CD8^{+} T CELLS IN IMMUNE THROMBOCYTOPENIA

THE CHARACTERIZATION OF $CD8^+$ T CELLS AS A POTENTIAL MECHANISM OF DISEASE IN IMMUNE THROMBOCYTOPENIA

By JOHN VRBENSKY, B.Sc. (Honours)

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McMaster University, Hamilton, Ontario, Canada

TITLE: The Characterization of $CD8^{+}$ T Cells as a Potential Mechanism of Disease in Immune Thrombocytopenia

AUTHOR: John Vrbensky, B.Sc. (Honours), McMaster University

SUPERVISORS: Dr. Ishac Nazy

Dr. Donald Arnold

Dr. John Kelton

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Lay Abstract

Platelets are small blood cells that are involved in minimizing blood loss at the site of a wound by forming a plug. In a disease called immune thrombocytopenia (ITP), patients have a low platelet count, which can result in bleeding. The bleeding symptoms of ITP decrease the quality of life for ITP patients and can be life-threatening in rare cases. It is believed that ITP is caused by proteins produced by the immune system called antibodies. I found that the antibodies that cause ITP can only be detected in half of all ITP patients. Therefore, there are probably additional causes of ITP. It is suspected that $CD8^+$ T cells might cause ITP in some patients. $CD8^+$ T cells are part of the immune system and they typically destroy other cells that are cancerous or infected by viruses. $CD8^+$ T cells might also destroy healthy cells, like platelets. My goal was to characterize $CD8^+$ T cells in order to determine their role in ITP. I found that $CD8^+$ T cells from ITP patients can target platelets, and that healthy people have these $CD8^+$ T cells as well. In regard to $CD8^+$ T cells that target platelets, the difference between ITP patients and healthy people appears to be related to immune system regulation and $CD8^+$ T cell activity. In the future, we should focus on understanding how platelet-specific $CD8^+$ T cells can cause a low platelet count in order to improve the clinical management of ITP.

Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by a low platelet count (less than 100 x 10^9 platelets/L) and an increased risk of bleeding. ITP is difficult to diagnose and manage due to the deficiencies in our understanding of the pathophysiological mechanisms leading to thrombocytopenia. Anti-platelet autoantibodies are believed to be the primary mechanism of thrombocytopenia in ITP. In this thesis, I demonstrate that autoantibodies can only be detected in half of all ITP patients; therefore, other mechanisms should be investigated. $CD8^+$ T cells have been implicated as a mechanism of disease in ITP, but platelet-specific $CD8^+$ T cells have yet to be identified. I have characterized $CD8^+$ T cells in ITP patients and found that platelet-specific $CD8^+$ T cells can be detected in ITP patients. These platelet-specific $CD8^+$ T cells can also be detected in healthy individuals, so they are not specific to ITP. However, regulatory defects were observed in ITP patients and $CD8^+$ T cell activity was elevated in ITP patients relative to healthy individuals and thrombocytopenic non-ITP patients. Investigating whether platelet-specific $CD8^+$ T cells can actively participate in platelet destruction and underproduction will be an essential step towards better understanding the role of $CD8^+$ T cells as a disease mechanism in ITP, which will lead to improvements in the management of ITP.

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List of Abbreviations and Symbols

AB: A and B antigens

ABT-737: a proapoptotic small molecule

ACA: antigen capture assay

ACK: ammonium-chloride-potassium

AIHA: autoimmune hemolytic anemia

AITP: autoimmune thrombocytopenic purpura

APC: antigen presenting cell, or allophycocyanin

APC Cy7: allophycocyanin cyanine fluorophore conjugate

AS: ankylosing spondylitis

ASA: aspirin

ASH: American Society of Hematology

AZA: azathioprine

BD: Becton Dickinson

BMMNCs: bone marrow mononuclear cells

BV: brilliant violet

CCCP: carbonyl cyanide 3-chlorophenylhydrazone

CD: cluster of differentiation

CFSE: carboxyfluorescein succinimidyl ester

CI: confidence interval

c-Mpl: TPO receptor

CSP: cyclosporine

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

CXCL: chemokine ligand

CXCR: chemokine receptor

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

ddH2O: double distilled water

DEX: dexamethasone

DNA: deoxyribonucleic acid

EBV: Epstein-Barr virus

ELISA: enzyme-linked immunosorbent assay

ELISpot: enzyme-linked immunospot

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FLICA: fluorochrome inhibitor of caspase

FMO: fluorescence minus one

FSC: forward scatter

GP: glycoprotein

HC: healthy control

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

ITP: immune thrombocytopenia

ID: identification

Ig: immunoglobulin

IL: interleukin

IFN: interferon

IVIg: intravenous immunoglobulin

JC-1: a mitochondrial membrane potential probe

m: membrane-bound

mAb: monoclonal antibody

MACE: modified antigen capture ELISA

MAIPA: monoclonal antibody-specific immobilization of platelet antigen

MHC: major histocompatibility complex

MMF: mycophenolate mofetil

MP: mycophenolate

mRNA: messenger ribonucleic acid

Neu: neuraminidase

NK: natural killer

Non-ITP: non-immune thrombocytopenia

OD: optical density

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PE: phycoerythrin

PECy5: phycoerythrin cyanine fluorophore conjugate

PPP: platelet poor plasma

PRED: prednisone

PRP: platelet rich plasma

P/S: penicillin/streptomycin

PS: phosphatidylserine

RITUX: rituximab

RPMI: Roswell Park Memorial Institute media

s: soluble

SD: standard deviation

SSC: side scatter

T1D: type 1 diabetes

TC: thrombocytopenic control (with non-ITP)

TCR: T cell receptor

Tc/T_C: cytotoxic T cell

TGF: transforming growth factor

Th/T_H: helper T cell

TPO: thrombopoietin

T_{reg}: T regulatory cell

Ts cell: T suppressor cell

TT: tetanus toxoid

TXA: tranexamic acid

VCAM: vascular cell adhesion molecule

VLA: very late antigen

WARF: warfarin

α: alpha

β: beta

γ: gamma

δ: delta

κ: kappa

^{3}H: tritium

I^{2}: inconsistency

M: male

F: female

n: number

°: degree

%: percent

<: less than

>: greater than

±: plus or minus

=: equal

µM: micromolar

g: gram

µg: microgram

ng: nanogram

pg: picogram

U: units

p: probability

R^2: square of the correlation coefficient

R: receptor

L: ligand or litre

mL: millilitre

μL: microlitre

ΔΨm: mitochondrial membrane depolarization

Declaration of Academic Achievement

John Vrbensky takes primary responsibility for the content of this thesis and its associated publications. John Vrbensky designed and conducted the experiments, collected, analyzed, and interpreted the data, performed statistical analysis, created the figures and tables, wrote, edited, and finalized the thesis chapters. The contributions from co-authors are described in further detail within each chapter.

CHAPTER 1 (INTRODUCTION)

T Cell-Mediated Autoimmunity in Immune Thrombocytopenia

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Author's Preface

$CD8^+$ T cells have been implicated in the pathogenesis of ITP and other autoimmune disorders. This chapter reviews our current understanding of the role of $CD8^+$ T cells in ITP.

The goals of chapter 1 are to:

1) Demonstrate that platelets are potential targets of $CD8^+$ T cells.
2) Summarize the findings from studies that have investigated $CD8^+$ T cells in ITP.
3) Review the laboratory methods that have been used to detect autoreactive $CD8^+$ T cells in other autoimmune diseases.

This chapter demonstrates that our understanding of the role of $CD8^+$ T cells in ITP is limited since platelet-specific $CD8^+$ T cells have not yet been discovered. The laboratory methods used to detect autoreactive $CD8^+$ T cells in other autoimmune diseases will be useful to detect platelet-specific $CD8^+$ T cells in ITP, which is the main goal of Chapter 4.

Note to the reader: this chapter contains references to the publications from chapters 2 and 3, which were published before chapter 1.

Abstract

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count and an increased risk of bleeding. In addition to anti-platelet autoantibodies, $CD8^+$ T cells have been implicated as a mechanism of platelet destruction. The current evidence for the existence of platelet-specific $CD8^+$ T cells in ITP is inconclusive. The purpose of this review is to summarize the studies that investigated $CD8^+$ T cells in ITP and to review the methods that have been used to detect autoreactive $CD8^+$ T cells in other autoimmune diseases.

Introduction

In patients with immune thrombocytopenia (ITP), the circulating platelet count falls below 100 x 10^9 platelets/L, which can cause hemostatic impairment and an increased propensity for bleeding [1]. An immune response towards platelet autoantigens results in premature platelet destruction in the spleen and platelet underproduction from bone marrow megakaryocytes [2]. The destruction and underproduction of platelets are thought to be mediated by autoantibodies that target platelet glycoproteins (GP); mainly GPIIbIIIa and GPIbIX. In a systematic review and meta-analysis, we showed that the most sensitive direct assays that measure autoantibodies on the platelet surface detect anti-GPIIbIIIa or anti-GPIbIX in only 53% of ITP patients [3]. This suggests that other mechanisms besides autoantibodies can contribute to the low platelet count in ITP. One proposed immune mechanism of disease in ITP involves $CD8^+$ T cells [4]. It

remains uncertain whether autoantibodies and CD8$^+$ T cells represent two mutually exclusive or complementary mechanisms of ITP (Figure 1). The objective of this review is to summarize the studies which have examined the role of CD8$^+$ T cells in ITP. We will also discuss other autoimmune diseases in which autoantigen specific CD8$^+$ T cells have been observed in order to improve our understanding of CD8$^+$ T cells as a potential disease mechanism in ITP.

Platelets as Potential Targets of CD8$^+$ T Cells

CD8$^+$ T Cell Activation and Target Cell Interaction

CD8$^+$ T cells are responsible for eliminating transformed and virally infected cells, and they can also be autoreactive. Autoreactive T cells can be generated through random T cell receptor (TCR) gene rearrangement towards any combination of self-peptide and major histocompatibility complex (MHC), but these autoreactive T cells are normally deleted or suppressed in healthy individuals by central and peripheral tolerance mechanisms [5]. In order for a CD8$^+$ T cell to recognize a target cell, the antigen target must first be processed by antigen-presenting cells (APC) and target cells into short peptide fragments. APCs collect antigens from the local microenvironment, and in healthy cells the peptides are derived from endogenous proteins that are degraded during normal protein turnover. Peptides are displayed by MHC class I and II on the cell surface for recognition by CD8$^+$ and CD4$^+$ T cells, respectively. MHC class I is expressed on all nucleated cells and presents peptides 8–10 amino acids in length.

MHC class II can accommodate peptides 13–25 amino acids long, and it is expressed by professional APCs (dendritic cells, macrophages, and B cells) as well as non-professional APCs such as epithelial cells in the gut and lung [6, 7].

At any given time, only a subset of all $CD8^+$ T cells are activated. The process of $CD8^+$ T cell activation requires three signals: 1) antigen presentation from the APC in the context of MHC I, which is recognized by the TCR, 2) co-stimulation (CD80 or CD86 on the APC binding to CD28 on the T cell), and 3) activating cytokines (e.g. IL-12 and IFNα/β) [8]. After activation, effector $CD8^+$ T cells have the ability to destroy target cells expressing cognate peptide-MHC I complexes without co-stimulation. Once an effector $CD8^+$ T cell recognizes a target cell, the $CD8^+$ T cell induces target cell apoptosis through the release of cytotoxic granules containing perforin and granzyme or through the ligation of death receptors [9-11]. In apoptotic cells, caspases are activated, phosphatidylserine (PS) gets flipped to the outer surface of the cell, and the mitochondrial membrane potential becomes depolarized. The final stage of apoptosis in nucleated cells is characterized by DNA fragmentation into 200 base pair segments [12].

Platelets Contain Apoptotic Machinery and Express MHC I

Even though platelets are anucleate, they contain apoptotic machinery and can undergo apoptotic processes [13]. Various apoptotic events that can be triggered within platelets include caspase activation, surface exposure of PS, and

the loss of mitochondrial membrane potential [14, 15] (Figure 2). These events can be measured by flow cytometry using fluorochrome inhibitor of caspase (FLICA) probes, Annexin V, and JC-1, respectively [12, 16]. Platelet apoptosis can be triggered artificially *in vitro* with calcium ionophore, ABT-737, high dose thrombin (10-100 nM), or carbonyl cyanide 3-chlorophenylhydrazone (CCCP) [13, 17, 18].

Platelets contain MHC class I mRNA, and thus they are capable of *de novo* MHC I biosynthesis and surface expression [19]. In addition, platelets have the components required for antigen processing including an active proteasome, endoplasmic reticulum, Golgi apparatus, transporter associated with antigen processing, calnexin, and calreticulin. Furthermore, platelets can phagocytose full-length soluble protein then process and cross-present the peptide fragments on MHC I [20]. Platelets also express accessory molecules involved in T cell activation such as CD86, CD40, and DC-SIGN [20-22]. Platelets obtain the cellular components that allow them to interact with T cells from megakaryocytes, which have been shown to act as APCs in a murine model of ITP [23]. Since platelets are able to process and present antigen in the context of MHC I, it is possible for them to interact with $CD8^{+}$ T cell receptors.

MHC I is encoded by three human leukocyte antigen (HLA) genes, HLA-A, HLA-B, and HLA-C, which can increase an individual's susceptibility for autoimmunity. For example, ankylosing spondylitis (AS) is an inflammatory arthritis that is strongly associated with human leukocyte antigen (HLA)-B27

[24]. Approximately 90% of patients with AS possess HLA-B27 [25]. Accordingly, autoreactive $CD8^+$ T cells that are HLA-B27-restricted have be found in patients with AS [26]. In contrast, there are no clear associations between ITP and HLA-A, B, or C. Some studies have reported an increased frequency of certain HLA genes in ITP patients, including HLA-B8 and HLA-B12 [27], HLA-A28 [28], and HLA-A2 [29]; however, most studies were unable to find an association with HLA-A, B, or C in ITP [30-34]. Even though an individual's HLA repertoire does not appear to predispose them to ITP, it defines the peptides that are presented within MHC complexes, and therefore specifies the potential T cell response to platelet antigens. In a study by Hopkins *et al*, platelet peptides associated with MHC I were eluted, and the 9 amino acid sequence GPRGALSLL from GPIbβ was detected from 4 of 5 ITP patients. These ITP patients were not pre-selected based on their HLA type, yet all 4 patients had the HLA-B7 allele in common. The sequence GPRGALSLL from GPIbβ was also detected in 2 out of 3 pre-selected HLA-$B7^+$ healthy individuals but not on any HLA-$B7^-$ individuals, suggesting that the presentation of GPIbβ on platelet MHC I is associated with HLA-B7 even though it is not specific for ITP [35]. The presence of MHC I-associated GPIbβ peptide on the platelet surface suggests that autoreactive GPIbβ-specific $CD8^+$ T cells might be present in HLA-$B27^+$ individuals.

Thus, platelets are equipped with the cellular components required for $CD8^+$ T cells to recognize and destroy them by apoptosis. In the following

section, we summarize and evaluate the current evidence for $CD8^+$ T cell-mediated platelet destruction in ITP.

Evidence for $CD8^+$ T Cell-Mediated Platelet Destruction in ITP

In a study by Olsson *et al*, a DNA microarray screen demonstrated that $CD3^+$ T cells from patients with active ITP had elevated expression of cytotoxic genes such as perforin and granzymes A and B. They also demonstrated that exogenously stimulated $CD8^+$ T cells from active ITP patients induced autologous platelet lysis, as measured by the release of indium-111 (^{111}In) from radiolabeled platelets. These results suggested that $CD8^+$ T cells play a role in immune-mediated platelet destruction in ITP [4]. In subsequent studies, a similar approach was employed by incubating platelets from ITP patients with autologous $CD8^+$ T cells which were triggered to degranulate with anti-CD3. Several studies measured platelet apoptosis through the binding of Annexin V to exposed PS, or through mitochondrial membrane depolarization using JC-1. One of these studies supported the observations made by Olsson *et al* by demonstrating that more platelets expressed phosphatidylserine when incubated with exogenously stimulated $CD8^+$ T cells from ITP patients compared to $CD8^+$ T cells from controls and compared to platelets alone [36]. Another study showed that in the presence of stimulated $CD8^+$ T cells, platelet apoptosis was elevated in the autoantibody negative subset of ITP patients compared to autoantibody positive ITP patients, which might suggest that autoantibodies and $CD8^+$ T cells represent mutually exclusive mechanisms of platelet destruction in ITP [37]. It was also

shown that dexamethasone [37] or IL-27 [38] could prevent platelet apoptosis in the presence of autologous $CD8^+$ T cells, implying that $CD8^+$ T cell-mediated platelet destruction can be attenuated. Recently, $CD8^+$ T cell-mediated platelet apoptosis was inhibited by the cytidine antimetabolite analogue decitabine. However, the platelets used in this study were sourced from healthy donors, so the significance of this result is uncertain since it was not an autologous approach [39].

It has been suggested that $CD8^+$ T cells can induce platelet desialylation, the process through which terminal sialic acids are cleaved from the platelet surface, and can thereby promote platelet clearance in ITP [40]. $CD8^+$ T cells express the sialidases Neu1 and Neu3 [41]. In the presence of autologous $CD8^+$ T cells, platelets showed signs of lysis, apoptosis, and desialylation, both *in vitro* and in a CD61 knockout murine model of ITP, in which the desialylated platelets were subsequently cleared by the liver [40]. The evidence for $CD8^+$ T cell-mediated platelet destruction in ITP is summarized in Table 1.

