *Blood Coagul. Fibrinolysis* **23,** 45–50 (2012).

57. Breunis, W. B. *et al.* Copy number variation of the activating FCGR2C gene predisposes to idiopathic thrombocytopenic purpura. *Blood* **111,** 1029–1038 (2008).

58. Fujimoto, T. T., Inoue, M., Shimomura, T. & Fujimura, K. Involvement of Fc gamma receptor polymorphism in the therapeutic response of idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **115,** 125–130 (2001).

59. Cooper, N. *et al.* Platelet-associated antibodies, cellular immunity and FCGR3a genotype influence the response to rituximab in immune thrombocytopenia. *Br. J. Haematol.* **158,** 539–547 (2012).

60. Anis, S. K., Abdel Ghany, E. A., Mostafa, N. O. & Ali, A. A. The role of PTPN22 gene polymorphism in childhood immune thrombocytopenic purpura. *Blood Coagul. Fibrinolysis* **22,** 521–525 (2011).

61. Chen, Z. *et al.* STAT1 single nucleotide polymorphisms and susceptibility to immune thrombocytopenia. *Autoimmunity* **48,** 305–312 (2015).

62. Ma, L. *et al.* Effects of CD70 and CD11a in Immune Thrombocytopenia Patients. *J. Clin. Immunol.* **31,** 632–642 (2011).

63. Pehlivan, M. *et al.* Investigation of TNF-alpha, TGF-beta 1, IL-10, IL-6, IFN-gamma, MBL, GPIA, and IL1A gene polymorphisms in patients with idiopathic thrombocytopenic purpura. *Platelets* **22,** 588–595 (2011).

64. Saitoh, T. *et al.* Interleukin-17F gene polymorphism in patients with chronic immune thrombocytopenia. *Eur. J. Haematol.* **87,** 253–258 (2011).

65. Zhou, H. *et al.* CD72 gene expression in immune thrombocytopenia. *Platelets* 1–8 (2011). doi:10.3109/09537104.2011.633646

66. Chen, Z. *et al.* Foxp3 methylation status in children with primary immune thrombocytopenia. *Hum. Immunol.* **75,** 1115–1119 (2014).

67. Qian, B.-H. *et al.* Increased miR-155 expression in peripheral blood mononuclear cells of primary immune thrombocytopenia patients was correlated with serum cytokine profiles. *Acta Haematol.* **133,** 257–263 (2015).

68. Long, L. *et al.* Upregulated microRNA-155 expression in peripheral blood mononuclear cells and fibroblast-like synoviocytes in rheumatoid arthritis. *Clin. Dev. Immunol.* **2013,** 296139 (2013).

69. Dai, R. *et al.* Identification of a common lupus disease-associated microRNA expression pattern in three different murine models of lupus. *PloS One* **5,** e14302 (2010).

70. Paraboschi, E. M. *et al.* Genetic association and altered gene expression of mir-155 in multiple sclerosis patients. *Int. J. Mol. Sci.* **12,** 8695–8712 (2011).

71. Corral-Fernández, N. E. *et al.* Dysregulated miR-155 expression in peripheral blood mononuclear cells from patients with type 2 diabetes. *Exp. Clin. Endocrinol. Diabetes Off. J. Ger. Soc. Endocrinol. Ger. Diabetes Assoc.* **121,** 347–353 (2013).

72. Zhao, H. *et al.* Reduced MIR130A is involved in primary immune thrombocytopenia via targeting TGFB1 and IL18. *Br. J. Haematol.* **166,** 767–773 (2014).

73. Lanier, L. L. Nk Cell Recognition. *Annu. Rev. Immunol.* **23,** 225–274 (2005).

74. Moretta, A. *et al.* P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J. Exp. Med.* **178,** 597–604 (1993).

75. Valiante, N. M. *et al.* Functionally and Structurally Distinct NK Cell Receptor Repertoires in the Peripheral Blood of Two Human Donors. *Immunity* **7,** 739–751 (1997).

76. Thielens, A., Vivier, E. & Romagné, F. NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Curr. Opin. Immunol.* doi:10.1016/j.coi.2012.01.001

77. Vilches, C. & Parham, P. KIR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity. *Annu. Rev. Immunol.* **20,** 217–251 (2002).

78. Campbell, K. S. & Purdy, A. K. Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations. *Immunology* **132,** 315–325 (2011).

79. Martin, M. P. *et al.* Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. *J. Immunol. Baltim. Md 1950* **169,** 2818–2822 (2002).

80. van der Slik, A. R. *et al.* KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes* **52,** 2639–2642 (2003).

81. Nikitina-Zake, L., Rajalingham, R., Rumba, I. & Sanjeevi, C. B. Killer cell immunoglobulin-like receptor genes in Latvian patients with type 1 diabetes mellitus and healthy controls. *Ann. N. Y. Acad. Sci.* **1037,** 161–169 (2004).

82. Momot, T. *et al.* Association of killer cell immunoglobulin-like receptors with scleroderma. *Arthritis Rheum.* **50,** 1561–1565 (2004).

83. García-León, J. A. *et al.* Killer cell immunoglobulin-like receptor genes in Spanish multiple sclerosis patients. *Mol. Immunol.* **48,** 1896–1902 (2011).

84. Jelčić, I. *et al.* Killer immunoglobulin-like receptor locus polymorphisms in multiple sclerosis. *Mult. Scler. J.* **18,** 951–958 (2012).

85. Hiby, S. E. *et al.* Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J. Exp. Med.* **200,** 957–965 (2004).

86. Alter, G. *et al.* Differential Natural Killer Cell–mediated Inhibition of HIV-1 Replication Based on Distinct KIR/HLA Subtypes. *J. Exp. Med.* **204,** 3027–3036 (2007).