Limitations

The studies discussed above provide no evidence for the existence of autoreactive platelet-specific $CD8^+$ T cells in ITP. $CD8^+$ T cells were exogenously stimulated using anti-CD3 [4, 36-40] which results in degranulation regardless of TCR specificity and without the typical prerequisite of target cell recognition [42, 43] (Figure 3A). Cells treated with soluble perforin and granzyme can undergo

apoptosis [44]. Therefore, it is unlikely that the results from these studies were due to the effects of platelet-specific $CD8^+$ T cells. We propose that a similarly designed study could demonstrate platelet-specificity of $CD8^+$ T cells by co-incubating autologous platelets and $CD8^+$ T cells from ITP patients without artificially stimulating the T cells with anti-CD3 (Figure 3B).

Other $CD8^+$ T Cell Abnormalities in ITP

Several studies have demonstrated various abnormalities with $CD8^+$ T cells in ITP patients. Recently, we showed that the cytotoxic potential of $CD8^+$ T cells is elevated in a subset (36%) of ITP patients. In response to exogenous TCR stimulation with anti-CD3, more $CD8^+$ T cells from ITP patients expressed CD107a, a marker of degranulation, compared to $CD8^+$ T cells from healthy individuals with normal platelet counts [43]. These results suggest that $CD8^+$ T cell priming occurs to a greater extent in ITP patients compared to healthy individuals. The increased cytotoxic potential of $CD8^+$ T cells in ITP might explain why platelets from ITP patients were destroyed or desialylated to a greater extent in previous studies that stimulated $CD8^+$ T cells with anti-CD3, since artificially triggered degranulation would lead to a greater concentration of perforin and granzyme in the ITP group compared to the healthy group. Evidence of this increased cytotoxic potential in ITP is consistent with an earlier study which measured circulating granzyme A and B levels in ITP patients. In this study, it was found that both granzyme A and B were elevated in the plasma of ITP patients compared to healthy controls, suggesting that there is a greater extent

of cell-mediated cytotoxicity occurring in ITP patients [45]. Therefore, $CD8^+$ T cells are highly active and capable of cytotoxicity in ITP, but it is not entirely evident that $CD8^+$ T cell cytotoxicity is directed towards platelets.

Other studies investigating $CD8^+$ T cells in ITP have found abnormalities in the number and function of $CD8^+$ T cell subsets. One example of a $CD8^+$ T cell subset that was recently studied is the $CD8^+CD28^-$ T suppressor cell (Ts cell), which tolerizes APCs and secretes immunosuppressive cytokines such as IL-10 and TGF-β. In patients with active ITP, Ts cells were present at a lower frequency compared to healthy individuals. The Ts cells from ITP patients were less capable of suppressing $CD4^+$ and $CD8^+$ T cell proliferation and inducing tolerogenic APCs, which might partially explain the lack of immune tolerance to platelet autoantigens in ITP [46]. In another study, the frequencies of two other $CD8^+$ T cell subsets, Tc1 and Tc17 cells, were measured. Tc1 cells are the 'classic' cytotoxic lymphocytes, capable of exerting cytotoxic effects on target cells and producing interferon γ (IFN-γ), while Tc17 cells produce IL-17 and do not destroy target cells. It was found that the frequency of both the Tc1 and Tc17 subsets were elevated in ITP patients compared to controls [47], although a platelet-specific response was not investigated.

$CD8^+$ T Cells at Sites of Platelet Destruction and Production

$CD8^+$ T cell abnormalities have been observed at potential sites of platelet destruction such as the spleen where opsonized platelets are cleared, or in the

bone marrow where platelets are produced. In one study, splenic $CD8^+$ T cells were examined in ITP patients who required a splenectomy after failing to respond to rituximab, the anti-CD20 monoclonal antibody that depletes B cells [48]. The authors hypothesized that a lack of response to rituximab was due to the actions of splenic $CD8^+$ T cells. The frequency of granzyme B^+, IFN-γ^+, and $CD27^-CD28^-$ $CD8^+$ T cells were increased in rituximab non-responders compared to patients not treated with rituximab and with splenectomized controls, which is consistent with an activated $CD8^+$ T cell effector memory phenotype in rituximab non-responders. Rituximab non-responders also exhibited an oligoclonal TCR Vβ repertoire, which shows that they have a population of $CD8^+$ T cells with a more restricted set of TCRs that recognize specific antigens, however there was no direct evidence that these restricted TCRs were platelet specific [48]. Regulatory T cell deficiencies in number and suppressive function have been noted in ITP patients, and these deficiencies were corrected in rituximab responders [49]. Another study found that bone marrow $CD8^+$ T cells from ITP patients express a greater level of the trafficking markers VLA-4 and CX3CR1 compared to healthy individuals. VLA-4 (integrin α4β1) binds to vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells during inflammatory conditions and promotes lymphocyte extravasation. CX3CR1 is the receptor for fractalkine (CX3CL1), a chemoattractant chemokine. The increased expression of these lymphocyte trafficking markers appeared to be associated with $CD3^+$ cell accumulation in the

bone marrow [50]. However, platelet or megakaryocyte specificity of $CD8^+$ T cells was not investigated.

In another study, $CD8^+$ T cells were examined in the bone marrow and the authors hypothesized that $CD8^+$ T cells from ITP patients were capable of destroying megakaryocytes and thereby inhibiting thrombopoiesis [51]. Bone marrow mononuclear cells (BMMNCs) were isolated and cultured in different groups: BMMNCs alone, BMMNCs depleted of $CD8^+$ T cells, and BMMNCs supplemented with $CD8^+$ T cells. Megakaryocyte count, megakaryocyte apoptosis, and platelet production were measured in these different conditions. Compared to non-hematological control patients, ITP patients had an increased number of bone marrow megakaryocytes at baseline, with decreased platelet production and a decreased frequency of apoptotic megakaryocytes. As the number of $CD8^+$ T cells increased in the ITP bone marrow cultures, the number of megakaryocytes also increased, while platelet production and the frequency of apoptotic megakaryocytes decreased. This suggested that $CD8^+$ T cells in ITP patients suppressed megakaryocyte apoptosis, which promoted megakaryocytopoiesis while paradoxically preventing thrombopoiesis [51]. However, megakaryocytes in the later stages of apoptosis (Annexin V^+ Propidium $Iodide^+$) may not have been accounted for. This study also provided the most substantial evidence for $CD8^+$ T cell mediated pathology in ITP to date by looking for platelet-specific $CD8^+$ T cells. The approach involved culturing $CD8^+$ T cells with irradiated PBMCs to act as APCs, and with autologous platelets as a source

of platelet antigens. $CD8^+$ T cell proliferation was measured with ^{3}H-thymidine. $CD8^+$ T cells from ITP patients proliferated vigorously after incubation with APCs and autologous platelets, while the same extent of proliferation did not occur in controls, suggesting that ITP patients have platelet-specific $CD8^+$ T cells [51]. However, since the background level of proliferation was not reported in this study, it is unclear whether these platelet-specific $CD8^+$ T cells also exist in healthy individuals or not. This study did not include a thrombocytopenic control group. Investigations of the mechanisms of disease in ITP would benefit from a disease control group in order to better dissect the pathophysiological differences between ITP and non-ITP. Another consideration of this study by Li *et al* is that whole platelets were used as a source of platelet antigens; therefore in addition to platelets, soluble proteins, viruses, and other pathogens ingested by platelets may have triggered the proliferation of $CD8^+$ T cells that were not platelet-specific [52-54].

Autoantigen-Specific $CD8^+$ T Cells in Other Autoimmune Diseases

Type 1 diabetes (T1D) is a disease in which insulin production is reduced due to an autoimmune attack towards insulin-producing β cells in the pancreas [55]. $CD8^+$ T cells specific for β cell autoantigens such as insulin have been detected in the pancreas from patients with T1D [56], and preproinsulin-specific $CD8^+$ T cell clones isolated from type 1 diabetics can destroy β cells from HLA-matched organ donors [57]. The immunological techniques used to detect

autoreactive $CD8^+$ T cells in patients with T1D will be useful for detecting autoreactive $CD8^+$ T cells in ITP, and these techniques will be discussed herein.

There are four methods that are used frequently to detect autoantigen-specific $CD8^+$ T cells: the enzyme-linked immunospot (ELISpot) assay, MHC tetramers, activation markers, and proliferation-based assays (Figure 4). In an ELISpot, PBMCs are stimulated with autoantigen-derived peptides, triggering the secretion of a cytokine such as IFN-γ from autoreactive $CD8^+$ T cells, which is captured on a plate coated with anti-IFN-γ. The endpoint of an ELISpot is a coloured spot that forms on the plate through an enzyme-substrate reaction which indicates the presence of an antigen-specific T cell [58]. It can be inferred that the spots are derived from $CD8^+$ T cells by the depletion of $CD8^+$ T cells, by adding monoclonal antibodies that block CD8 or MHC I, or by stimulating the PBMC culture with MHC I-restricted peptides [59]. MHC tetramers consist of four biotinylated complexes of MHC class I, β_2-microglobulin, and a synthetic peptide of interest. The biotinylated MHC-peptide monomers are tetramerized with streptavidin to increase their avidity for TCRs. The streptavidin is conjugated to a fluorophore, which allows for cell identification by flow cytometry. With this method, the peptide of interest must be identified beforehand, usually for its ability to bind to the MHC class I binding groove, and individuals whose T cells are assessed with tetramers must be HLA-typed prior to testing. Since MHC tetramers only bind to TCRs that recognize a specific peptide-MHC combination, MHC tetramers can be used to identify T cells specific to any antigen of interest

[60]. Activation markers such as CD69, CD137, and CD154 are upregulated on the surface of activated $CD4^+$ and $CD8^+$ T cells and they can be detected using flow cytometry. These activation markers can be used to detect antigen-specific T cells after a relatively short *in vitro* stimulation (<24 hours) with the antigen of interest [61]. In proliferation assays, peptides are presented to T cells by APCs, and proliferation indicates the presence of a T cell with a TCR that is specific to the target antigen. Proliferation can be measured several ways, most commonly through the dilution of carboxyfluorescein succinimidyl ester (CFSE) or by the incorporation of ^{3}H-thymidine [62]. CFSE-based assays can be used in combination with monoclonal antibodies in order to differentiate between $CD4^+$ and $CD8^+$ T cell proliferation in PBMC cultures.

Together, ELISpots, MHC tetramers, activation markers, and proliferation assays have been used to identify an array of autoreactive $CD8^+$ T cell target antigens in autoimmune diseases, including melanocyte-derived autoantigens in vitiligo [63, 64] and numerous pancreatic autoantigens in T1D (including insulin, preproinsulin, islet amyloid polypeptide precursor protein, glutamic acid decarboxylase, and islet-specific glucose-6-phosphatase catalytic subunit-related protein) [59, 65-68]. In ITP, proliferation assays have been employed to detect GPIIbIIIa specific $CD4^+$ T cells and to generate $CD4^+$ T cell lines from ITP patients that responded to GPIIbIIIa [69, 70].

Employing the techniques discussed above may translate into improvements in our understanding of ITP, since the identification of autoreactive

T cells has had clinical implications for T1D. For example, monitoring autoantigen-specific $CD4^+$ T cell proliferation and MHC $tetramer^+$ $CD8^+$ T cells identified patients with T1D who would continue to require insulin after islet transplantation [68]. Therapies that are commonly used to treat ITP patients can have direct effects on T cells. For example, dexamethasone induces the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which downregulates T cell activation [71]. We postulate that by measuring anti-platelet autoantibodies and platelet-specific T cells simultaneously in ITP patients, clinical responses to therapy might be predicted and our understanding of the mechanisms of ITP will be improved.

Conclusions

ITP is a heterogenous autoimmune disease characterized by immune mediated platelet destruction and underproduction. Platelets can interact with $CD8^+$ T cell receptors through MHC I, and they can also undergo apoptosis; thus, platelets are a potential target for $CD8^+$ T cells. Numerous studies have shown that $CD8^+$ T cells from ITP patients can be artificially triggered to destroy platelets, and several $CD8^+$ T cell abnormalities have been observed in ITP patients. However, our understanding of the role of $CD8^+$ T cells in ITP is limited since direct evidence of platelet-specific $CD8^+$ T cells is lacking. Our summary of the laboratory methods used to detect autoantigen-specific $CD8^+$ T cells in other autoimmune diseases provides a foundation for future efforts to identify platelet-specific $CD8^+$ T cells. Once platelet-specific $CD8^+$ T cells are identified, an

evaluation of their ability to destroy platelets and megakaryocytes will be a significant step towards understanding their role in ITP. The investigation of $CD8^+$ T cell-mediated megakaryocyte destruction should be a high priority since each megakaryocyte produces thousands of platelets. Therefore, it will be important to investigate $CD8^+$ T cells derived from the bone marrow of ITP patients. Platelet-specific $CD8^+$ T cell immunophenotyping will also provide valuable insights since $CD8^+$ T cell subsets (e.g. $T_c1/T_c2/T_c17$) produce unique cytokines with varying effects on megakaryocyte maturation and platelet production. It will also be important to assess changes in the characteristics of platelet-specific $CD8^+$ T cells in response to immunosuppressive agents that are commonly used to treat ITP. In the future, the discovery of platelet-specific $CD8^+$ T cells will advance our understanding of the underlying mechanisms of ITP which will ultimately lead to improved treatment strategies.

Tables

Table 1: The Evidence in Support of CD8$^+$ T Cells as a Mechanism of Platelet Destruction in ITP

<table>
<tr><th>Observation</th><th>Compared To</th><th>Reference(s)</th><th>Implication</th><th>Commentary</th></tr>
<tr><td>Exogenously stimulated CD8$^+$ T cells from active ITP patients induced enhanced destruction of autologous platelets.</td><td>Healthy individuals

ITP in remission (reference 4 only)</td><td>4, 36, 40</td><td>CD8$^+$ T cell mediated platelet apoptosis is a potential mechanism of platelet destruction.</td><td rowspan="4">In all of these studies, the CD8$^+$ T cells were exogenously stimulated with anti-CD3, which induces degranulation (the release of cytotoxic granule contents) while bypassing the usual prerequisite of ligation between TCRs and peptide-MHC I complexes.

Therefore, it is unclear whether or not these CD8$^+$ T cell observations were platelet-specific.</td></tr>
<tr><td>Platelet destruction by exogenously stimulated CD8$^+$ T cells was greatest in autoantibody negative ITP patients.</td><td>Autoantibody positive ITP patients

Healthy individuals</td><td>37</td><td>CD8$^+$ T cells are a driving mechanism of thrombocytopenia in ITP patients who do not have anti-platelet autoantibodies.</td></tr>
<tr><td>Exogenously stimulated CD8$^+$ T cells from active ITP patients induced enhanced desialylation of autologous platelets.</td><td>Healthy individuals</td><td>40</td><td>Platelet desialylation can be induced by CD8$^+$ T cells, which leads to platelet clearance in the liver.</td></tr>
<tr><td>Dexamethasone, IL-27, or decitabine can prevent platelet destruction mediated by exogenously stimulated CD8$^+$ T cells.</td><td>No treatment/ buffer</td><td>37-39</td><td>CD8$^+$ T cell mediated platelet destruction can be counteracted.</td></tr>
</table>

Figures Legends

Figure 1: The Immunopathogenesis of ITP. Autoantibodies specific to platelet glycoproteins (primarily GPIIbIIIa and GPIbIX) promote platelet clearance by splenic macrophages and by inducing platelet desialylation, and they can bind to megakaryocytes in the bone marrow. Anti-platelet autoantibodies are detectable in only 53% of ITP patients. $CD8^+$ T cells may be responsible for platelet destruction by inducing apoptosis or desialylation, and they may also promote platelet underproduction by targeting megakaryocytes. However, platelet-specific $CD8^+$ T cells have yet to be identified so their role in ITP remains uncertain.

Figure 2: Platelets Possess the Machinery Required to be Monitored and Destroyed by $CD8^+$ T Cells. $CD8^+$ T cells induce apoptosis of their target cells after they recognize cognate peptide-MHC I complexes. Platelets express MHC I on the cell surface and they contain other machinery necessary for antigen processing and presentation. Although they are anucleate and therefore cannot undergo DNA fragmentation (the hallmark of apoptosis in nucleated cells), platelets can undergo other cellular processes that occur during apoptosis such as caspase activation, mitochondrial membrane depolarization ($\Delta\Psi m$), and phosphatidylserine (PS) exposure. Therefore, platelets have the capacity to interact with and be destroyed by $CD8^+$ T cells.

Figure 3: Platelets as Potential Targets of CD8$^+$ T Cell-Mediated Cytotoxicity in ITP. A) CD8$^+$ T cells stimulated with anti-CD3 release their cytotoxic granule contents, regardless of the specificity of their TCR and without the prerequisite engagement between the TCR and MHC I-peptide complex on the target cell. So far, all of the studies that have investigated the interaction between CD8$^+$ T cells and platelets in ITP have used this approach, which is not platelet-specific. B) Autoreactive CD8$^+$ T cells can destroy target cells following the engagement of the TCR and MHC I-peptide complex, without the need for exogenous stimulation with anti-CD3. Genuinely autoreactive CD8$^+$ T cells with the capacity to destroy platelets or megakaryocytes have not yet been detected in ITP.

Figure 4: Methods to Detect Autoantigen-Specific CD8$^+$ T Cells. The techniques depicted in this figure will be useful to detect autoreactive CD8$^+$ T cells in ITP. mAb: monoclonal antibody; MHC: major histocompatibility complex; TCR: T cell receptor. A) The ELISpot assay indicates the presence of an autoreactive T cell by the presence of a coloured spot that forms as a result of an enzyme-substrate reaction. B) MHC tetramers contain a specific peptide of interest. The streptavidin molecule used for tetramerization of the peptide-MHC complexes is labelled with a fluorescent marker. MHC tetramers directly bind to TCRs with a high degree of specificity. C) Activation markers can be detected on stimulated T cells using flow cytometry. Some examples of activation markers include CD69, CD137, and CD154. D) In proliferation assays, autoreactive T

cells proliferate in response to the presentation of autoantigens. T cells are labelled with CFSE and the dye is distributed between daughter cells after each round of cell division (^{3}H-thymidine assay not shown).

Figures

Figure 1

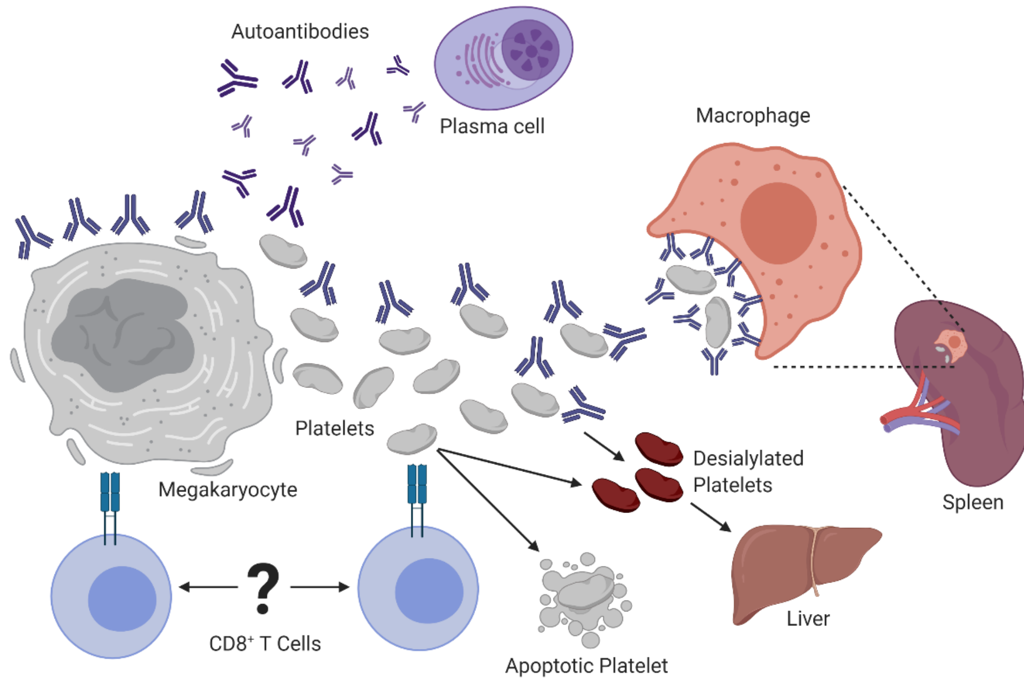


Figure 2

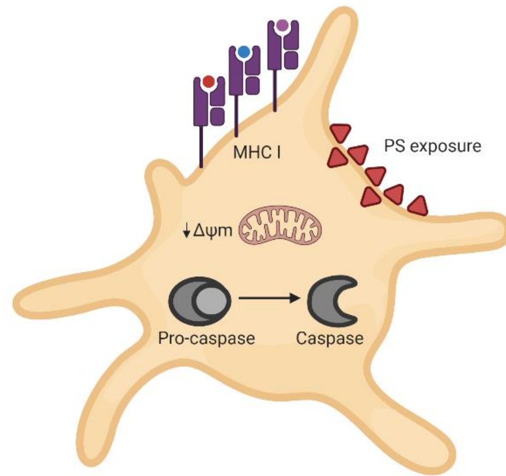


Figure 3

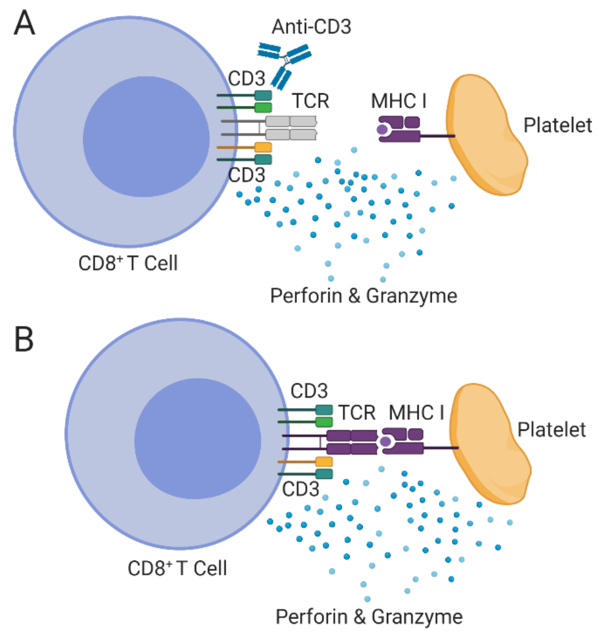
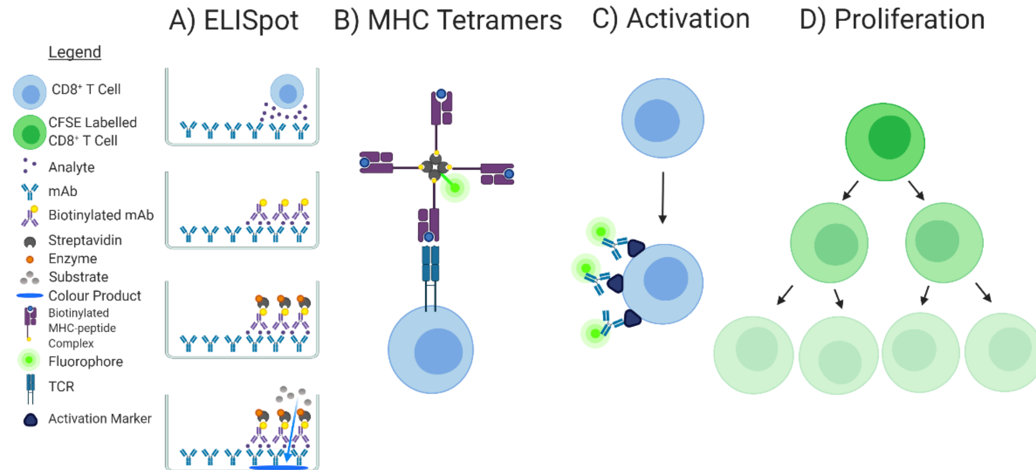


Figure 4



Conflicts of Interest

None of the authors have conflicts of interest to declare.

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Authorship Contributions

JRV takes primary responsibility for the paper. JRV conceptualized and created the manuscript. All authors reviewed and approved the final version of the manuscript.

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CHAPTER 2

The Sensitivity and Specificity of Platelet Autoantibody Testing in Immune Thrombocytopenia: a Systematic Review and Meta-Analysis of a Diagnostic Test

Vrbensky JR, Moore JE, Arnold DM, Smith JW, Kelton JG, Nazy I. The sensitivity and specificity of platelet autoantibody testing in immune thrombocytopenia: a systematic review and meta-analysis of a diagnostic test. J Thromb Haemost. 2019 May;17(5):787-794. doi: 10.1111/jth.14419. Epub 2019 Mar 20. PMID: 30801909.

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Author's Preface

Anti-platelet autoantibodies can cause thrombocytopenia by promoting platelet destruction and by reducing platelet production. Estimates regarding the proportion of ITP patients with detectable autoantibodies have varied widely. The goal of chapter 2 is to conduct a systematic review and meta-analysis of platelet autoantibody testing for the diagnosis of ITP. This findings in this chapter demonstrate that autoantibodies to platelet glycoproteins IIbIIIa and IbIX are detectable in about half of all ITP patients, which suggests that other mechanisms are involved in the pathogenesis of ITP. $CD8^{+}$ T cells represent one possible alternate mechanism, which will be explored further in chapters 3 and 4.

Essentials

- The diagnosis of ITP is based on a platelet count <100x10^9/L and exclusion of other causes.
- There are no standard tests or biomarkers to diagnose ITP.
- The sensitivity of platelet autoantibody testing is low (53%). The specificity is high (>90%).
- A positive autoantibody test can be useful to rule in ITP but a negative does not rule out ITP.

Summary

Background: Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count and an increased risk of bleeding. The sensitivity and specificity of platelet autoantibody tests is variable and their utility is uncertain.

Objective: The purpose of this study was to perform a systematic review and meta-analysis of platelet autoantibody tests in the diagnosis of ITP.

Methods: Ovid Medline, PubMed, and Web of Science were searched from inception until May 31, 2018. Two reviewers independently assessed studies for eligibility and extracted data. Studies that reported testing results for anti-platelet autoantibodies on platelets (direct tests) or in plasma/serum (indirect tests) for 20 or more ITP patients were included.

Results: Pooled estimates for sensitivity and specificity were calculated using a random effects model. Pooled estimates for the sensitivity and specificity of direct anti-platelet autoantibody testing for either anti-glycoprotein (GP)IIbIIIa or anti-GPIbIX were 53% (95% CI, 44-61%) and 93% (95% CI, 81-99%), respectively. For indirect testing, the pooled estimates for the sensitivity and specificity were 18% (95% CI, 12-24%) and 96% (95% CI, 87-100%), respectively.

Conclusions: The serological investigation of ITP has a high specificity but low sensitivity. A positive autoantibody test can be useful for ruling in ITP, but a negative test does not rule out ITP.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count, which can be asymptomatic or life-threatening. Primary ITP is defined as isolated thrombocytopenia, whereas secondary ITP occurs in association with other conditions such as systemic lupus erythematosus or antiphospholipid syndrome. There is no gold standard test for the diagnosis of ITP other than a platelet count below $100x10^9/L$[1,2], and no other biomarker has proven to be clinically useful. Thus, patients must undergo a series of investigations so that other causes of thrombocytopenia are excluded, and the diagnosis of ITP is made inferentially. This lack of certainty results in misdiagnosis[3] and empirical treatment strategies.

Autoantibody-mediated platelet destruction is the central hypothesis in our understanding of the pathophysiology of ITP. Autoantibodies have also been implicated in platelet underproduction, as they have been shown to target megakaryocytes in the bone marrow[4]. Most commonly, these autoantibodies target major platelet antigens including glycoprotein (GP)IIbIIIa and GPIbIX. Various test methods have been developed to detect glycoprotein-specific platelet autoantibodies either bound to the platelet surface (direct tests) or in plasma or serum (indirect tests). These assays include the monoclonal antibody-specific immobilization of platelet antigen (MAIPA)[5], microtiter well assay[6], the enzyme-linked immunosorbent assay (ELISA)[7], and the modified antigen capture ELISA (MACE)[8], among others. A review of the technical differences among some of these assays has been published recently[9]. However, testing for anti-platelet autoantibodies is not currently recommended for the diagnosis of ITP[10]. In this study, we evaluated the performance characteristics of direct and indirect anti-platelet autoantibody tests for the diagnosis of ITP based on the published literature.

Methods

Data Sources and Search Strategy

Ovid Medline, PubMed, and Web of Science were searched for studies on platelet autoantibody testing in ITP published until May 31, 2018. The search was restricted to the English language and original data. Key terms for ITP and the relevant test methods were combined for the search (see Supplemental Material).

Eligibility Criteria and Study Selection

Eligibility criteria were applied by two reviewers who independently assessed each study. Studies were included only when at least 20 ITP patients were included, and autoantibodies against anti-GPIIbIIIa, anti-GPIbIX (or both) of the IgG isotype were measured with a direct test. Results of indirect testing were only included from studies that also reported the results of direct testing to limit the variability in test methods. The results of commercial assays were not included. We excluded studies that pre-screened patients for anti-platelet autoantibodies. Conference abstracts were also excluded. Studies were included when both reviewers agreed that they met all eligibility criteria. Disagreements were resolved by consensus.

Data Extraction and Quality Assessment

Data was collected from primary studies in duplicate and independently, and included patient demographics, ITP disease characteristics, study design, autoantibody detection methods, positive threshold values, and the number of

autoantibody positive and negative patients and controls. If a study used more than one method to detect autoantibodies, the results of the more common technique were extracted for analysis. The two reviewers compared extracted data to identify errors and to ensure accuracy. Corresponding authors were contacted when additional data was needed. The laboratory and diagnostic techniques were assessed by one reviewer. The features used to assess study quality were: i) the use of standard consensus diagnostic criteria for primary ITP (platelet count $<100x10^9$/L and the exclusion of other causes of thrombocytopenia); ii) a description of the antibody testing result threshold; iii) a description of the disease stage of patients; iv) consecutive enrollment; and v) prospective data collection.

Statistical Analysis

Cohen's kappa coefficient (κ) was calculated to assess agreement between the reviewers during the study selection phase, and to assess the agreement between direct and indirect assays. The pooled estimates for sensitivity and specificity were calculated using a random effects model (DerSimonian-Laird). Stats Direct software version 3.1.17 was used to conduct statistical analysis, and forest plots were generated using GraphPad Prism version 7.04.

Data Synthesis

The pooled estimates for the sensitivity of autoantibody testing included the results from patients with primary ITP, defined as thrombocytopenia in the absence of a secondary cause. Platelet count thresholds $<100x10^9$/L or

$<150x10^9/L$ were considered acceptable. The pooled estimates for specificity included the results from patients with non-immune thrombocytopenia, while the results from healthy controls were excluded. The pooled estimates for sensitivity and specificity were reported with 95% confidence intervals (CI). The inconsistency (I^2 statistic) of results across studies was calculated as an indicator of heterogeneity. When there was only one study in a category, the sensitivity or specificity from that study was reported with a 95% CI. The hypotheses for subgroup analysis were generated *a priori*.

Results

Study Selection

We identified 2948 citations through our literature search. Agreement was strong (κ=0.8) for the initial study selection, and very strong (κ=0.915) for final article inclusion. After the exclusion of duplicate publications and ineligible studies, 18 studies were included (n=1170 ITP patients, n=225 controls with non-immune thrombocytopenia) (Table 1 and Figure 1a). The criteria used to diagnose ITP patients in each study are outlined in Table S1. The diagnoses of the control patients with non-immune thrombocytopenia are listed in Table S2. Six of the 18 eligible studies reported results of both direct and indirect autoantibody tests.

Study Quality

Of the 18 eligible studies, 8 (44%) used the standard consensus diagnostic criteria for ITP (platelet count $<100x10^9/L$ plus the exclusion of other causes of

thrombocytopenia). Thirteen studies (72%) reported the autoantibody test threshold for a positive result as the optical density (OD), or as a reference to the control group OD. Eight studies (44%) reported the disease stage at the time of testing using ‘newly diagnosed’, ‘persistent’, ‘chronic’, or ‘acute’. Consecutive patient enrollment and prospective data collection were reported in 3 (17%) and 15 (83%) studies, respectively (Figure 1b).

The Performance Characteristics of Autoantibody Testing

The pooled estimates for the sensitivity and specificity of direct anti-platelet autoantibody testing for either anti-GPIIbIIIa or anti-GPIbIX were 53% (95% CI, 44-61%) and 93% (95% CI, 81-99%), respectively. The pooled estimates for the sensitivity and specificity of indirect testing were 18% (95% CI, 12-24%) and 96% (95% CI, 87-100%), respectively (Figure 2). The results for each glycoprotein-specific autoantibody test are listed in Table 2. In the 8 studies that used the standard consensus diagnostic criteria for ITP, there was no difference in the sensitivity of autoantibody testing compared to all 18 eligible studies combined (data not shown). In the subgroup of studies (n=6) that used a strict cut-off (OD>3 standard deviations above the mean of controls) as the threshold for a positive test result, the pooled estimates for the sensitivity and specificity of direct testing for either anti-GPIIbIIIa or anti-GPIbIX were 58% (95% CI, 42-73%) and 94% (95% CI, 77-100%), respectively. The pooled estimates for the sensitivity and specificity of indirect testing were 21% (95% CI, 13-32%) and 96% (95% CI, 87-100%), respectively (Table 3).

In the subgroup of studies (n=6) that performed direct and indirect assays simultaneously, the pooled estimates for the sensitivity of anti-GPIIbIIIa testing were 41% (95% CI, 33-49%) for direct testing and 24% (95% CI, 11-40%) for indirect testing. One study[11] performed both direct and indirect assays for anti-GPIbIX testing; the sensitivity was 39% (95% CI: 22-59%) for direct testing, and 8% (95% CI: 2-21%) for indirect testing. The pooled sensitivity estimate for direct testing of either autoantibody was 45% (95% CI: 35-55%), and the pooled sensitivity estimate for indirect testing was 18% (95% CI: 12-24%) (Table 4). The agreement (κ) between direct and indirect assays was <0.3. In each individual study, the sensitivity was always higher using the direct assay, with the exception of one study[8].

An additional analysis was conducted using two studies that included pediatric ITP patients only[12,13]. These studies were not part of the original group of eligible studies because they used indirect assays alone, without a direct assay. Both of these studies tested for autoantibodies against GPIIbIIIa, and only one tested for autoantibodies against GPIbIX. Neither of these studies reported the specificity. In these two studies, the pooled estimates for sensitivity were 57% (95% CI, 39-74%) for anti-GPIIbIIIa and 53% (95% CI: 36-69%) for anti-GPIbIX testing.