87. Martin, M. P. *et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.* **31,** 429–434 (2002).

88. Suppiah, V. *et al.* IL28B, HLA-C, and KIR Variants Additively Predict Response to Therapy in Chronic Hepatitis C Virus Infection in a European Cohort: A Cross-Sectional Study. *PLoS Med* **8,** e1001092 (2011).

89. Naumova, E. *et al.* Genetic polymorphism of NK receptors and their ligands in melanoma patients: prevalence of inhibitory over activating signals. *Cancer Immunol. Immunother. CII* **54,** 172–178 (2005).

90. Wu, X. *et al.* KIR2DS4 and its variant KIR1D are associated with aGVHD, CMV, and OS after sibling-related HLA matched transplantation for patients whose donors belong to the KIR gene haplotype A. *Biol. Blood Marrow Transplant. J. Am. Soc. Blood Marrow Transplant.* (2015). doi:10.1016/j.bbmt.2015.10.004

91. Sobecks, R. M. *et al.* Impact of KIR and HLA Genotypes on Outcomes after Reduced-Intensity Conditioning Hematopoietic Cell Transplantation. *Biol. Blood Marrow Transplant. J. Am. Soc. Blood Marrow Transplant.* **21,** 1589–1596 (2015).

92. Oevermann, L. *et al.* KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood* **124,** 2744–2747 (2014).

93. Yeung, D. T. *et al.* KIR2DL5B genotype predicts outcomes in CML patients treated with response-directed sequential imatinib/nilotinib strategy. *Blood* **126,** 2720–2723 (2015).

94. Caocci, G. *et al.* Killer immunoglobulin-like receptors can predict TKI treatment-free remission in chronic myeloid leukemia patients. *Exp. Hematol.* (2015). doi:10.1016/j.exphem.2015.08.004

95. Hoteit, R. *et al.* KIR genotype distribution among patients with multiple myeloma: Higher prevalence of KIR 2DS4 and KIR 2DS5 genes. *Meta Gene* **2,** 730–736 (2014).

96. Ruggeri, L. *et al.* Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. *Science* **295,** 2097–2100 (2002).

97. Leung, W. *et al.* Determinants of antileukemia effects of allogeneic NK cells. *J. Immunol. Baltim. Md 1950* **172,** 644–650 (2004).

98. Boulad, N. *et al.* The Influence Of KIR Haplotype In ITP Incidence, Treatment Response and Bleeding Symptoms. *Blood* **122,** 2316–2316 (2013).

99. El-beblawy, N. M. S., Elbarbary, N. S., Kamal, T. M. & Mahmoud, P. M. A Study of Human Killer Cell Immunoglobulin-Like Receptor and Multidrug Resistance Gene Polymorphisms in Children With Immune Thrombocytopenia. *Clin. Appl. Thromb.* 1076029615576738 (2015). doi:10.1177/1076029615576738

100. George, J. N. *et al.* Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. *Blood* **88,** 3–40 (1996).

101. Keen-Kim, D. *et al.* Overrepresentation of rare variants in a specific ethnic group may confuse interpretation of association analyses. *Hum. Mol. Genet.* **15,** 3324–3328 (2006).

102. Ashouri, E., Ghaderi, A., Reed, E. F. & Rajalingam, R. A novel duplex SSP-PCR typing method for KIR gene profiling. *Tissue Antigens* **74,** 62–67 (2009).

103. Maxwell, L. D., Ross, O. A., Curran, M. D., Rea, I. M. & Middleton, D. Investigation of KIR diversity in immunosenescence and longevity within the Irish population. *Exp. Gerontol.* **39,** 1223–1232 (2004).

104. Gourraud, P.-A., Meenagh, A., Cambon-Thomsen, A. & Middleton, D. Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. *Immunogenetics* **62,** 729–740 (2010).

105. Carrington, M. & Norman, P. The KIR Gene Cluster. (2003). at <http://www.ncbi.nlm.nih.gov/books/NBK10134/#A27>

106. Nowak, I. *et al.* Does the KIR2DS5 Gene Protect from Some Human Diseases? *PLoS ONE* **5,** (2010).

107. Khakoo, S. I. *et al.* HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **305,** 872–874 (2004).

108. Middleton, D. & Gonzelez, F. The extensive polymorphism of KIR genes. *Immunology* **129,** 8–19 (2010).

109. Howe, E. C., Wlodarski, M., Ball, E. J., Rybicki, L. & Maciejewski, J. P. Killer immunoglobulin-like receptor genotype in immune-mediated bone marrow failure syndromes. *Exp. Hematol.* **33,** 1357–1362 (2005).

110. van der Meer, A. *et al.* KIR2DS5 is associated with leukemia free survival after HLA identical stem cell transplantation in chronic myeloid leukemia patients. *Mol. Immunol.* **45,** 3631–3638 (2008).

111. Rajagopalan, S. & Long, E. O. Understanding How Combinations of HLA and KIR Genes Influence Disease. *J. Exp. Med.* **201,** 1025–1029 (2005).

112. Uhrberg, M. *et al.* Human diversity in killer cell inhibitory receptor genes. *Immunity* **7,** 753–763 (1997).

113. Mandelboim, O. *et al.* Natural killer activating receptors trigger interferon gamma secretion from T cells and natural killer cells. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3798–3803 (1998).

Appendix A

Final submitted manuscript of:

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Conflicts of interest

The authors Louise Seymour, Jamie Nourse, Pauline Crooks and Leesa Wockner confirm there are no conflicts of interest. Robert Bird, Huyen Tran and Maher Gandhi have accepted sponsored travel to overseas conferences from Roche.

ABSTRACT

Immune thrombocytopenia (ITP) is an autoimmune disorder of unknown aetiology, characterised by an isolated low platelet count in the absence of other identifiable causes. Genes influencing activation of the immune system have been identified as influencing predisposition. Killer cell immunoglobulin-like receptors (KIR) control T-cell and Natural Killer cell function via inhibitory and activating signalling pathways. The inhibitory KIR2DL3, KIR3DL2 and KIR3DL1 are up-regulated in the T-cells of patients with ITP in remission relative to those with active disease, and an association of KIR2DS2/KIR2DL2 with ITP has also been reported. No comprehensive KIR analysis in ITP has been reported. We performed genotyping of all currently known KIR genes using sequence specific primer polymerase chain reaction (SSP-PCR) on a cohort of 83 adult patients with ITP (chronic/persistent or relapsed primary ITP identified by defined criteria) and 106 age matched healthy white volunteers. Non-white patients were not included in the analysis. There was an over-representation of KIR2DS3 (known to be in linkage disequilibrium with KIR2DS2/2DL2) and under-representation of KIR2DS5 (also protective against other immune mediated disorders) in adult ITP (odds ratio 0.16, C.I. 0.08-0.32, $P<0.001$). By multivariable binary logistic regression to adjust for age, sex and the effects of other KIR genes, the presence of KIR2DS2/2DL2 with KIR2DS5 abrogated the risk of KIR2DS2/2DL2 and the protective benefit of KIR2DS5. Further studies are required to establish the mechanistic basis for these observations and their potential impact on ITP therapy.

INTRODUCTION

Immune thrombocytopenia (ITP) is a disorder characterised by isolated thrombocytopenia due to autoimmune mediated platelet destruction occurring in both paediatric and adult populations. Paediatric ITP is generally precipitated by an acute infection and is usually self-limiting. In contrast, in adult ITP, many patients relapse or progress to chronic ITP indicating a distinct pathophysiology. Pathogenesis of ITP is a complex multifactorial process involving an immune mediated peripheral destruction of platelets and insufficient bone marrow production. Further understanding of the immunobiology of adult chronic and relapsed ITP is required in order to develop novel targeted therapies.

There are a number of polymorphisms associated with genetic predisposition to adult ITP (1). Our laboratory has demonstrated that the FCgamma receptor polymorphism FCGR3a-V158 was overrepresented in adult patients with ITP compared with healthy white volunteers. This study also demonstrated that the presence of Killer cell immunoglobulin-like immunoglobulin receptor (KIR) genes KIR2DS2/KIR2DL2 was associated with increased susceptibility to adult ITP independently of FCGR3a-158 polymorphisms (2). Olsson *et al*. demonstrated that the inhibiting KIR2DL3, KIR3DL2 and KIR3DL1 are known to be up-regulated in the T-cells of patients with ITP in remission relative to those with active disease (3).

KIRs are a family of polymorphic and highly homologous genes that regulate effector functions of NK cells and a subset of $CD4^+$ and $CD8^+$ T lymphocytes (that lack CD28 expression). They are subdivided based on the number of extracellular Immunoglobulin domains (2D or 3D) and can be inhibitory or activating. Activating KIRs lack the immuno tyrosine inhibitory motif (ITIM) present in the intracellular domain of inhibitory KIRs due to a carboxy-terminal truncation of the protein, and have thus been named as short forms of the receptors. KIR genotype expression varies between different immune effector cells, and determines whether there is phenotypic dominance of KIR-mediated inhibition over activation. Genetic studies strongly implicate the presence of certain KIR genotypes, KIR haplotypes or KIR/HLA compound genotypes in susceptibility and outcome to diverse disease processes such as psoriasis/psoriatic arthritis (4), rheumatoid arthritis/vasculitis, type I diabetes (5,6), scleroderma (7), multiple sclerosis (8,9), pre-eclampsia (10), resistance to HIV (11,12)and HCV viral infection (13), coronary artery disease, and melanoma (14). Rituximab (an anti-CD20 monoclonal antibody) is an established treatment of adult ITP (15). The distribution of KIR genes in adult ITP may influence antibody mediated cellular cytotoxicity (16) and hence the response to antibody-

mediated therapies. Prospective studies are required to establish their impact on rituximab response in adult chronic/persistent and relapsed ITP.

To our knowledge, no prior comprehensive KIR gene association study has been performed in adult ITP. Therefore, we performed this laboratory sub study on samples obtained from a large prospective study to assess the influence of all currently known KIR genes (with the exception of KIR3DL3 which is present in all haplotypes) on susceptibility in a well-defined cohort of chronic and relapsed adult ITP and age matched healthy volunteers in order to explore any disease associations with the presence or absence of KIR genes and haplotypes.

METHODS

Patients and healthy volunteer recruitment

One hundred and twenty-four patients with chronic and relapsed ITP were accrued from multiple centres in Australia as part of a sponsored clinical trial (ML20948; NCT00475423). DNA was available for 95 patients that consented for participation in this laboratory sub-analysis. Diagnosis was made according to the then current American Society of Hematology guidelines (17). Patients were only eligible if they met stringent criteria. To ensure that only primary ITP was included, patients with lymphoproliferative disorders, multi-system autoimmune disorders, drug-induced thrombocytopenia or seropositivity for Human Immunodeficiency virus, Hepatitis B or C were excluded. Newly diagnosed ITP patients were not included. Patient characteristics including ethnicity were collected prospectively as part of the trial protocol. The study was approved by the relevant Hospital/Research Institute Ethics Committees and was performed in accordance with the Declaration of Helsinki. Signed informed consent was obtained from all patients in the study.

Patients were divided into two groups. The first denoted as ‘persistent/chronic ITP’ was defined as ITP for more than 3 months requiring ongoing therapy to maintain a platelet count of ≥ 30 x 10^9/L. This definition is similar to the phases of disease as laid out by the International Working Group (IWG) (18) . The second group was termed ‘relapsed ITP’. These were patients with an initial response as defined per IWG: namely a platelet count ≥ 30x 10^9/L and at least 2-fold increase in the baseline count with absence of bleeding, without the requirement for ongoing therapy. Relapse in these patients was defined as a platelet count falling to below 30 x 10^9/L. It included patients with one or multiple relapses; for the former, relapse had to occur within 12 months of diagnosis. One hundred and six healthy adult volunteers, with no history of haematological or autoimmune

disorders of any kind, served as a control cohort. Gene and polymorphism frequencies differ with ethnicity (19), therefore for accurate interpretation of results, we ensured that our control population were all white. Study of healthy controls was approved by the Queensland Institute of Medical Research Ethics Committee and signed informed consent was obtained from all participants.