Tables

Table 1 Autoantibody testing studies in patients with immune thrombocytopenia

Study	Reference	Method	Patients, *n*	Controls, *n*	Controls	Antibody Testing Result Threshold (OD)
Brighton, 1996	[21]	MAIPA	66 Direct 76 Indirect	51 Direct 53 Indirect	Non-ITP	>3 SD above normal
Chan, 2003	[22]	ACA	59 Direct	31 Direct	Non-ITP	0.2
Chen, 2012	[23]	MAIPA	64 Direct	33 Direct	Non-ITP	>3 SD above normal
Crossley, 1997	[24]	MAIPA	23 Direct 37 Indirect	–	–	1.3× normal
Fabris, 2002	[25]	MACE	42 Direct	39 Direct	Non-ITP	>3 SD above normal
Fabris, 2004	[26]	MACE	50 Direct	–	–	>3 SD above normal
Gaiger, 1994	[27]	MAIPA	40 Direct 45 Indirect	–	–	>6 SD above normal
He, 2013	[28]	MAIPA	50 Direct	–	–	NR
Kosugi, 1996	[8]	MACE	37 Direct 57 Indirect	–	–	NR
Kosugi, 2001	[29]	MACE	47 Direct 80 Indirect	–	–	>3 SD above normal
Meyer, 2006	[11]	MAIPA	28 Direct 39 Indirect	–	–	0.2
Najaoui, 2012	[30]	MAIPA	240 Direct	–	–	0.15
Panzer, 2006	[31]	MAIPA	42 Direct	–	–	NR
Panzer, 2007	[32]	MAIPA	40 Direct	–	–	NR
Porcelijn, 2018	[33]	MAIPA	60 Direct	43 Direct	Non-ITP	>3 SD above normal (0.13)
Tomer, 2005	[34]	Immunobead	62 Direct	–	–	Fluorescence ratio > 1.3*
Warner, 1999	[35]	ACA	56 Direct	26 Direct	Non-ITP	0.2
Zhao, 2015	[36]	MAIPA	71 Direct	–	–	NR

Direct assays detect platelet glycoprotein-specific autoantibodies on the platelet surface. Indirect assays detect these autoantibodies in plasma or serum samples. Non-ITP includes thrombocytopenic patients with a disease that is not primary or secondary ITP. ACA, antigen capture assay; ITP, immune thrombocytopenia; MACE, modified antigen capture enzyme-linked immunosorbent assay; MAIPA, monoclonal antibody specific immobilization of platelet antigen; NR, not reported; OD, optical density; SD, standard deviation. *This threshold is not an OD.

Table 2 The sensitivity and specificity of autoantibody testing in ITP

	Anti-GPIIbIIIa		Anti-GPIbIX		Either autoantibody	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Direct	48% (39–56%) n = 929 15 studies I^2: 85%	92% (79–99%) n = 133 4 studies I^2: 79%	37% (30–45%) n = 637 9 studies I^2: 68%	98% (91–100%) n = 69 2 studies I^2: 40%	53% (44–61%) n = 622 9 studies I^2: 77%	93% (81–99%) n = 159 4 studies I^2: 83%
Indirect	24% (11–40%) n = 213 4 studies I^2: 85%	—	8% (2–21%) n = 39 1 study	—	18% (12–24%) n = 160 3 studies I^2: 0%	96% (87–100%) n = 53 1 study

The pooled estimates of the sensitivity and specificity are reported (with 95% confidence intervals) from all 18 eligible studies. The number of studies that contributed to the pooled estimates are listed. I^2, the inconsistency of results across studies (listed below each statistic when applicable); *n*, the number of patients or controls used to calculate the pooled estimates.

Table 3 A subgroup analysis on the sensitivity and specificity of autoantibody testing in ITP in studies with an OD > 3 SD above normal as the threshold for a positive test result

	Anti-GPIIbIIIa		Anti-GPIbIX		Either autoantibody	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Direct	45% (31–60%) n = 221 4 studies I^2: 80%	93% (65–99%) n = 76 2 studies I^2: 91%	45% (18–73%) n = 110 2 studies I^2: 90%	100% (92–100%) n = 43 1 study	58% (42–73%) n = 218 4 studies I^2: 83%	94% (77–100%) n = 133 3 studies I^2: 89%
Indirect	14% (7–23%) n = 80 1 study	—	—	—	21% (13–32%) n = 76 1 study	96% (87–100%) n = 53 1 study

The pooled estimates of the sensitivity and specificity are reported (with 95% confidence intervals) from 6 studies. The number of studies that contributed to the pooled estimates are listed. I^2, the inconsistency of results across studies (listed below each statistic when applicable); *n*, the number of patients or controls used to calculate the pooled estimates.

Table 4 Direct vs. indirect autoantibody testing

	Direct	Indirect
Sensitivity		
Anti-GPIIbIIIa	41% (33–49%) *n* = 135 4 studies I^2: 0%	24% (11–40%) *n* = 213 4 studies I^2: 85%
Anti-GPIbIX	39% (22–59%) 1 study *n* = 28	8% (2–21%) 1 study *n* = 39
Either autoantibody	45% (35–55%) *n* = 134 3 studies I^2: 26%	18% (12–24%) *n* = 160 3 studies I^2: 0%

This subgroup analysis included studies that performed direct and indirect testing together (n = 6 studies in total). The pooled estimates of sensitivity are reported (with 95% confidence intervals). The number of studies that contributed to the pooled estimates are listed. I^2, the inconsistency of results across studies (listed below each statistic when applicable); *n*, the number of patients or controls used to calculate the pooled estimates.

Figure Legends

Figure 1: Study selection and quality assessment. (A) Results of the database search and study selection process. (B) A quality assessment of the 18 eligible studies included in this review. The 'standard consensus diagnosis' refers to a platelet count <100 x 10^9 L^{-1} and the exclusion of secondary causes of ITP. The inclusion of an 'antibody testing result threshold' refers to OD cutoffs or a reference to the control group OD. A description of the disease stage of patients

refers to the inclusion of the standardized terms for ITP: ‘newly diagnosed’, ‘persistent’, ‘chronic’, or ‘acute’ in the description of the patients in each study.

Figure 2: Autoantibody testing results in ITP. Forest plots of the sensitivity and specificity for autoantibody testing (either anti-GPIIbIIIa or anti-GPIbIX). The sensitivity and specificity (circles) are reported for each study with 95% CIs (solid lines). The pooled estimates (open diamonds with vertical dashed lines) are also reported. n represents the number of individuals in each study. (A–B) The sensitivity and specificity of direct testing. (C–D) The sensitivity and specificity of indirect testing.

Figures

Figure 1

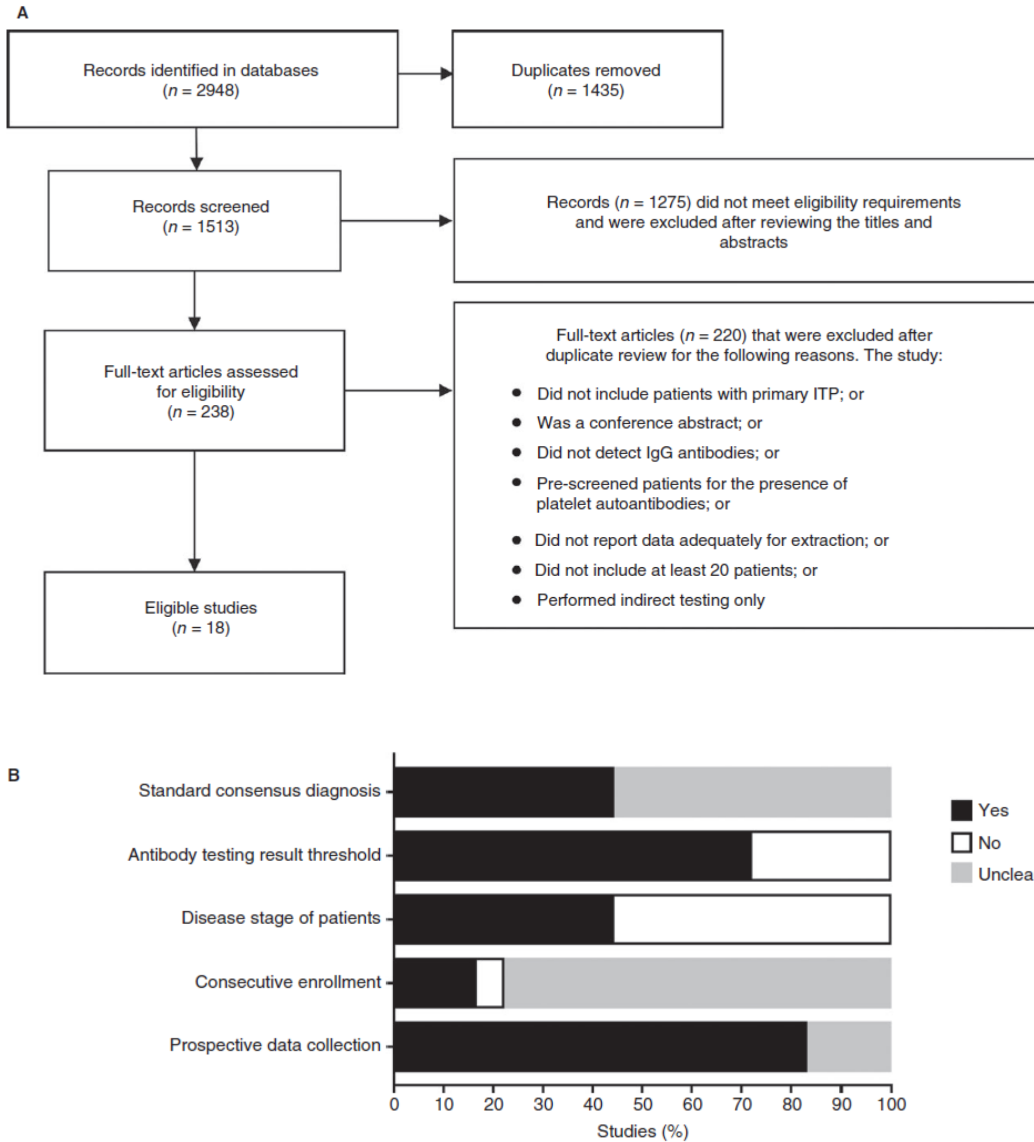
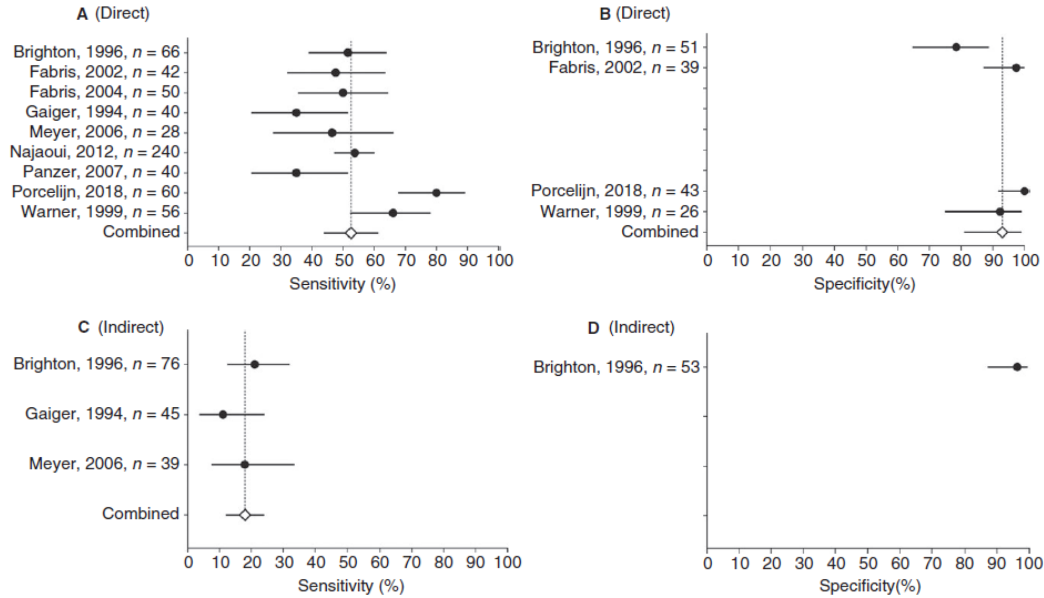


Figure 2



Discussion

We provide pooled estimates for the sensitivity and specificity of autoantibody testing in ITP from 18 studies, consisting of 1170 ITP patients. Overall, the pooled estimates for the sensitivity of anti-platelet autoantibody testing was low (53%), whereas the specificity of these tests was high (>90%). The degree of heterogeneity in these estimates was high, and the patients in these studies were heterogeneous in respect to the duration and severity of ITP, splenectomy status, and treatment history. Subgroup analyses based on these patient characteristics were not possible because clinical data was often reported for the entire cohort of patients within studies. A large multi-center prospective

study with uniform inclusion and exclusion criteria would be valuable in the future. In the subgroup analysis on the studies that used an OD>3SD above normal as the threshold for a positive test result, the sensitivity was improved without compromising the specificity. Based on this result, we suggest that an OD>3SD above normal should be used to establish a threshold to improve the standardization of anti-platelet autoantibody assays across laboratories.

We reasoned that a subgroup analysis on the studies that performed direct and indirect tests together would allow for a comparison between direct and indirect testing while controlling for the patients tested, and for laboratory conditions. The subgroup analysis on the six studies that fulfilled this requirement suggests that direct assays measuring autoantibodies on the platelet surface are more sensitive than indirect assays which measure circulating anti-platelet autoantibodies in the plasma or serum. This result is consistent with the results from the analysis on all 18 eligible studies. It is also consistent with a report published by the Platelet Immunology Scientific Subcommittee of the International Society on Thrombosis and Haemostasis on the standardization of platelet autoantibody testing in ITP. This subcommittee recommended direct glycoprotein-specific assays over indirect assays because the direct assays are more sensitive[14]. We also performed a separate analysis on two pediatric ITP studies because of the differences between adult and pediatric ITP[2]. Although there were only two pediatric ITP studies, and these studies used indirect test methods only, our analysis provides a more comprehensive estimate of the

sensitivity of anti-platelet autoantibody testing in pediatric ITP compared to the individual studies alone.

Diagnostic tests used to detect autoantibodies in autoimmune hemolytic anemia (AIHA) are clinically useful because the sensitivity and specificity of these tests are high[15]. Overall, the specificity for anti-platelet autoantibody testing in primary ITP is high, but the sensitivity is low. Therefore, these assays are useful for ruling in ITP when they are positive, but they cannot be used to exclude ITP when they are negative. Several reasons might explain the low sensitivity of these tests. It is possible that anti-GPIIbIIIa and anti-GPIbIX are not present in all patients with ITP and other mechanisms, such as autoreactive $CD8^+$ T cells, cause thrombocytopenia in patients without autoantibodies[16]. Other autoantibodies could also be involved such as those specific to thrombopoietin (TPO), its receptor c-Mpl, or the complex formed when TPO binds c-Mpl[17], although these targets are not generally tested. It is also possible that immunosuppressive treatments such as rituximab prevent autoantibody production[18]. The timing of blood collection from patients requiring treatment should be considered to avoid confounding the results of autoantibody testing. Technical problems could also prevent the detection of anti-platelet autoantibodies, especially if the affinity of the autoantibody for the antigen is low or if the autoantibody titer is low[19]. One of the more likely reasons for the low sensitivity of these assays is that the clinical diagnosis of ITP is difficult. It was recently shown that as many as 1 in 7 thrombocytopenic patients are initially misdiagnosed[3]. This is consistent with our

current study, in which it was unclear in 56% of the eligible studies whether or not the standard diagnostic criteria for primary ITP was used. In the 18 eligible studies, it is possible that some patients were misdiagnosed with primary ITP when instead they had an underlying condition leading to thrombocytopenia.

The current guidelines from the American Society of Hematology (ASH) suggest that antibody tests are not useful for the diagnosis of ITP[10]. Our results show that autoantibody testing can help rule in ITP. The diagnosis of primary ITP is currently one of exclusion, and there are no readily available biomarkers to assist with this diagnosis besides the platelet count. A more accurate clinical definition of ITP is necessary. In a recent study, we observed that a very low platelet count nadir ($<20x10^9$/L) or a platelet count response to treatment with intravenous immunoglobulin or corticosteroids increased the likelihood of diagnostic agreement among hematologists[20]. In the future, distinguishing autoantibody-mediated ITP from other causes of ITP could lead to therapeutic insights.

Strengths and Limitations

Strengths of this study include the data collection, review, and extraction processes, which were performed independently by two reviewers. Interrater reliability was high, indicating the high likelihood that the right articles were included in this review. The sensitivity and specificity of tests for autoantibodies specific to other platelet glycoproteins were not included in this review.

Conclusions

This meta-analysis shows that autoantibody testing in ITP patients has a high specificity, but a low sensitivity. Although the sensitivity is low in both direct and indirect assays, direct assays have a superior sensitivity compared to indirect assays. Overall, anti-platelet autoantibody testing is useful for ruling in ITP. The low sensitivity of autoantibody detection assays suggests that ITP is caused by multiple mechanisms besides anti-platelet autoantibodies.

Addendum

JR Vrbensky and JE Moore take primary responsibility for the paper. JR Vrbensky, JE Moore, JW Smith, and I Nazy designed the research. JR Vrbensky and JE Moore performed the research and collected the data. JR Vrbensky and JE Moore performed statistical analysis. All authors interpreted the data. All authors wrote the manuscript. All authors read and approved the final version of the manuscript before submission to the Journal of Thrombosis and Haemostasis.

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Disclosure of Conflicts of Interest

None of the authors have conflicts of interest to declare. The funding organization(s) of the individual authors had no role in the design of the study, the collection/ management/ analysis/ interpretation of the data, or in the preparation/ review/ approval of the manuscript.

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Supplemental Material

Methods

Search Strategy

The disease and assay keywords were combined using ‘or’ and ‘and’. Asterisks were used to search multiple endings on key terms and any terms more than one word long were combined by brackets when searched in Web of Science. The final search algorithm was: MAIPA OR Monoclonal antibody-specific immobilization of platelet antigens OR Flow Cytometr* OR ACA OR Antigen Capture Assay* OR Direct assay* OR Indirect assay* OR ELISA* OR Enzyme-linked immunosorbent assay* OR Antigen Capture ELISA OR ACE OR MACE AND ITP OR Purpura OR Thrombocytopenic OR idiopathic thrombocytopenic purpura* OR idiopathic thrombocytopaenic purpura* OR idiopathic thrombocytopenia* OR idiopathic thrombocytopaenia* OR immune thrombocytopenic purpura* OR immune thrombocytopaenic purpura* OR immune thrombocytopenia* OR immune thrombocytopaenia* OR AITP OR autoimmune thrombocytopenic purpura* OR autoimmune thrombocytopaenic purpura* OR autoimmune thrombocytopenia* OR autoimmune thrombocytopaenia* NOT Review [Publication Type]) AND English [language].