Sequence Specific Primer directed Polymerase chain reaction (SSP-PCR)

DNA was extracted from cryopreserved peripheral blood cell pellets (patients) or buccal scrapes (healthy volunteers) using standard procedures and analysis performed in batches. All samples were analysed in the same laboratory. Of the 16 currently known KIR genes, we analysed 13 of these. KIR3DL3 was omitted from the analysis as it is present in 100% of the population. KIR2DL2 and KIR2DS2 were previously analysed by this laboratory (2) so were not repeated. The 13 remaining KIR genes were analysed by a modified version of published SSP-PCR in a set of 7 multiplex PCR reactions using the same primers (20). Each PCR was performed in a reaction volume of 15 □l with a final concentration of 1.5x PCR buffer II (10 mM Tris–HCl and 50 mM KCl), 200 □M of each dNTP, 0.6 □M of each forward and reverse primer (with exception of 3DP1F82 and 3DP1F83 were used at 0.3 □M final concentration), 2 mM of MgCl2, 0.5 unit of AmpliTaq DNA polymerase and 10 ng genomic DNA. PCR amplification was performed using an ABI 9700 Gene-Amp PCR System using the set-specific thermal cycling conditions as previously published (20). Following amplification, 5 □l of the products (for sets 1,2,4,5,6,7) and 10 □L for set 3 were electrophoresed in 3% agarose gel containing ethidium bromide at low voltage for 40 minutes. A low range ladder was also included.

Four of the seven duplex PCR sets performed include primers targeting a framework KIR gene (sets 2, 4 and 6) and serve as an internal positive control to confirm the successful PCR amplification in each of these reactions. The remaining four PCR sets (sets 1, 3, 5 and 7) target at least one frequently occurring KIR gene, and therefore, most individuals are likely positive for one of the two genes tested in each of these sets. There was at least one of these KIR genes positive in all our reactions in both ITP patients and controls.

Human Leukocyte Antigen (HLA) Allotyping

Tier 1 HLA-C allotyping was performed by ProImmune Limited (Oxford, United Kingdom) on 71 patient samples using a PCR-SSOP method. HLA typing was not

performed on the control samples and 12 of the patient samples due to insufficient quantities of DNA available.

Statistical analysis

To identify potential associations between disease status and KIR genotypes, Pearson's chi-squared test was performed. Alternatively, when expected counts were small, Fisher's exact test was used. Binary logistic regression was used to assess the effect genes had in combination. Genes in linkage disequilibrium were not included in the same model. In addition, effect sizes were adjusted for both age and gender. Unadjusted and adjusted odds ratios are reported. All analysis was performed in SPSS v.19. An alpha level of 0.05 was considered statistically significant.

RESULTS

Study population

Ethnicity was available for all 95 patients, with 83 (84%) of patients identifying themselves as white. Due to gene and gene polymorphism frequencies differing with ethnicity, only the white cases were analysed. The mean age of patients was 50.5 years (range 18–85) with 47 (57%) females, 37 (45%) with chronic/persistent ITP and 44 (53%) with relapsed ITP, two people (2.4%) had primary/acute refractory ITP. In the 106 healthy white controls, 72 (68%) were women and mean age was 40.5 years (range 23–68 years).

Genotype and allele frequencies.

We have previously shown that the distributions observed in the healthy controls for the KIR2DS2/2DL2 genes were in agreement with those previously reported and did not deviate from the Hardy Weinberg Equilibrium (P>0.1)(2,21). The distribution of KIR genes in both cases and healthy participants are shown in Table 1. The KIR gene frequencies did not differ between adult ITP patients and controls with the exception of KIR2DS3 (odds ratio [OR] =2.28, C.I. 1.19-4.35, P=0.012), KIR2DS5 and KIR2DL5 (OR=0.16, C.I. 0.08-0.32, P<0.001 and OR=0.47, C.I. 0.25-0.88, P=0.017 respectively). From this we deduce that KIR2DS3 confers an increased risk and KIR2DS5 and KIR2DL5 confer protection (Figure 1). There were no significant differences noted between KIRs and the subtypes of ITP (chronic/persistent and relapsed ITP) so for the rest of the analysis these subtypes have been combined and referred to as ITP.

A Linkage Disequilibrium (LD) plot demonstrating the LD present between the genes in our analysis is shown in Figure 2. From this it can be seen that our results are

consistent with, and validated the LD data of Gourraud (22). Based on our LD findings and the test of association in Table 2, we can further explain the significant associations demonstrated in Table 1. The genes that show a conferral of risk (KIR2DS2/KIR2DL2 and KIR2DS3) are highly positively associated. The genes that confer protection are weakly negatively associated with KIR2DS2 (KIR2DL5; p=0.05) or not associated at all (KIR2DS5; p=0.24). KIR2DL5 and KIR2DS5 are both positively associated (Chi-squared test; p-value<0.001; data not shown), however as KIR2DL5 has a weak negative association with KIR2DS2/KIR2DL2 and KIR2DS3, we would expect it to correlate with another gene that has a protective effect regardless of any biological relationship. As KIR2DL5 confers a more moderate degree of protection to adult ITP and as it is both associated with genes on the centromeric and telomeric region it is not an appropriate candidate for further modelling. However, KIR2DS5 is independently protective of ITP when adjusted for age, sex and KIR2DS2/2DL2 genotype (OR=0.16, C.I. 0.07-0.34, P<0.001).

KIR Haplotypes

Using the known defined KIR haplotypes (23), study participants were allocated haplotypes based on whether or not they only carried the 9 genes present on the haplotype A (*KIR3DL3–2DL3–2DP1–2DL1–3DP1–2DL4–3DL1–2DS4–3DL2)*. If they only carried these genes they were considered homozygous for the haplotype A and allocated the genotype A/A. Since haplotype B can contain the same genes as haplotype A, it is only possible to say that an individual contains at least one B haplotype. Hence, all other participants were considered to carry at least one B haplotype (either BB or AB) and were allocated the genotype B/-.

79% of patients with adult ITP were genotype B/- and 92% of healthy participants had this genotype. Logistic regression demonstrates that genotype B/- confers protection against ITP (adjusted for age/gender, OR=0.31, C.I. 0.12-0.84, P=0.021). Given that KIR2DS5 is part of haplotype B, we then investigated whether the protective effect of haplotype B is due to the presence of KIR2DS5. When both KIR2DS5 and genotype B/- are considered in conjunction, the effect of KIR2DS5 dominates the effect of genotype B/- rendering it non-significant (OR= 1.3, C.I. 0.4-4.27, P=0.66). This demonstrates that KIR2DS5 is driving the protective effect of genotype B/- more so than the other genes in genotype B/-.

Compound genotypes

As previously shown, KIR2DL2/2DS2 is significantly related to increased risk of developing ITP; more so than just KIR2DL2 and KIR2DS2 alone (2). Including KIR2DL2/2DS2 in our analysis, omits the need to include KIR2DS3 (as it exhibits strong LD with KIR2DL2 and KIR2DS2). Table 3 shows that there is increased risk of ITP in the KIR2DS2/2DL2 positive population that are KIR2DS5 negative (adjusted for age/gender, OR=4.48, C.I. 1.17–17.15, P=0.029), and also that there is a decreased risk of ITP (protective) in those that are KIR2DS5 positive and KIR2DS2/2DL2 negative. Interestingly, in those positive for KIR2DS2/2DL2 and KIR2DS5 both the independent risk and protective effects appear to be ameliorated (P=NS).

HLA-C Allotypes

The ligand for KIR2DS5 is unknown. However, as the HLA-C2 allotype has been associated with protection against ankylosing spondylitis and acute rejection of renal allograft (24), we investigated the distribution of HLA-C allotypes in our ITP cohort. HLA-C allotypes were allocated into relevant KIR ligand groups, C1 and C2, based on the table published by Khakoo *et al.* (25). In the patient samples tested, we found that 44% had the genotype C1C1, 8% C2C2 and 48% C1C2. This distribution of allotypes was not significantly different (p=0.35) to the “healthy” population (i.e. those with a resolved hepatitis C infection) reported in Khakoo *et al.* (25). As such there is no evidence to suggest there is an interaction between ITP and HLA-C allotype. Furthermore there was no association found between HLA-C type and KIR2DS5 positivity in patients with ITP (P=0.77).

DISCUSSION

In this study, we performed the first prospective case-control genetic association study comparing the comprehensive KIR repertoire with the presence of adult ITP. Analysis was restricted to a cohort of well-defined chronic/persistent and relapsed ITP patients. KIR genotype was compared with healthy control participants. Data were adjusted for the effects of age and sex. We identified a number of significant associations, including notably the novel finding of the protective influence of the presence of KIR2DS5. Logistic regression analysis showed the protective effect conferred by KIR2DS5 dominates the effect of the B-haplotype. We also demonstrated certain KIR genes associated with increased risk of the development of ITP. Previously we found an association of the compound genotype KIR2DS2/2DL2 with ITP and extended to this in the current study by showing that KIR2DS3 also confers increased risk (2). It has previously been described by

Middleton (26) that KIR2DS3 can map to both centromeric and telomeric regions of the KIR gene cluster which could in theory affect the interpretation of its LD relationship with KIR2DS2 and KIR2DL2. Although we did not determine the allelic position of the KIR2DS3 gene in our patient and control groups, we did determine through LD analysis that KIR2DS3 is in strong LD with KIR2DS2 and KIR2DS3 and the increased risk seen with this was not independent.

For comparison of KIR frequencies we tested 106 white volunteers. Those with a history of autoimmunity or cancer were specifically excluded. The frequency of haplotype B in our white control population (92%) was higher than that in the published literature of white populations (mean 75%) (25). The majority of published KIR frequency studies are from European populations, and there is no frequency data of KIRs in white Australians available. This highlights the importance of ethnic variability when studying KIR associations.

Although KIR disease association studies have been carried out in a variety of autoimmune, infectious and malignant diseases, the protective effect of KIR2DS5 has been shown in only a few other diseases (ankylosing spondylitis, acute rejection of renal allograft) (24). Howe and colleagues also showed a non-significant trend with KIR2DS5 being underrepresented in patients with the bone marrow failure syndromes Aplastic Anaemia and Paroxysmal Nocturnal Haemoglobinuria (27). KIR2DS5 is also associated with leukemia free survival after HLA identical hematopoietic stem cell transplantation (28). The mechanism for the protective effect of the activating KIR2DS5 seen in ITP is not known, but may involve altering the balance of inhibitory and activating KIRs to favour induction of immune tolerance and the subsequent reduced risk of developing autoimmunity (24). MHC class I (particularly HLA-C) molecules are known to be ligands for specific KIRs (29). However, no ligand has been identified for KIR2DS5. It has been suggested that there is an association between HLA-C2 and protection against ankylosing spondylitis (24), but no evidence of an interaction between KIR2DS5 and any HLA class I molecule has be demonstrated. It may be that associations of KIR2DS5 and ITP are due to LD and actually reflect other KIR/HLA interactions (24). We have, however demonstrated that in adult ITP, the protective effect of KIR2DS5 is independent and not associated with LD or HLA-C allotype.