Results

Table S1: The Diagnostic Criteria for ITP in All Eligible Studies.

Study	ITP Diagnosis
Brighton, 1996	Platelets <140x10^9/L twice within 48 hours; nonpalpable spleen; at least normocellular bone marrow aspirate with normal or increased megakaryopoeisis; no other clinical or laboratory features to suggest another cause for thrombocytopenia.
Chan, 2003	Thrombocytopenia in the absence of splenomegaly and the absence of any other cause of thrombocytopenia; no known familial history of low platelets.
Chen, 2012	2010 International consensus guidelines.
Crossley, 1997	Cited McMillan, 1981.
Fabris, 2002	Isolated thrombocytopenia (platelets <100×10^9/L); normal or increased number of bone marrow megakaryocytes; normal spleen size and exclusion of secondary causes of thrombocytopenia.
Fabris, 2004	Acquired and isolated thrombocytopenia with platelet counts of 100x10^9/L or lower; normal or increased number of bone marrow megakaryocytes; normal spleen size; exclusion of secondary immune and drug-related thrombocytopenia; exclusion of laboratory features of Epstein-Barr virus, hepatitis B virus, hepatitis C virus, and HIV infections; normal thyroid function test.
Gaiger, 1994	Documented thrombocytopenia 50x10^9/L or less on at least two occasions; normal or increased numbers of megakaryocytes in bone marrow aspirate; normal spleen size; absence of a preceding viral infection or drug ingestion known to induce immune thrombocytopenia; absence of clinical and serological indications for a collagen-vascular disorder or any other disease known to be associated with immune or non-immune thrombocytopenia; and a disease duration of more than 6 months.
He, 2013	American Society of Hematology 1996 guidelines.
Kosugi, 1996	Cited Tsubakio, 1981.
Kosugi, 2001	American Society of Hematology 1996 guidelines.
Meyer, 2006	American Society of Hematology 1996 guidelines.
Najaoui, 2012	Thrombocytopenic patients with suspected ITP. Patients with an enlarged spleen, aplastic anemia, leukemia, lymphoma, myelodysplastic syndrome, solid tumors, liver cirrhosis, recent cardiac surgery, bone marrow ⁄ blood stem cell transplantation, sepsis, and drug-induced thrombocytopenia, or unexplained leukopenia were excluded.
Panzer, 2006	American Society of Hematology 1996 guidelines.
Panzer, 2007	American Society of Hematology 1996 guidelines.
Porcelijn, 2018	American Society of Hematology 2011 guidelines.
Tomer, 2005	American Society of Hematology 1996 guidelines.
Warner, 1999	Thrombocytopenia; normal bone marrow megakaryocytes; absence of splenomegaly; and the absence of secondary causes of thrombocytopenia.
Zhao, 2015	American Society of Hematology 2011 guidelines.

Table S2: The Diagnosis of Control Patients with Non-Immune Thrombocytopenia

Study	Non-ITP Controls
Brighton, 1996	Aplastic anemia, leukemia, non-Hodgkin's lymphoma, multiple myeloma, myelodysplasia, chronic liver disease and hypersplenism, drug-associated (methotrexate, chloramphenicol), sepsis and laboratory disseminated intravascular coagulation, hypersplenism, May-Hegglin anomaly, megaloblastosis, myelofibrosis, polycythemia rubra vera, renal osteodystrophy, hemophagocytic syndrome.
Chan, 2003	Prostate cancer, leukaemia, anaemia, thrombocythaemia, systemic lupus erythematosus, haemochromatosis, Bernard Soulier disease (BSS), hypersplenism and myelodysplasia.
Chen, 2012	Aplastic anemia, myelodysplastic syndrome, and acute leukemia.
Fabris, 2002	Thrombocytopenia due to chemotherapy for lymphoma or acute leukemia, bone marrow transplant, lympho- or myeloproliferative disease, (chronic myeloid leukemia, myelofibrosis, chronic lymphocytic leukemia, or non-Hodgkin's lymphoma), myelodysplastic syndrome, myelophthisis secondary to solid cancer, plasma cell dyscrasia (Waldeström's syndrome or multiple myeloma), thrombotic thrombocytopenic purpura, liver disease with splenomegaly, megaloblastic anemia, pseudothrombocytopenia, familial macrothrombocytopenia or platelet function defects of uncertain etiology.
Porcelijn, 2018	Hematological malignancy, gestational thrombocytopenia, viral infection, drug-induced thrombocytopenia, aplastic anemia, hepato-splenomegalic pooling, pseudothrombocytopenia, microangiopathy.
Warner, 1999	Aplastic anaemia, pancytopenia secondary to chemotherapy (acute leukaemia, multiple myeloma, and lymphoma), post polycythaemia myeloid metaplasia, Bernard-Soulier syndrome, incidental thrombocytopenia of pregnancy, thrombocytopenia secondary to alcohol, platelet function defect of uncertain aetiology, thrombocytopenia of unknown cause, HIV-associated thrombocytopenia, and hepatitis-related thrombocytopenia.

CHAPTER 3

Increased Cytotoxic Potential of CD8$^+$ T Cells in Immune Thrombocytopenia

Vrbensky JR, Arnold DM, Kelton JG, Smith JW, Jaffer AM, Larché M, Clare R, Ivetic N, Nazy I. Increased cytotoxic potential of CD8$^+$ T cells in immune thrombocytopenia. *Br J Haematology*. 2020; 188(5):e72-e76. doi: 10.1111/bjh.16334. Epub 2019 December 18. PMID: 31850531.

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Author's Preface

Anti-platelet autoantibodies are detectable in only half of all ITP patients, so other mechanisms are likely involved in ITP. $CD8^+$ T cells have been implicated as one such alternate mechanism of disease, but our understanding of the role of $CD8^+$ T cells in ITP is limited.

The goals of chapter 3 are to:

1) Measure the frequency of circulating $CD8^+$ T cells in ITP.
2) Characterize the cytotoxic potential of $CD8^+$ T cells in ITP, as indicated by the surface expression of CD107a upon degranulation.
3) Investigate associations between autoantibodies and the cytotoxic potential of $CD8^+$ T cells in ITP.

The findings in this chapter demonstrate that the cytotoxic potential of $CD8^+$ T cells is elevated in a subset of ITP patients. Determining whether this coincides with the destruction of platelets or megakaryocytes will first require identification of platelet-specific $CD8^+$ T cells, which will be the focus of chapter 4.

Note to the reader: In the original publication in the British Journal of Haematology, the methods of this study appeared in the supplemental information section. For this thesis chapter, the methods have been included in the main body of the text.

Introduction

Patients with immune thrombocytopenia (ITP) have low platelets and an increased bleeding risk, depending on disease severity (Rodeghiero, et al 2009). It is well known that ITP patients produce autoantibodies that enhance platelet clearance (Warner, et al 1999). However, platelet autoantibodies are undetectable in half of all ITP patients, suggesting that other mechanisms contribute to the pathogenesis of ITP (Vrbensky, et al 2019). Platelets are susceptible to immunosurveillance by $CD8^+$ T cells since they express major histocompatibility complex class I (MHC I) (Chapman, et al 2012). Thus, $CD8^+$ T cell-mediated platelet destruction is a possible disease mechanism in ITP. Characterizing $CD8^+$ T cells in ITP will improve our understanding of disease mechanisms and our approach to disease management. When an effector $CD8^+$ T cell recognizes its cognate peptide presented by MHC I, it degranulates and induces apoptosis of the target cell (Stinchcombe and Griffiths 2007). The luminal side of cytotoxic granules are coated with lysosome-associated membrane protein-1 (LAMP-1, CD107a), which is expressed on the $CD8^+$ T cell outer membrane upon degranulation. Effector and effector memory $CD8^+$ T cells are the only circulating subsets capable of degranulation and cytotoxicity (Wolint, et al 2004); consequently, CD107a can be used to indicate the cytotoxic potential of $CD8^+$ T cells (Betts, et al 2003). We hypothesized that $CD8^+$ T cells have a greater cytotoxic potential in ITP patients who do not have detectable autoantibodies compared to patients with autoantibodies.

Methods

Patients

We studied patients from the McMaster ITP registry [9] with primary ITP in the chronic stage of disease (n=22; Table 1), non-immune thrombocytopenia (non-ITP; n=8; Table S1), and healthy controls with normal platelet counts (n=19). Peripheral blood mononuclear cells (PBMCs) were isolated from each individual. At the time of testing, platelet counts from all patients with ITP and non-ITP were $<100 \times 10^9$/L. Suspected non-ITP patients with an anti-platelet autoantibody were excluded. This study was approved by the Hamilton Integrated Research Ethics Board. Informed consent was obtained from all participants.

PBMC Isolation

20 mL of blood was collected in acid citrate dextrose vacutainers. PBMCs were isolated by centrifugation on Histopaque 1077. PBMCs were washed once with PBS supplemented with 10% heat-inactivated fetal bovine serum and 1 U/mL heparin, then washed again without heparin. Red blood cells were lysed with ammonium-chloride-potassium. PBMCs were resuspended at 2×10^6 cells/mL in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. PBMCs were rested overnight at 37°C before measuring the T cell frequencies and their cytotoxic potential.

Autoantibodies

The direct antigen capture assay (ACA) was used to measure platelet-bound autoantibodies against glycoproteins (GP) IIbIIIa and IbIX [26]. Optical density (OD) readings were recorded at 405 nm using a Tecan Sunrise plate reader. A positive result was defined as an OD >0.21.

T Cells and CD107a

PBMCs (1x10^6/mL) were labelled with anti-CD3 FITC and anti-CD4 PE or anti-CD8 PE for 30 minutes at 4°C in order to measure the number of $CD4^+$ and $CD8^+$ T cells using a Cytoflex flow cytometer. For CD107a detection, a 96-well cell culture plate was coated overnight with 50 µL of anti-CD3 (5 µg/mL, HIT3a clone) or phosphate buffered saline (PBS). The following day PBMCs (2x10^6 cells/mL) were incubated, along with BD Golgi Stop containing monensin (1:100 in RPMI) and anti-CD107a FITC, for 5 hours at 37°C. The dose and duration of anti-CD3 stimulation were optimized to induce maximal degranulation (Figure S2 and S3). The cells were labelled with 100 µL of Zombie Violet viability dye (1:1600) for 15 minutes at room temperature to exclude non-viable cells. Next, the cells were labelled with anti-CD3 PE and anti-CD8 PECy5 for 30 minutes at 4°C. Antibody titrations were performed to optimize cell labelling. 30 000 events were collected within a lymphocyte gate on a Cytoflex flow cytometer. Isotype-matched antibodies were used to determine the fluorescence due to specific binding and compensation matrices were applied to all samples. The

gating strategy is shown in Figure S4. Flow cytometry analysis was performed with FlowJo (v10).

Statistical Analysis

Age and platelet counts are reported as the median with the range. All other values are reported as the mean ± standard deviation (SD). The number of $CD4^+$ and $CD8^+$ T cells was compared between groups using *t*-tests. The expression of CD107a was compared between groups using Mann-Whitney tests. The healthy control range and the non-ITP range were defined as the mean + 2 SD. Clinical data was evaluated in ITP patients using linear regression and Fisher's exact test. Statistical analysis was performed with GraphPad Prism 7; *p*-values <0.05 were considered significant.

Results

The median platelet counts for patients with ITP, non-ITP, and healthy controls were 38 (range: 3-82x10^9/L), 54 (range: 21-67x10^9/L) and 214 (range: 182-411x10^9/L), respectively. Of 22 ITP patients, 15 were ever positive for anti-GPIIbIIIa or anti-GPIbIX (Table 1).

T cells were comprised of 64%±16% $CD4^+$ and 35%±16% $CD8^+$ cells in ITP, which was comparable to non-ITP (68%±13% $CD4^+$, p=0.56 and 31%±13% $CD8^+$ cells, p=0.55) and healthy controls (62%±11% $CD4^+$, p=0.54 and 37%±10% $CD8^+$ cells, p=0.61) (Figure 1A). PBMCs were stimulated with anti-CD3 in the presence of anti-CD107a FITC and monensin (Figure 1B-C). The

percentage of $CD3^+CD8^+$ cells that expressed CD107a after stimulation was higher in ITP patients (18%±13%) compared to healthy controls (10%±4%, p=0.04) (Figure 1D). Re-testing demonstrated assay reproducibility up to 14 months later (Figure S1). The percentage of $CD3^+CD8^+$ cells that expressed CD107a was above the healthy control range in 8/22 ITP patients and there was an inverse correlation between CD107a and the platelet count (R^2=0.73, p=0.007) for these 8 patients (Figure 1E). There was no correlation between CD107a expression and platelet count in the ITP group as a whole. There was no difference in the cytotoxic potential between treated and untreated patients (p=0.54). CD107a expression was similar in ITP (18%±13%) and non-ITP (13%±10%, p=0.35), and was above the non-ITP range in 3/22 ITP patients. In the non-ITP group, CD107a expression was highest in a patient with autoimmune hepatitis. Autoantibody-negative ITP patients were not more likely to have high CD107a expression compared to autoantibody-positive patients (OR: 0.6, 95% CI: 0.1-3.5).

Tables

Table I. Characteristics of ITP patients.

Patient	Age	Sex	Platelets ($\times 10^9$/l)	Platelet nadir ($\times 10^9$/l)	Anti-GPIIbIIIa	Anti-GPIbIX	Either autoantibody	Treatments at time of testing
ITP 1*δ	53	F	3	1	+	+	+	Romiplostim, IVIg, Dex, Csp, Aza, Mp
ITP 2	57	M	22	8	–	–	–	IVIg
ITP 3	25	M	34	1	–	–	–	IVIg
ITP 4	70	M	45	20	–	–	–	None
ITP 5	38	F	66	12	+	+	+	None
ITP 6*δ	82	M	23	14	–	+	+	None
ITP 7*	48	F	45	26	+	–	+	IVIg
ITP 8	26	M	24	3	–	–	–	Ritux, Pred, Txa
ITP 9	65	F	37	7	+	–	+	None
ITP 10	67	F	13	3	–	–	–	Revolade, Danazol
ITP 11	70	M	61	6	+	+	+	None
ITP 12*	87	M	38	2	+	+	+	Mp
ITP 13*	70	F	82	5	+	–	+	None
ITP 14*	29	F	35	35	–	–	–	None
ITP 15*	22	M	48	2	–	–	–	None
ITP 16	56	F	13	5	+	+	+	None
ITP 17	35	F	79	1	+	+	+	Mp
ITP 18*δ	63	M	43	2	+	+	+	Eltrombopag
ITP 19	55	F	24	1	+	+	+	Mp
ITP 20	84	F	49	9	+	+	+	Danazol, Dex
ITP 21	71	F	60	9	+	+	+	Csp, Mp, Pred
ITP 22	66	F	24	2	+	+	+	Revolade, Pred
	60		38	5	14+/22 (64%)	12+/22 (55%)	15+/22 (68%)	

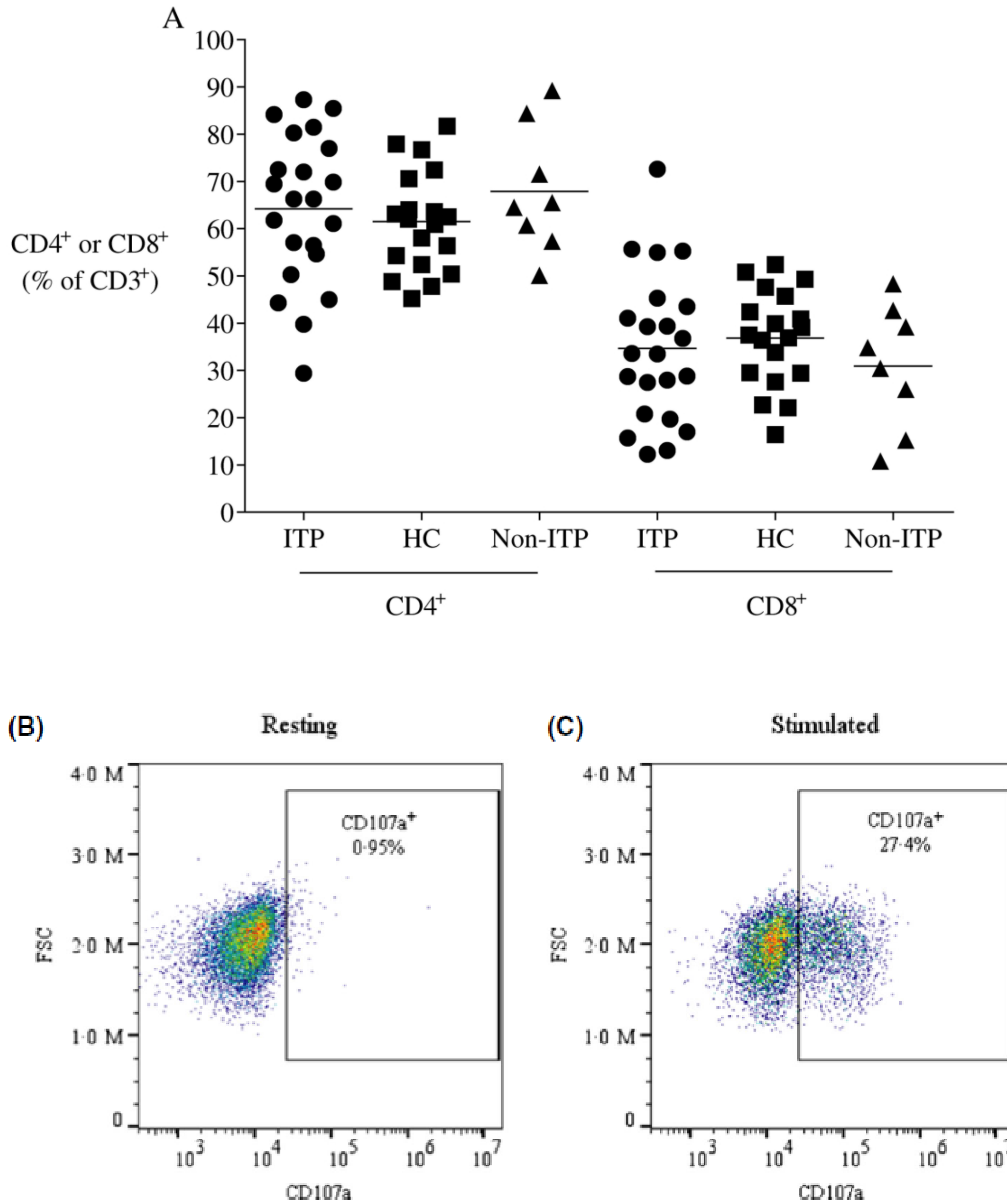
*: CD107a expression was above the healthy control range (mean + two standard deviations from the healthy controls) for this patient. δ: CD107a expression was above the non-ITP range (mean + two standard deviations from non-ITP) for this patient. Age and platelet count summaries are listed as the median. (+): The patient was historically positive at least once in the direct ACA for the autoantibody. (-): The patient has always tested negative in the direct ACA for the autoantibody. IVIg, intravenous immunoglobulin; Dex, dexamethasone; Csp, cyclosporine; Aza, azathioprine; Mp, mycophenolate; Ritux, rituximab; Pred, prednisone; Txa, tranexamic acid.