This study adds to the accumulating evidence that KIRS contributes to the pathogenesis of adult ITP. A pathogenetic model for the association of KIR genotype with ITP is unclear. KIR proteins are expressed in both NK and effector T-cells. The activation of these cells is controlled by a balance between a genetically determined repertoire of

activating and inhibitory KIRs (30). It has been demonstrated that in both cell types, activating KIRs are involved in direct cell-mediated cytotoxicity although through different mechanisms. Activating KIRs in NK cells results in the direct activation of the cell, however in cytotoxic $CD8^+$ T-cells, activating KIRS act as co-stimulatory receptors that boost the response to T-cell receptor (TCR) cross linking and ultimate cell activation (31).

In a ground-breaking study by Olsson *et al*, autologous T-cells from patients with active chronic ITP (but not from those with ITP in remission) were shown to mediate platelet destruction (3). By contrast, autologous NK cells were incapable of inducing direct platelet cytolysis. Moreover, the inhibitory KIR2DL3, KIR3DL2 and KIR3DL1 were up-regulated in the T-cells of patients with ITP in remission relative to those with active disease (3). Olsson selected the KIRs to be investigated by using $CD3^+$ T-cell microarray to investigate for preferential gene expression in 8 patients with active ITP, 6 patients with ITP in remission and 6 healthy controls. Although this technique did not identify KIR2DS5 as a potential protective candidate, the numbers were small and comprehensive KIR genotyping not performed. Similarly, our study was not designed to measure levels of gene expression within T- or NK-cells. Along these lines, it remains to be definitively established whether KIR2DL2/KIR2DS2 and KIR2DS5 genes influence T-cell-mediated platelet cytotoxicity. Alternatively, they may influence indirect destruction of autoantibody bound platelets by NK-cells. Although it is known that in-vitro KIR manipulation can modulate ADCC (32), the role of NK-cell-mediated ADCC in ITP has yet to be determined.

In summary, in a comparison of relevant healthy control volunteers and a tightly defined cohort of adult ITP patients, the presence of KIR2DS5 was independently associated with protection against ITP. By multivariable binary logistic regression to adjust for age, sex and the effects of other KIR genes, the compound genotype of KIR2DS2/2DL2 with KIR2DS5 abrogated the risk of KIR2DS2/2DL2 and the protective benefit of 2DS5. These findings shed new light on the immunobiology of adult persistent/chronic and relapsed ITP. Identification of the HLA ligand to KIR2DS5 will assist in establishing the mechanistic basis for these observations. Further studies are required to determine the impact of KIRS on ITP therapy.

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References

1. Abdel-Hamid SM, Al-Lithy HN. B Cell Activating Factor Gene Polymorphisms in Patients With Risk of Idiopathic Thrombocytopenic Purpura. Am J Med Sci. 2011 Jul;342(1):9–14.

2. Nourse JP, Lea R, Crooks P, Wright G, Tran H, Catalano J, et al. The KIR2DS2/2DL2 genotype is associated with adult persistent/chronic and relapsed immune thrombocytopenia independently of FCGR3a-158 polymorphisms. Blood Coagul Fibrinolysis. 2012 Jan;23(1):45–50.

3. Olsson B, Andersson P-O, Jernarings M, Jacobsson S, Carlsson B, Carlsson LMS, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Nat Med. 2003 Aug 24;9(9):1123–4.

4. Martin MP, Nelson G, Lee J-H, Pellett F, Gao X, Wade J, et al. Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. J Immunol Baltim Md 1950. 2002 Sep 15;169(6):2818–22.

5. Van der Slik AR, Koeleman BPC, Verduijn W, Bruining GJ, Roep BO, Giphart MJ. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. Diabetes. 2003 Oct;52(10):2639–42.

6. Nikitina-Zake L, Rajalingham R, Rumba I, Sanjeevi CB. Killer cell immunoglobulin-like receptor genes in Latvian patients with type 1 diabetes mellitus and healthy controls. Ann N Y Acad Sci. 2004 Dec;1037:161-9.

7. Momot T, Koch S, Hunzelmann N, Krieg T, Ulbricht K, Schmidt RE, et al. Association of killer cell immunoglobulin-like receptors with scleroderma. Arthritis Rheum. 2004 May;50(5):1561–5.

8. García-León JA, Pinto-Medel MJ, García-Trujillo L, López-Gómez C, Oliver-Martos B, Prat-Arrojo I, et al. Killer cell immunoglobulin-like receptor genes in Spanish multiple sclerosis patients. Mol Immunol. 2011 Sep;48(15–16):1896–902.

9. Jelčić I, Hsu KC, Kakalacheva K, Breiden P, Dupont B, Uhrberg M, et al. Killer immunoglobulin-like receptor locus polymorphisms in multiple sclerosis. Mult Scler J. 2012 Jul 1;18(7):951–8.

10. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CWG, Carrington M, Trowsdale J, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. J Exp Med. 2004 Oct 18;200(8):957–65.

11. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, et al. Differential Natural Killer Cell–mediated Inhibition of HIV-1 Replication Based on Distinct KIR/HLA Subtypes. J Exp Med. 2007 Nov 26;204(12):3027–36.

12. Martin MP, Gao X, Lee J-H, Nelson GW, Detels R, Goedert JJ, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet. 2002 Aug;31(4):429–34.

13. Suppiah V, Gaudieri S, Armstrong NJ, O'Connor KS, Berg T, Weltman M, et al. IL28B, HLA-C, and KIR Variants Additively Predict Response to Therapy in Chronic Hepatitis C Virus Infection in a European Cohort: A Cross-Sectional Study. PLoS Med. 2011;8(9)

14. Naumova E, Mihaylova A, Stoitchkov K, Ivanova M, Quin L, Toneva M. Genetic polymorphism of NK receptors and their ligands in melanoma patients: prevalence of inhibitory over activating signals. Cancer Immunol Immunother CII. 2005 Feb;54(2):172–8.