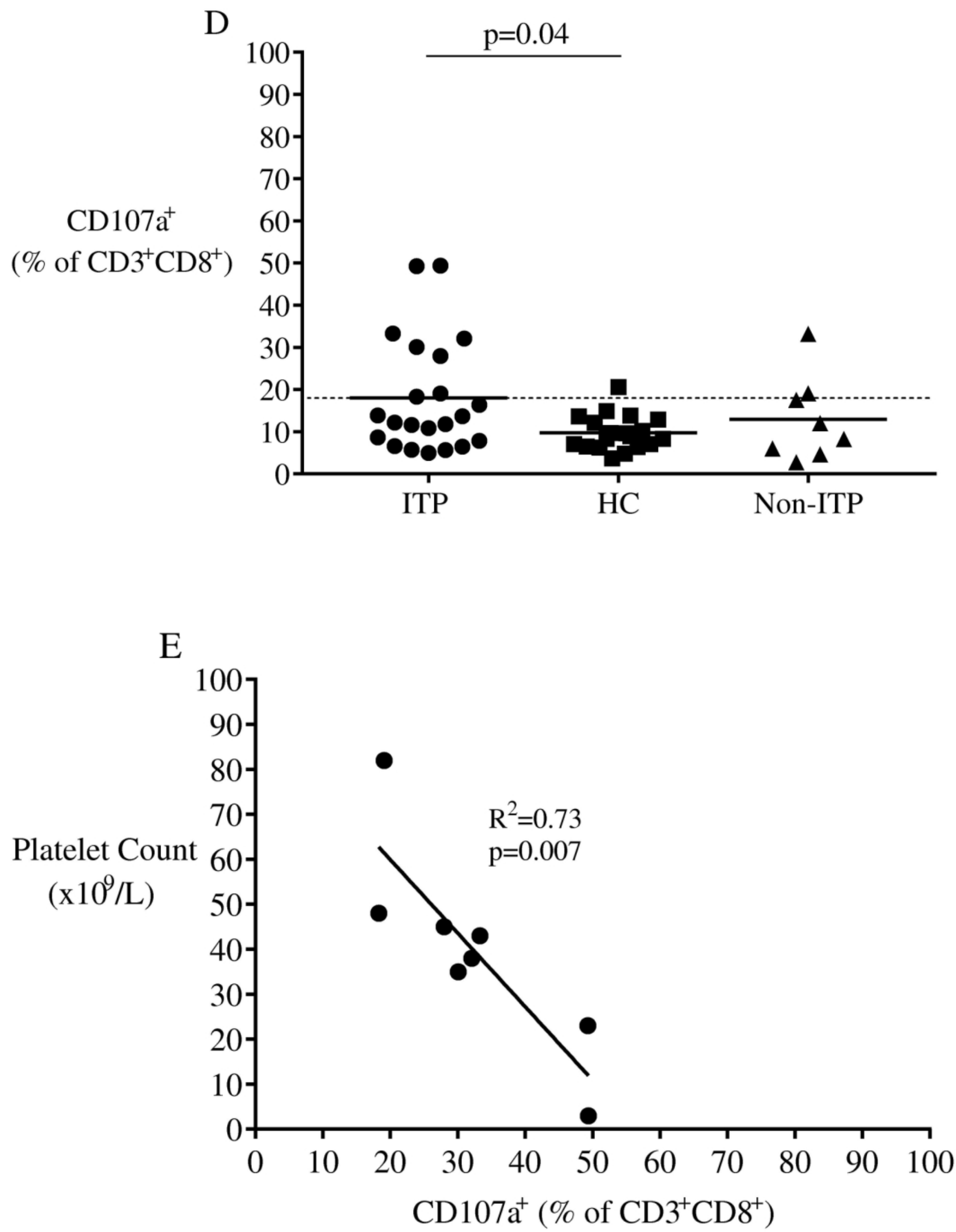
Figure Legends

Figure 1: $CD8^+$ T Cells in ITP patients. (A) The frequency of $CD4^+$ and $CD8^+$ T cells was similar between ITP patients (ITP), patients with non-immune thrombocytopenia (Non-ITP), and healthy controls (HC). The absolute $CD4^+$ and $CD8^+$ T cell count was similar in all groups (not shown). (B–D) PBMCs were stimulated with plate-bound anti-CD3 (5 µg/ml) for 5 h in the presence of anti-CD107a FITC and monensin. CD107a expression was measured as a marker of degranulation on $CD3^+CD8^+$ cells (B) at rest and (C) after stimulation with anti-CD3. (D) The extent of degranulation after stimulation with anti-CD3 was highest in the ITP group. CD107a was expressed on <1% of resting $CD3^+CD8^+$ cells in all patients and controls (not shown). The healthy control range (mean + 2 SD = 18%) is depicted as a dashed line. (E) Platelet count and CD107a expression were inversely correlated in the group of eight ITP patients who expressed CD107a above the healthy control range ($R^2 = 0.73$, $p = 0.007$).

Figures

Figure 1




D
p=0.04
100
90
80
70
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20
10
0
CD107a+
(% of CD3+CD8+)
ITP
HC
Non-ITP
E
100
90
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30
20
10
0
Platelet Count
(x10^9/L)
R^2=0.73
p=0.007
0
10
20
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50
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70
80
90
100
CD107a+ (% of CD3+CD8+)

Discussion

Since anti-platelet autoantibodies are only detectable in half of all ITP patients, other disease mechanisms are likely involved (Vrbensky, et al 2019); therefore, we studied $CD8^+$ T cells in ITP. We found that the relative numbers of $CD4^+$ and $CD8^+$ T cells were normal in ITP, which is in contrast with previously reported results (Li, et al 2007). In order to characterize the cytotoxic potential of $CD8^+$ T cells, we measured CD107a after stimulation with anti-CD3 and found that CD107a was expressed by more $CD8^+$ T cells in a subset of ITP patients compared to healthy controls. These findings are consistent with studies showing elevated CD107a expression by $CD8^+$ T cells in Churg-Strauss syndrome (Boita, et al 2012) and in a murine model of type I diabetes (Graham, et al 2011). Our study is the first to use CD107a expression to characterize $CD8^+$ T cells in ITP patients, which has also been utilized to characterize ITP natural killer cells (Ebbo, et al 2017).

In our study, CD107a expression was comparable in autoantibody-negative and autoantibody-positive patients. This suggests that autoantibody-mediated ITP cannot be distinguished from $CD8^+$ T cell-mediated ITP by CD107a expression alone. Our observations could be attributed to the fact that CD107a expression was measured on the total $CD8^+$ T cell population, since stimulation with anti-CD3 is independent of T cell receptor (TCR) recognition of peptides presented on MHC I. It is possible that CD107a expression is elevated in platelet specific $CD8^+$ T cells from autoantibody-negative ITP patients compared to

autoantibody positive patients, but to date, no studies have identified platelet-specific TCRs. We found an inverse correlation between CD107a and the platelet count in the subgroup of ITP patients with elevated CD107a, suggesting a relationship between the cytotoxic potential of $CD8^+$ T cells and disease severity. However, this correlation is limited by the low number of patients, and it is unclear if it is disease-specific.

A strength of our study is that patients with non-ITP were included for comparison with ITP patients. Since CD107a expression in ITP patients was comparable with non-ITP, it is unclear whether the enhanced cytotoxic potential of $CD8^+$ T cells in ITP is disease-specific. A limitation of our study is that $CD8^+$ T cell-mediated killing of platelets was not measured. It was previously shown that $CD8^+$ T cells stimulated with anti-CD3 were capable of inducing autologous platelet lysis (Olsson, et al 2003, Zhang, et al 2006) and desialylation (Qiu, et al 2016). However, these effects could have been due to the exogenous stimulation of $CD8^+$ T cells with anti-CD3 as opposed to TCR-mediated recognition of platelet peptides on MHC I.

In conclusion, we found that $CD8^+$ T cells have an enhanced cytotoxic potential in a subset of ITP patients. There was no correlation between the cytotoxic potential of $CD8^+$ T cells and the presence or absence of platelet autoantibodies. Whether or not ITP patients have $CD8^+$ T cells specific to platelet autoantigens remains to be determined. Identifying autoreactive $CD8^+$ T cells will

improve our understanding of the pathophysiology of ITP and our ability to manage the disease.

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Authorship Contributions

JRV takes primary responsibility for the paper. JRV, DMA, ML, RC, and IN designed the research. JRV and JWS performed the research. JRV, JWS, and AMJ collected data. JRV performed statistical analysis. All authors analyzed and interpreted the data. JRV, IN, and DMA wrote the manuscript. All authors reviewed the manuscript.

Conflicts of Interest Disclosures

None of the authors have conflicts of interest to declare.

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Supporting Information

List of Reagents and Suppliers

Acid citrate dextrose vacutainers (BD Biosciences, NJ, USA)

Histopaque 1077 (Sigma Aldrich, ON, Canada)

Heat-inactivated fetal bovine serum (Gibco, NY, USA)

Heparin (Fresenius Kabi Canada Ltd, ON, Canada)

Ammonium-chloride-potassium (Gibco, NY, USA)

RPMI (Gibco, NY, USA)

Penicillin/streptomycin (Gibco, NY, USA)

L-glutamine (Gibco, NY, USA)

Anti-CD3 FITC (BD Biosciences, ON, Canada)

Anti-CD4 PE (BD Biosciences, ON, Canada)

Anti-CD8 PE (BD Biosciences, ON, Canada)

96-well cell culture plate (Eppendorf, Hamburg, Germany)

Anti-CD3 (HIT3a clone) (BD Biosciences, ON, Canada)

BD Golgi Stop containing monensin (BD Biosciences, ON, Canada)

Anti-CD107a FITC (BD Biosciences, ON, Canada)

Zombie Violet viability dye (Bio Legend, CA, USA)

Anti-CD3 PE (eBioscience Inc, CA, USA)

Anti-CD8 PECy5 (BD Biosciences, ON, Canada)

Isotype-matched antibodies (BD Biosciences, ON, Canada)

Table S1: Control Patients with Non-Immune Thrombocytopenia

Patient	Age	Sex	Platelets ($x10^9$/L)	Platelet Nadir ($x10^9$/L)	Either Autoantibody	Treatments at Time of Testing	Diagnosis
TC 1	66	M	67	64	-	None	Liver cirrhosis secondary to fatty liver
TC 2	72	F	47	33	-	None	Cryptogenic cirrhosis
TC 3*	45	M	21	16	-	None	Autoimmune hepatitis and cirrhosis, portal hypertension with massive splenomegaly
TC 4	56	M	21	21	-	None	Aplastic anemia and mild pancytopenia
TC 5	67	M	60	39	-	None	Hepatitis C-induced liver cirrhosis
TC 6*	30	M	64	62	-	None	Portal hypertension with splenomegaly
TC 7	32	M	60	53	-	None	Cryptogenic cirrhosis
TC 8	63	F	40	36	-	None	Liver cirrhosis secondary to non-alcoholic steatohepatitis, portal hypertension with splenomegaly
	60		**54**	**38**	**0+/8**		

*: indicates that a patient's CD107a expression was above the healthy control range (mean + 2 SD). The age and platelet count summaries are listed as the median. (-): The patient has always tested negative in the direct ACA for anti-GPIIbIIIa and anti-GPIbIX.

Supplemental Figures

Figure S1: Reproducibility of the CD107a Assay. PBMCs from ITP patients (n=3) and healthy controls (n=2) were stimulated with 5 ug/mL of anti-CD3 for 5 hours. CD107a expression was consistent up to 14 months later (p=0.93; paired t test). In this figure, the coded numbers identifying patients do not correspond to the patient identifiers in Table 1.

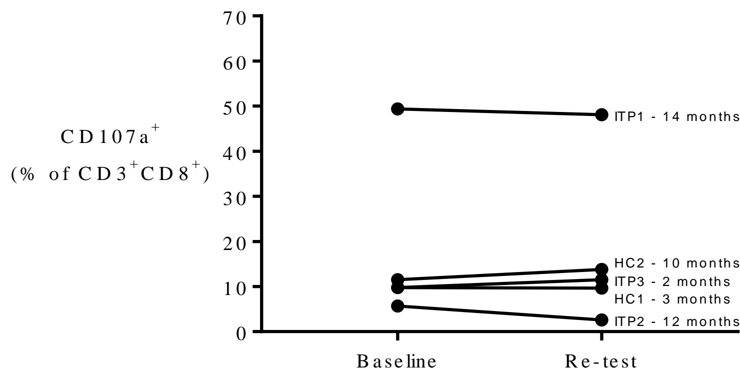


Figure S2: The Dose of Anti-CD3 was Optimized to Elicit Maximal Degranulation. PBMCs from healthy donors (n=2) were stimulated with varying doses of anti-CD3 for 5 hours. CD107a expression was measured on $CD3^+CD8^+$ cells. 5 ug/mL of anti-CD3 induced maximal degranulation ($CD107a^+$).

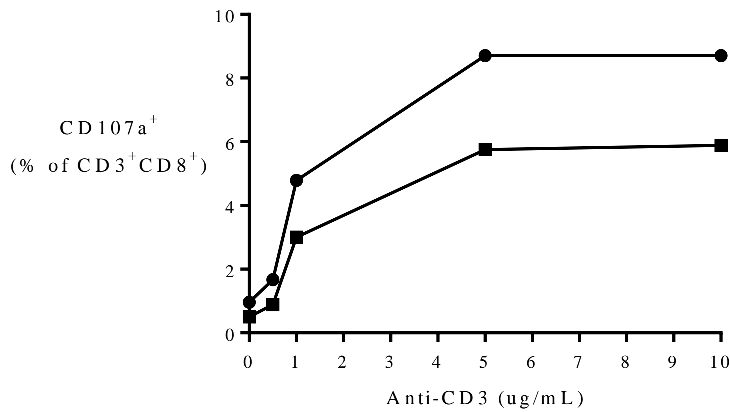


Figure S3: The Duration of Stimulation with Anti-CD3 was Optimized to Elicit Maximal Degranulation. PBMCs from healthy donors (n=2) were stimulated with 5 ug/mL of anti-CD3 for varying amounts of time. CD107a expression was measured on $CD3^{+}CD8^{+}$ cells. Maximal degranulation ($CD107a^{+}$) occurred after 5 hours of stimulation.

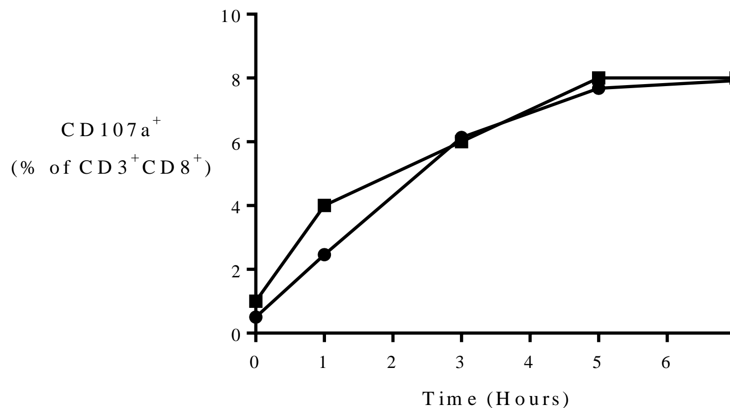
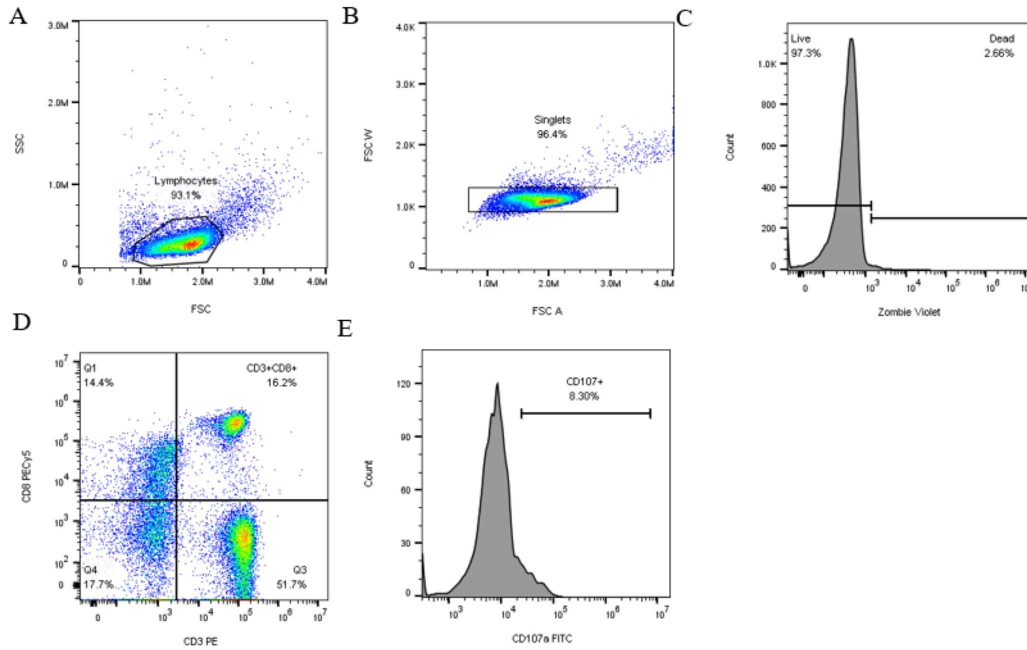


Figure S4: The Gating Strategy for the Measurement of CD107a on $CD3^{+}CD8^{+}$ cells. A) Lymphocytes were gated based on size (FSC) and internal complexity (SSC). B) Only single events ('singlets') were included. C) Only live

cells were included based on zombie violet viability dye. D) Live CD8$^+$ T cells (CD3$^+$CD8$^+$) were gated, located in the top right quadrant. D) CD107a expression was measured on the surface of live CD8$^+$ T cells.



CHAPTER 4

Platelet-Specific T Cells in Patients with Immune Thrombocytopenia

John R Vrbensky, Ishac Nazy, John G Kelton, Mark Larché, Rumi Clare, Hina Bhakta, Maria Roussakis, Viktoriya Rybenchuk, Donald M Arnold

Author's Preface

In chapter 3, it was shown that $CD8^+$ T cells in ITP patients are ready to destroy target cells, but whether they can specifically target platelets or megakaryocytes is uncertain. In order to test this, we must first identify platelet-specific $CD8^+$ T cells.

The goals of chapter 4 are to:

1) Establish a flow cytometric assay that can detect antigen-specific T cell proliferation.
2) Use the proliferation assay to detect platelet-specific $CD8^+$ T cells in ITP patients.
3) Analyze associations between platelet-specific $CD8^+$ T cells and clinical/ laboratory characteristics of ITP.
4) Assess $CD8^+$ T cell regulation and activity in ITP patients.

The findings in this chapter demonstrate that platelet-specific $CD8^+$ T cells can be detected in ITP patients and healthy individuals. T cell regulation is diminished and $CD8^+$ T cell activity is elevated in ITP patients compared to healthy individuals and thrombocytopenic patients with non-ITP. These results provide the justification and the foundation for the isolation of platelet-specific

$CD8^+$ T cells, which can be further characterized in the future for their ability to destroy platelets and/or megakaryocytes.

Note to the reader: At the time of this thesis submission to McMaster University, a manuscript corresponding with this chapter is being prepared to submit for publication.

Abstract

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count and an increased susceptibility for bleeding. $CD8^+$ T cells, which typically destroy virally infected or transformed cells, have been implicated in the destruction of platelets and megakaryocytes in ITP. To date, the evidence for platelet-specific $CD8^+$ T cells in patients with ITP is lacking. Herein we demonstrate that $CD8^+$ T cells from ITP patients and healthy individuals can proliferate in response to platelet glycoproteins IIbIIIa and Ibα. T cell regulation was impaired in ITP patients as indicated by low plasma TGF-β and elevated IL-6 compared to plasma from healthy individuals and thrombocytopenic patients with non-ITP. $CD8^+$ T cell activity was high in ITP patients as indicated by elevated plasma levels of sCD137, granzyme A, and granzyme B compared to healthy individuals and thrombocytopenic patients with non-ITP. Altogether, these results suggest that autoreactive platelet-specific $CD8^+$ T cells can be found in health and disease, and that $CD8^+$ T cell activity is dysregulated in ITP.

Introduction

Primary immune thrombocytopenia (ITP) is a bleeding disorder characterized by isolated thrombocytopenia (platelet count $<100 \times 10^9$/L)[1,2]. Since platelets are essential for primary hemostasis, patients with ITP have an increased risk of bleeding. The low platelet count in ITP is attributed to increased platelet destruction in the

spleen and liver and platelet underproduction due to the destruction or dysfunction of bone marrow megakaryocytes[3]. The prevailing mechanism of thrombocytopenia in ITP is autoantibody-mediated platelet destruction and underproduction. These autoantibodies target glycoproteins (GP) IIbIIIa and IbIX on platelets and megakaryocytes, which function as receptors for fibrinogen[4] and von Willebrand factor[5], respectively. In a systematic review and meta-analysis, we showed that anti-GP IIbIIIa and anti-GP IbIX autoantibodies were detectable in only 53% of primary ITP patients, suggesting that other mechanisms leading to thrombocytopenia are possible in ITP[6]. $CD8^+$ T cells have been proposed as a potential mechanism of platelet destruction and underproduction in ITP[7,8]. Evidence of $CD8^+$ T cells that specifically recognize platelet antigens is required to support this hypothesis.

$CD8^+$ T cells recognize processed antigen in the context of major histocompatibility complex (MHC) class I, so it is possible for them to interact with megakaryocytes and platelets since these cells express MHC I[9,10]. $CD8^+$ T cells are responsible for killing virally infected or transformed cells, but they can also be autoreactive. In type 1 diabetes (T1D) for example, $CD8^+$ T cells specific for insulin can be found in the pancreas[11], and preproinsulin-specific $CD8^+$ T cell clones isolated from type 1 diabetics can recognize and destroy β

pancreatic cells from human leukocyte antigen (HLA)-matched organ donors[12].

We recently reviewed the common laboratory methods used to identify antigen-specific $CD8^+$ T cells and suggested that these methods would be useful to identify autoreactive $CD8^+$ T cells in ITP[13]. In the current study, we hypothesized that platelet-specific $CD8^+$ T cells would be detected in a subset of ITP patients using a proliferation-based assay. Platelet-specific $CD8^+$ T cells were expanded and detected in peripheral blood samples from ITP patients and healthy individuals. Cytokine profiling suggested that in ITP, T cell regulation was impaired and $CD8^+$ T cell activity was elevated compared to healthy individuals and thrombocytopenic patients with non-ITP. These results suggest that autoreactive platelet-specific $CD8^+$ T cells can be found in both health and disease, but in ITP, the dysregulation of $CD8^+$ T cells might be involved in disease pathogenesis.

Materials and Methods

Blood Samples

Peripheral blood samples (40 mL) drawn into sterile heparinized vacutainers were used for cell isolation; 30 mL of blood drawn into acid citrate dextrose were used for anti-platelet

autoantibody testing; 2 mL of blood collected in ethylenediaminetetraacetic acid were used to measure the platelet count on a Coulter A^{c}T diff cell counter. ITP patients (n=11) were recruited from the McMaster ITP registry[14] and diagnosed with primary ITP based on a circulating platelet count <100x10^9/L in the absence of other potential causes[1]. Healthy individuals (n=10) with normal platelet counts were included as controls for T cell proliferation and plasma cytokine analysis. Thrombocytopenic patients with non-ITP (n=5) were included as controls for plasma cytokine analysis only. Informed consent was obtained from all individuals in accordance with the Hamilton Integrated Research Ethics Board.

Anti-Platelet Autoantibodies

The direct antigen capture assay (ACA) was used to measure platelet-bound autoantibodies against GP IIbIIIa and GP IbIX, as previously described[15]. The platelet count was adjusted to 300 000 platelets/µL, and samples with very low platelets were resuspended in the minimum volume required for testing (500 µL). Optical density (OD) readings were recorded at 405 nm (490 nm reference) using a BioTek 800 TS microplate reader. A positive result was defined as an OD > 0.21.

T Cell Proliferation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as previously described[16], then washed with Roswell Park Memorial Institute (RPMI) media. In order to be able to detect T cell proliferation, the PBMCs were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) for 20 minutes in a 37°C water bath while protected from light. Next, RPMI supplemented with 5% pooled human AB serum was added for 5 minutes to stop CFSE labelling. The cells were resuspended at 1×10^6 cells/mL in RPMI media supplemented with 5% pooled human AB serum, 100 U/mL penicillin, 100 µg/mL streptomycin (P/S), and 2 mM L-glutamine. After CFSE labelling, 5×10^5 PBMCs (0.5 mL) were added per well in a 24-well tissue culture plate. For background proliferation (in media), phosphate buffered saline (PBS) and ddH_2O were added as buffer controls. As a positive control for total T cell proliferation, PBMCs were stimulated with 10 µL of anti-CD3/anti-CD28 beads and 10 ng/mL recombinant human IL-2. As a positive control for antigen-specific proliferation, PBMCs were stimulated with 1 µg/mL of tetanus toxoid (TT). In order to detect platelet-specific T cell proliferation, PBMCs were stimulated with 1 µg/mL of either GP IIbIIIa or GP Ibα.

T cell proliferation was assessed with a Cytoflex flow cytometer 9 days after cell stimulation. The cells were washed with PBS before

labelling with zombie violet viability dye for 15 minutes at room temperature in the dark. Next, the cells were surface labelled with fluorophore-conjugated antibodies in staining buffer for 30 minutes at 4°C in the dark (anti-CD3 PE, anti-CD4 APC Cy7, anti-CD8 APC, anti-CD14 BV421, and anti-CD20 BV421). Proliferation was expressed as the percentage of $CFSE^{low}$ cells (below the minimal CFSE signal in the background control) out of $CD3^{+}CD4^{+}$ or $CD3^{+}CD8^{+}$ cells that were alive, $CD14^{-}$, and $CD20^{-}$. Representative plots of T cell proliferation are shown in Figure 1A, and the gating strategy is shown in Figure S1. The $CFSE^{low}$ signal was normalized to the mean + 3 standard deviations (SD) of the background, set to 1%. The mean of two replicates was reported for T cell proliferation in response to media, tetanus toxoid, or anti-CD3/anti-CD28 beads plus IL-2. The maximum signal from six replicates stimulated with platelet GPs was reported, due to the low frequency of autoreactive T cells. Fluorescence minus one (FMO) controls were used to determine the fluorescence due to specific binding. Compensation matrices were created using single-stained cells. Data analysis was performed with FlowJo version 10.

Cytokine Multiplex

Platelet rich plasma (PRP) was centrifuged at 1900 g for 10 minutes to obtain platelet poor plasma (PPP), which was stored at -80°C until use. The concentration of TGF-β, IL-6, granzyme A, granzyme B,

and sCD137 were analyzed in PPP samples by the multiplex fluorescent bead assay (Eve Technologies in Alberta, Canada). Plasma from 18 samples (8 ITP patients, 5 healthy individuals, and 5 thrombocytopenic patients with non-ITP) were analyzed in duplicate measurements. Five of 8 ITP patients and 5 of 5 healthy individuals assessed for plasma cytokines were also tested in the T cell proliferation assay. Thrombocytopenic patients with non-ITP were included in the plasma cytokine analysis only. Any values below the detection limit were reported as the lowest observed cytokine concentration.

Statistical Analysis

Graph Pad Prism version 9.0.2 was used to perform statistical analysis and to create graphs. Age and platelet counts were reported as the median with the range. T cell proliferation and plasma cytokines were reported as the mean ± standard deviation. The D'Agostino-Pearson test was used to assess normality. Comparisons were made with t tests for normally distributed data; otherwise Wilcoxon/Mann-Whitney tests were used. Odds ratios were calculated using Fisher's exact test and reported with 95% confidence intervals (CI). Agreements between laboratory tests were calculated using Cohen's kappa (κ). Correlations (R^2) between T cell proliferation, cytokine levels, and platelet counts were also assessed. For all tests, p values <0.05 were considered significant.

Results

ITP Patient Characteristics

The median platelet count of the ITP patients was 39 x 10^9/L (range: 1-84 x 10^9/L). Nine of the ITP patients were female (82%). The median age was 49 years (range: 19-73 years). In the direct anti-platelet autoantibody test, 9 ITP patients tested positive for anti-GP IIbIIIa (82%), 8 were positive for anti-GP IbIX (73%), and 9 patients were positive for either anti-GP IIbIIIa or anti-GP IbIX (82%). The clinical characteristics of ITP patients are listed in Table 1; healthy individuals and thrombocytopenic control patients with non-ITP are listed in Tables S1 and S2, respectively.

Total and Tetanus-Specific T Cell Proliferation

We evaluated $CD4^+$ and $CD8^+$ T cell proliferation in response to anti-CD3/anti-CD28 beads plus IL-2 as a positive control for total T cell proliferation. All individuals who were tested (11/11 ITP patients and 10/10 healthy individuals) demonstrated $CD4^+$ (Figure 1B) and $CD8^+$ (Figure 1C) T cell proliferation to anti-CD3/anti-CD28 beads plus IL-2.

We evaluated $CD4^+$ and $CD8^+$ T cell proliferation in response to tetanus toxoid (TT) as a positive control for antigen-specific proliferation. Eight of 11 ITP patients (73%) and 9 of 10 healthy individuals (90%) showed a $CD4^+$ T cell response to TT. $CD4^+$ T cell proliferation in response to TT was 6.7% ± 6.4% in ITP patients (p=0.01* vs media) and 5.1% ± 5.8% in healthy individuals

(p=0.004** vs media; p=0.65 ITP vs healthy) (Figure 1D). Eight out of 11 ITP patients (73%) and 8 out of 10 healthy individuals (80%) showed a $CD8^+$ T cell response to TT. $CD8^+$ T cell proliferation in response to TT was 5.0% ± 6.8% in ITP patients (p=0.003** vs media) and 2.5% ± 2.5% in healthy individuals (p=0.01* vs media; p=0.51 ITP vs healthy) (Figure 1E). Three ITP patients and 1 healthy individual were re-tested once again up to 10 weeks after their initial assessment. In response to TT, the $CD4^+$ T cell responses were repeatable in 4/4 re-tests (100%) and the $CD8^+$ T cell responses were repeatable in 3/4 re-tests (75%) (Table 2).

Platelet-Specific T Cells in ITP Patients and Healthy Individuals

To test for autoreactive T cells, we evaluated $CD4^+$ and $CD8^+$ T cell proliferation in response to platelet GP IIbIIIa and GP Ibα. Four of 11 ITP patients (36%) and 5 out of 10 healthy individuals (50%) produced a $CD4^+$ T cell proliferative response to GP IIbIIIa. $CD4^+$ T cell proliferation in response to GP IIbIIIa was 1.1% ± 0.6% in ITP patients (p=0.04* vs media) and 1.1% ± 0.4% in healthy individuals (p=0.004** vs media; p=0.98 ITP vs healthy) (Figure 2A). Five of 11 ITP patients (45%) and 6 out of 10 healthy individuals (60%) showed a $CD8^+$ T cell proliferative response to GP IIbIIIa. $CD8^+$ T cell proliferation in response to GP IIbIIIa was 0.9% ± 0.5% in ITP patients (p=0.01* vs media) and 1.1% ± 0.3% in healthy individuals (p=0.002** vs media; p=0.31 ITP vs healthy) (Figure 2B). Three ITP patients and 1 healthy individual were re-tested up to 10

weeks after their initial test. In response to GP IIbIIIa, the $CD4^+$ T and $CD8^+$ T cell results were repeatable in 4/4 re-tests (100%) (Table 2).

Six of 11 ITP patients (54%) and 6 out of 10 healthy individuals (60%) showed a $CD4^+$ T cell proliferative response to GP Ibα. $CD4^+$ T cell proliferation in response to GP Ibα was 1.7% ± 1.3% in ITP patients (p=0.01* vs media) and 1.3% ± 0.9% in healthy individuals (p=0.002** vs media; p=0.65 ITP vs healthy) (Figure 2A). Nine of 11 ITP patients (82%) and 7 out of 10 healthy individuals (70%) showed a $CD8^+$ T cell proliferative response to GP Ibα. $CD8^+$ T cell proliferation in response to GP Ibα was 1.6% ± 1.0% in ITP patients (p=0.004** vs media) and 1.1% ± 0.4% in healthy individuals (p=0.002** vs media; p=0.14 ITP vs healthy) (Figure 2B). Three ITP patients and 1 healthy individual were re-tested up to 10 weeks after their initial test. In response to GP Ibα, the $CD4^+$ T and $CD8^+$ T cell results were repeatable in 3/4 re-tests (75%) (Table 2).