15. Stasi R. Pathophysiology and therapeutic options in primary immune thrombocytopenia. Blood Transfus Trasfus Sangue. 2011 Jul;9(3):262–73.

16. Hatjiharissi E, Xu L, Santos DD, Hunter ZR, Ciccarelli BT, Verselis S, et al. Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the FcγRIIIa-158 V/V and V/F polymorphism. Blood. 2007 Oct 1;110(7):2561–4.

17. George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, Ballem PJ, et al. Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. Blood. 1996 Jul 1;88(1):3–40.

18. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardization of Terminology, Definitions and Outcome Criteria in Immune Thrombocytopenic Purpura of Adults and Children: Report from an International Working Group. Blood. 2009 Mar 12;113(11):2386–93.

19. Keen-Kim D, Mathews CA, Reus VI, Lowe TL, Herrera LD, Budman CL, et al. Overrepresentation of rare variants in a specific ethnic group may confuse interpretation of association analyses. Hum Mol Genet. 2006 Nov 15;15(22):3324–8.

20. Ashouri E, Ghaderi A, Reed EF, Rajalingam R. A novel duplex SSP-PCR typing method for KIR gene profiling. Tissue Antigens. 2009 Jul;74(1):62–7.

21. Maxwell LD, Ross OA, Curran MD, Rea IM, Middleton D. Investigation of KIR diversity in immunosenescence and longevity within the Irish population. Exp Gerontol. 2004 Aug;39(8):1223–32.

22. Gourraud P-A, Meenagh A, Cambon-Thomsen A, Middleton D. Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. Immunogenetics. 2010 Sep 29;62(11-12):729–40.

23. Carrington M, Norman P. The KIR Gene Cluster [Internet]. 2003 [cited 2013 Feb 10]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK10134/#A27

24. Nowak I, Majorczyk E, Wiśniewski A, Pawlik A, Magott-Procelewska M, Passowicz-Muszyńska E, et al. Does the KIR2DS5 Gene Protect from Some Human Diseases? PLoS One. 2010 Aug 26;5(8).

25. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science. 2004 Aug 6;305(5685):872–4.

26. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. Immunology. 2010 Jan 1;129(1):8–19.

27. Howe EC, Wlodarski M, Ball EJ, Rybicki L, Maciejewski JP. Killer immunoglobulin-like receptor genotype in immune-mediated bone marrow failure syndromes. Exp Hematol. 2005 Nov;33(11):1357–62.

28. Van der Meer A, Schaap NPM, Schattenberg AVMB, van Cranenbroek B, Tijssen HJ, Joosten I. KIR2DS5 is associated with leukemia free survival after HLA identical stem cell transplantation in chronic myeloid leukemia patients. Mol Immunol. 2008 Aug;45(13):3631–8.

29. Rajagopalan S, Long EO. Understanding How Combinations of HLA and KIR Genes Influence Disease. J Exp Med. 2005 Apr 4;201(7):1025–9.

30. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. Immunity. 1997 Dec;7(6):753–63.

31. Mandelboim O, Kent S, Davis DM, Wilson SB, Okazaki T, Jackson R, et al. Natural killer activating receptors trigger interferon gamma secretion from T cells and natural killer cells. Proc Natl Acad Sci U S A. 1998 Mar 31;95(7):3798–803.

32. Borgerding A, Hasenkamp J, Engelke M, Burkhart N, Trümper L, Wienands J, et al. B-lymphoma cells escape rituximab-triggered elimination by NK cells through increased HLA class I expression. Exp Hematol. 2010 Mar;38(3):213–21.

Table 1: Distribution of genes amongst the cases and controls with accompanying unadjusted odds ratios.

gene	Case n=83 present (% of case)	Control n=106 present (% of controls)	odds ratio	95% CI	P value
KIR2DL2 (susceptibility)	50 (60.2%)	47 (44.3%)	1.90	(1.06 - 3.41)	0.030
KIR2DS2 (susceptibility)	50 (60.2%)	45 (42.5%)	2.05	(1.14 - 3.68)	0.015
KIR3DS1	34 (41.0%)	39 (36.8%)	1.19	(0.66 - 2.15)	0.559
KIR2DL1*	81 (97.6%)	106 (100.0%)	NA		0.192
KIR2DS5 (protective)	37 (44.6%)	88 (83.0%)	0.16	(0.08 - 0.32)	<0.001
KIR2DP1*	82 (98.8%)	103 (97.2%)	2.39	(0.24 - 23.39)	0.632
KIR2DL4 F/W	82 (98.8%)	106 (100.0%)	NA		0.439
KIR2DS3 (susceptibility)	31 (37.3%)	22 (20.8%)	2.28	(1.19 - 4.35)	0.012
KIR3DP1 (del)*# F/W	80 (96.4%)	104 (98.1%)	0.51	(0.08 - 3.14)	0.655
KIR3DP1 (undel)#	30 (36.1%)	27 (25.5%)	1.66	(0.89 - 3.10)	0.113
KIR2DS1	31 (37.3%)	50 (47.2%)	0.67	(0.37 - 1.20)	0.176
KIR2DL3	41 (49.4%)	61 (57.5%)	0.72	(0.40 - 1.28)	0.265
KIR2DL5 (protective)	51 (61.4%)	82 (77.4%)	0.47	(0.25 - 0.88)	0.017
KIR3DL1*	78 (94.0%)	102 (96.2%)	0.61	(0.16 - 2.35)	0.509
KIR2DS4 (undel)	32 (38.6%)	40 (37.7%)	1.04	(0.57 - 1.87)	0.908
KIR2DS4	69	96	0.51	(0.22 -	0.128

(del)			1.22)	
	(83.1%)	(90.6%)		
KIR3DL2* F/W	83 (100.0%)	105 (99.1%)	NA	1.000

* indicates situations where the Fisher's Exact Test was used to calculate the P value. In other cases, Pearson's Chi-squared test was used. F/W = Framework genes which are present in virtually all haplotypes. # Three of the six known KIR3DP1 alleles have deletions of exon 2 and part of its flanking introns 1 and 2 compared with the remaining three alleles. The genotyping method used was able to distinguish between the deleted and undeleted forms. KIR3DP1 in either the deleted or undeleted form is present in 100% of cases and controls.

Table 2: Test of association between presence and absence of KIR2DS2 in both cases and controls. KIR2DL2 and KIR2DS3 are significantly positively associated while KIR2DL5 is borderline significantly but negatively associated.

	KIR2DS2 absent (total = 94)		KIR2DS2 present (total = 95)		
KIR Gene	absent	present	absent	present	p-value
KIR2DL2	82 (87.2%)	12 (12.8%)	10 (10.5%)	85 (89.5%)	<0.001
KIR2DS3	87 (92.6%)	7 (7.4%)	49 (51.6%)	46 (48.4%)	<0.001
KIR2DL5	34 (36.2%)	60 (63.8%)	22 (23.2%)	73 (76.8%)	0.050
KIR2DS5	28 (29.8%)	66 (70.2%)	36 (37.9%)	59 (61.2%)	0.24

Table 3: Confidence intervals for the combinations of KIR2DS2/2DL2 compound genotype and KIR2DS5 demonstrates that the combination of KIR2DS2/2DL2 and KIR2DS5 largely negates the risk of KIR2DS2/2DL2 and the protective benefit of KIR2DS5 (adjusted for age and sex).

	Adjusted for Sex and Age		
	OR	95% CI	P-value
KIR2DS2/DL2-/2DS5-	1		
KIR2DS2/2DL2+ / 2DS5-	4.48	(1.17 - 17.15)	0.029
KIR2DS2/2DL2- / 2DS5+	0.24	(0.09 - 0.64)	0.004
KIR2DS2/2DL2+ / 2DS5+	0.40	(0.15 - 1.08)	0.069

Figure 1. Odds ratio of KIR genotypes in patients versus controls
Genetic associations of KIRs are shown as odds ratios with 95% CI. Those to the left of the central line confer protection over ITP and those to the right confer increased risk. Those conferring protection are KIR2DS5 (P<0.001) and KIR2DL5 (P=0.017) and those conferring risk include KIR2DS3 (P=0.012), KIR2DS2 (P=0.015) and KIR2DL2 (P=0.03).

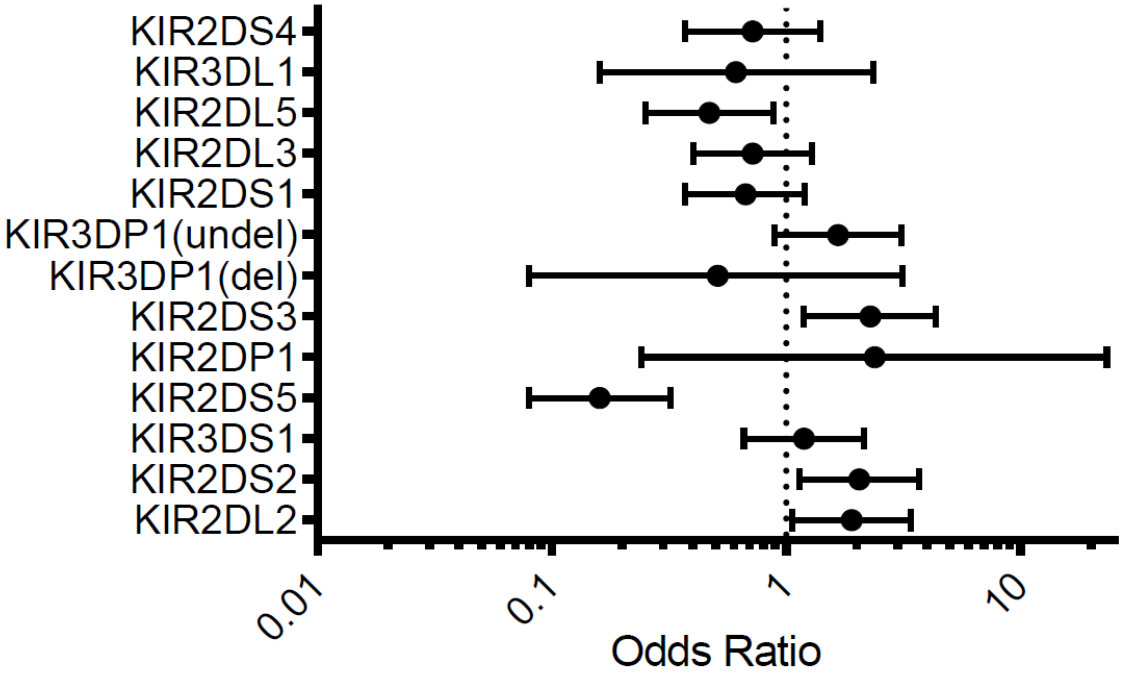

Figure 2. Linkage disequilibrium plot demonstrating LD between KIR subtypes.
The grey scale highlights the strength of the LD present between KIRs with the darkest grey demonstrating strong LD and the lighter grey, weak LD. LD estimates within the outlined regions are a result of pairwise LD comparisons to other KIR subtypes located in the same region. Those in the telomeric LD region (KIR2DS4, KIR2DS1, KIR2DS5, KIR3DS1, KIR3DL1) tend to be more highly associated with each other than to those on the centromeric LD region (KIR3DP1, KIR2DP1, KIR2DS3, KIR2DL2, KIR2DL3, KIR2DS2) and vice versa. KIR2DL4, KIR2DL1, and KIR3DL2 are not plotted, as they are present in at least 99% of subjects. Furthermore KIR2DL5 is not plotted as KIR2DL5A is on the telomeric region and KIR2DL5B is on the centromeric LD region.