Clinical and Laboratory Associations with Platelet-Specific T Cells

In order to investigate the association between disease severity and the presence of platelet-specific T cells, we compared platelet counts in ITP patients with and without platelet-specific T cells. Platelet counts were not statistically different in patients with or without T cells specific to GP IIbIIIa or GP Ibα. The median platelet count for patients with GP IIbIIIa $CD4^+$ T cells was 52 x 10^9/L (range: 9-84 x 10^9/L) vs 26 x 10^9/L (range: 1-60 x 10^9/L) without, p=0.27; with GP Ibα $CD4^+$ T cells was 11 x 10^9/L (range: 1-84 x 10^9/L) vs 39 x 10^9/L (range:

2-60 x 10^9/L) without, p=0.51; with GP IIbIIIa $CD8^+$ T cells was 52 x 10^9/L (range: 1-84 x 10^9/L) vs 26 x10^9/L (range: 2-60 x 10^9/L) without, p=0.44; with GP Ibα $CD8^+$ T cells was 26 x10^9/L (range: 1-84 x 10^9/L) vs 39 x 10^9/L (range: 3-60 x 10^9/L) without, p=0.91. In addition, the odds of severe thrombocytopenia (platelets <30 x 10^9/L) were not different among patients with or without platelet-specific $CD4^+$ or $CD8^+$ T cells (GP IIbIIIa $CD4^+$ T cells, OR: 0.5 (95% CI: 0.03-6.1, p>0.99); GP Ibα $CD4^+$ T cells, OR: 8.0 (95% CI: 0.60-117.9, p=0.24); GP IIbIIIa $CD8^+$ T cells, OR: 0.67 (95% CI: 0.08-5.8, p>0.99); GP Ibα $CD8^+$ T cells, OR: 2.0 (95% CI: 0.16-35.4, p>0.99)) (Figure 3A).

We compared direct autoantibody test results in ITP patients and found no difference among patients with or without platelet-specific T cells. The median anti-GP IIbIIIa OD for patients with GP IIbIIIa $CD4^+$ T cells was 0.36 vs 0.85 without (p=0.23), and with GP IIbIIIa $CD8^+$ T cells was 0.58 vs 0.55 without (p=0.93) (Figure 3B). The median anti-GP IbIX OD for patients with GP Ibα $CD4^+$ T cells was 0.37 vs 0.95 without (p=0.12), and with GP Ibα $CD8^+$ T cells was 0.48 vs 1.1 without (p=0.22) (Figure 3C). Direct autoantibody testing and platelet-specific $CD4^+$ or $CD8^+$ T cell proliferation demonstrated a lack of agreement. The presence of platelet-specific $CD4^+$ T cells to GP IIbIIIa or GP Ibα did not coincide with the presence of anti-GP IIbIIIa, κ= -0.08 (95% CI: -0.48-0.31) or anti-GP IbIX, κ= -0.14 (95% CI: -0.67-0.39), respectively. The absence of anti-GP IIbIIIa or anti-GP IbIX did not coincide with the presence of $CD8^+$ T

cells to GP IIbIIIa, κ= 0.04 (95% CI: -0.45-0.52) or GP Ibα, κ= 0.12 (95% CI: -0.09-0.40), respectively.

Plasma Cytokine Multiplex

We measured plasma levels of TGF-β, IL-6, granzyme A, granzyme B, and sCD137 as indicators of T cell regulation and $CD8^{+}$ T cell activity in ITP patients (n=8), healthy individuals (n=5), and thrombocytopenic patients with non-ITP (n=5). The mean plasma concentration of TGF-β was lower in ITP patients (1753 pg/mL ± 1189 pg/mL) compared to healthy individuals (5996 pg/mL ± 2498 pg/mL, p=0.003**) and non-ITP patients (3487 pg/mL ± 2254 pg/mL, p=0.04*) (healthy vs non-ITP, p=0.13) (Figure 4A). The mean plasma concentration of IL-6 was elevated in ITP patients (19.7 pg/mL ± 17.1 pg/mL) compared to healthy individuals (1.6 pg/mL ± 2.4 pg/mL, p=0.02*) and non-ITP patients (3.6 pg/mL ± 8.0 pg/mL, p=0.04*) (healthy vs non-ITP, p=0.40) (Figure 4B). The mean plasma concentration of granzyme A was elevated in ITP patients (1781 pg/mL ± 1661 pg/mL) compared to healthy individuals (304 pg/mL ± 373 pg/mL, p=0.04*) and non-ITP patients (194 pg/mL ± 130 pg/mL, p=0.01*) (healthy vs non-ITP, p=0.63) (Figure 4C). The mean plasma concentration of granzyme B was elevated in ITP patients (16.9 pg/mL ± 15.8 pg/mL) compared to healthy individuals (3.4 pg/mL ± 2.8 pg/mL, p=0.04*) and non-ITP patients (1.7 pg/mL ± 3.1 pg/mL, p=0.006**) (healthy vs non-ITP, p=0.22) (Figure 4D). The mean plasma concentration of sCD137 was elevated in ITP patients (39.0 pg/mL ± 23.0 pg/mL) compared to healthy individuals (14.4 pg/mL ± 4.0 pg/mL,

p=0.04*) and non-ITP patients (13.1 pg/mL ± 7.1 pg/mL, p=0.03*) (healthy vs non-ITP, p=0.74) (Figure 4E). There were no associations between plasma cytokine levels and T cell proliferation or platelet counts in ITP patients (not shown).

Tables

Table 1: Clinical Characteristics and Laboratory Results of ITP Patients

Patient ID	Age	Sex	Diagnosis	Platelet Count ($x10^9/L$)	Current Treatments	Anti-GP IIbIIIa (OD)	Anti-GP Ibα (OD)	$CD4^+$ GP IIbIIIa	$CD4^+$ GP Ibα	$CD8^+$ GP IIbIIIa	$CD8^+$ GP Ibα
1	72	M	Primary ITP	84	None	0.467	0.493	+	+	+	+
2	49	F	Primary ITP	52	TXA	-	-	+	+	+	+
3*	29	M	Primary ITP	9	IVIg	0.582	0.509	+	+	+	+
4*	67	F	Primary ITP	1	IVIg	0.773	0.48	-	+	+	+
5	19	F	Primary ITP	21	DEX	0.252	0.253	+	+	-	+
6	45	F	Primary ITP	54	IVIg	0.951	3.9	-	-	+	+
7*	65	F	Primary ITP	12	IVIg	-	-	-	+	-	+
8*	73	F	Primary ITP	39	IVIg	0.852	-	-	-	-	+
9	29	F	Primary ITP	2	IVIg	1.855	0.954	-	-	-	+
10	54	F	Primary ITP	39	AZA, PRED	2.711	1.351	-	-	-	-
11*	42	F	Primary ITP	60	IVIg	0.229	0.781	-	-	-	-

*: plasma cytokine analysis was conducted for these patients. F: female, M: male. AZA: azathioprine, PRED: prednisone, TXA: tranexamic acid, IVIg: intravenous immune globulin, DEX: dexamethasone. OD: optical density, reported as negative (-) when the OD was below the threshold of 0.21.

Table 2: The Repeatability of Antigen-Specific CD4+ and CD8+ T Cell Measurements Over Time

	Time Between Tests (weeks)	CD4+ T Cells						CD8+ T Cells					
		Tetanus		GP IIbIIIa		GP Ibα		Tetanus		GP IIbIIIa		GP Ibα	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
ITP 1	4	-	-	-	-	+	-	-	-	-	-	+	-
ITP 2	8	+	+	+	+	+	+	+	+	+	+	+	+
ITP 3	10	+	+	-	-	+	+	-	+	-	-	-	-
Healthy 1	5	+	+	+	+	+	+	+	+	-	-	-	-

Four individuals (3 ITP patients, 1 healthy) were re-tested for the presence of antigen specific T cells up to 10 weeks after their initial test. (+): a positive test result indicating the presence of antigen specific T cells. (-): a negative test result indicating the absence of antigen specific T cells. Note that the numbers assigned to individuals in Table 2 do not match those in Table 1 or Table S1.

Figure Legends

Figure 1: Detection of T Cell Proliferation. A) Representative plots showing $CD8^+$ T cell proliferation in response to antigens (GP IIbIIIa, GP Ibα, or tetanus toxoid) or αCD3 αCD28 beads plus IL-2. The percentage of $CFSE^{low}$ events indicates the extent of T cell proliferation over 9 days of stimulation. B-E) Positive controls for total proliferation (αCD3 αCD28 plus IL-2) and antigen-specific proliferation (tetanus toxoid; TT). The percentage of $CFSE^{low}$ cells was normalized to the mean + 3SD of the background (media). The threshold for a positive T cell response is indicated by the dashed line at 1%. White and black symbols indicate results that did or did not cross the threshold, respectively.

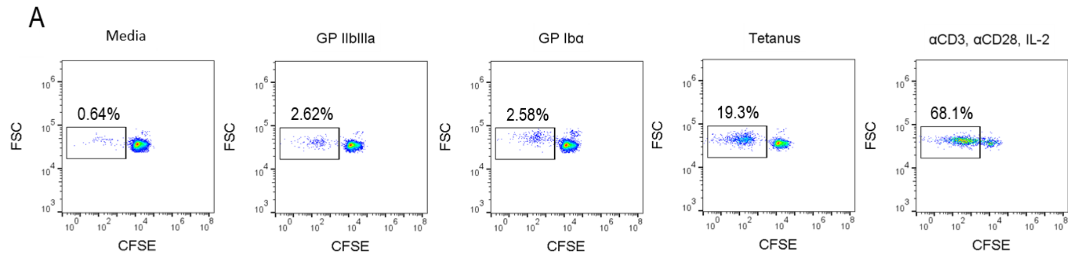
Figure 2: Platelet-Specific $CD4^+$ and $CD8^+$ T Cells in ITP Patients and Healthy Individuals. A) $CD4^+$ T cell responses to GP IIbIIIa and GP Ibα B) $CD8^+$ T cell responses to GP IIbIIIa and GP Ibα. The percentage of $CFSE^{low}$ events indicates the extent of T cell proliferation over 9 days of stimulation. The percentage of $CFSE^{low}$ cells was normalized to the mean + 3SD of the background (media). The threshold for a positive T cell response is indicated by the dashed line at 1%. White and black symbols indicate results that did or did not cross the threshold, respectively. T cell proliferation in response to platelet autoantigens was not statistically different between ITP patients and healthy individuals.

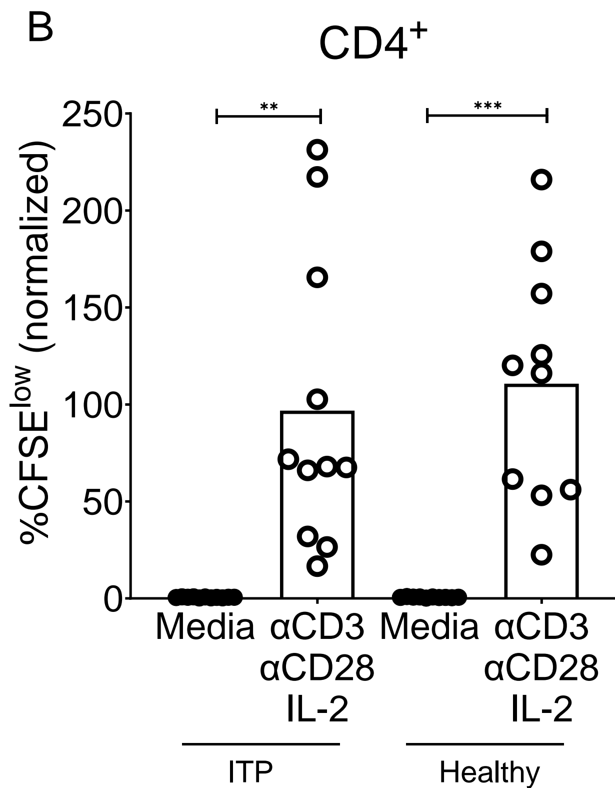
Figure 3: An Assessment of the Associations Between Platelet-Specific T Cells and Disease Characteristics of ITP Patients. A) Platelet counts were compared in patients with (+) and without (-) platelet-specific $CD4^+$ or $CD8^+$ T cells. Odds ratios for severe thrombocytopenia were based on platelet counts less than 30 x 10^9 platelets/L (dashed line). B and C) Direct autoantibody test results were compared in patients with (+) and without (-) platelet-specific $CD4^+$ or $CD8^+$ T cells. A positive result for anti-platelet autoantibodies was indicated by an OD > 0.21 (dashed line). ns: not statistically significant.

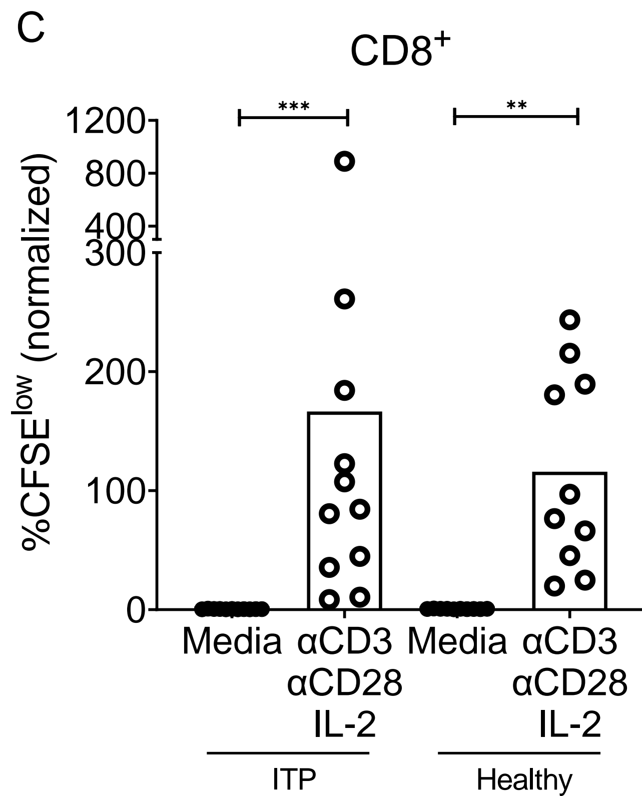
Figure 4: Plasma Cytokine Analysis. Plasma levels of TGF-β, IL-6, granzyme A, granzyme B, and sCD137 were measured in ITP patients, healthy individuals, and thrombocytopenic patients with non-ITP. A) TGF-β was lower in ITP patients compared to healthy individuals and non-ITP patients. B-E) IL-6, granzyme A, granzyme B, and sCD137 were higher in ITP patients compared to healthy individuals and non-ITP patients.

Figures

Figure 1




B
CD4+
%CFSElow (normalized)
250
200
150
100
50
0
**
***
Media
αCD3
αCD28
IL-2
Media
αCD3
αCD28
IL-2
ITP
Healthy


C
CD8+
***
**
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800
400
300
200
100
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Media
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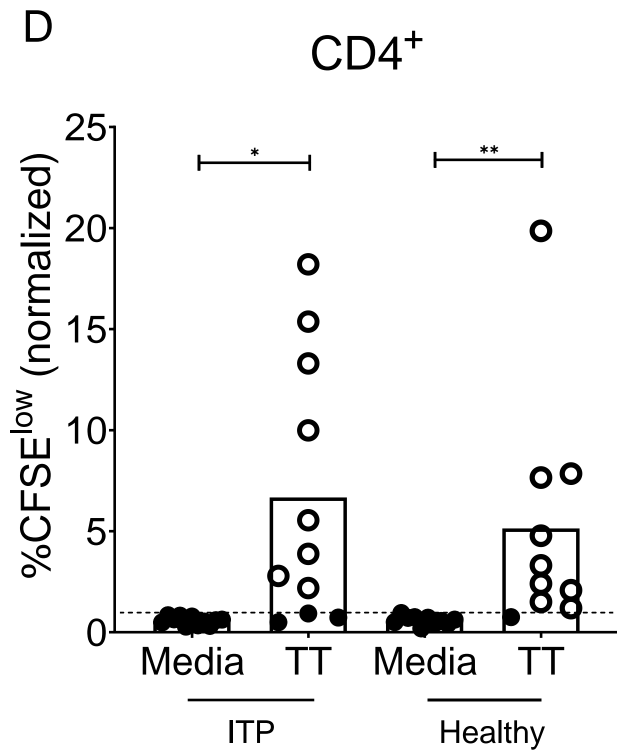
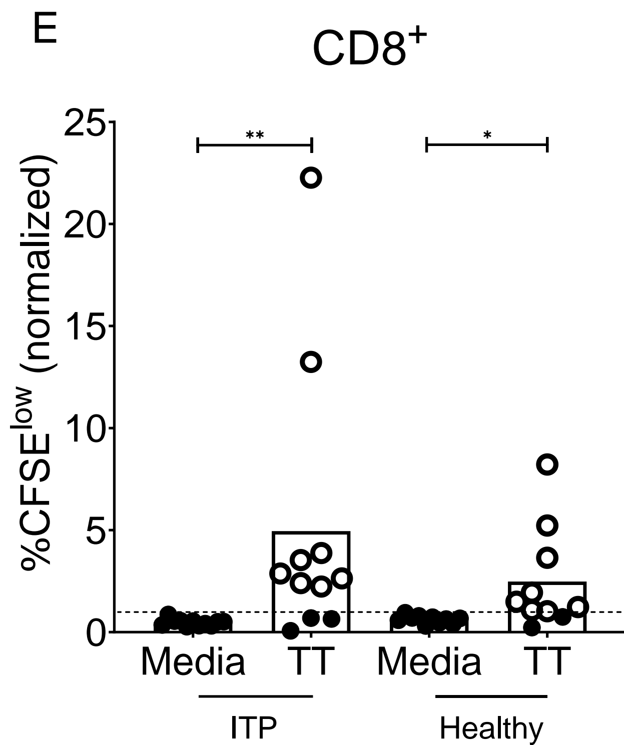
αCD28
IL-2
Media
αCD3
αCD28
IL-2
ITP
Healthy


D
CD4+
%CFSElow (normalized)
25
20
15
10
5
0
*
**
Media
TT
Media
TT
ITP
Healthy


E
CD8+
%CFSElow (normalized)
25
20
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**
*
Media
TT
Media
TT
ITP
Healthy

Figure 2

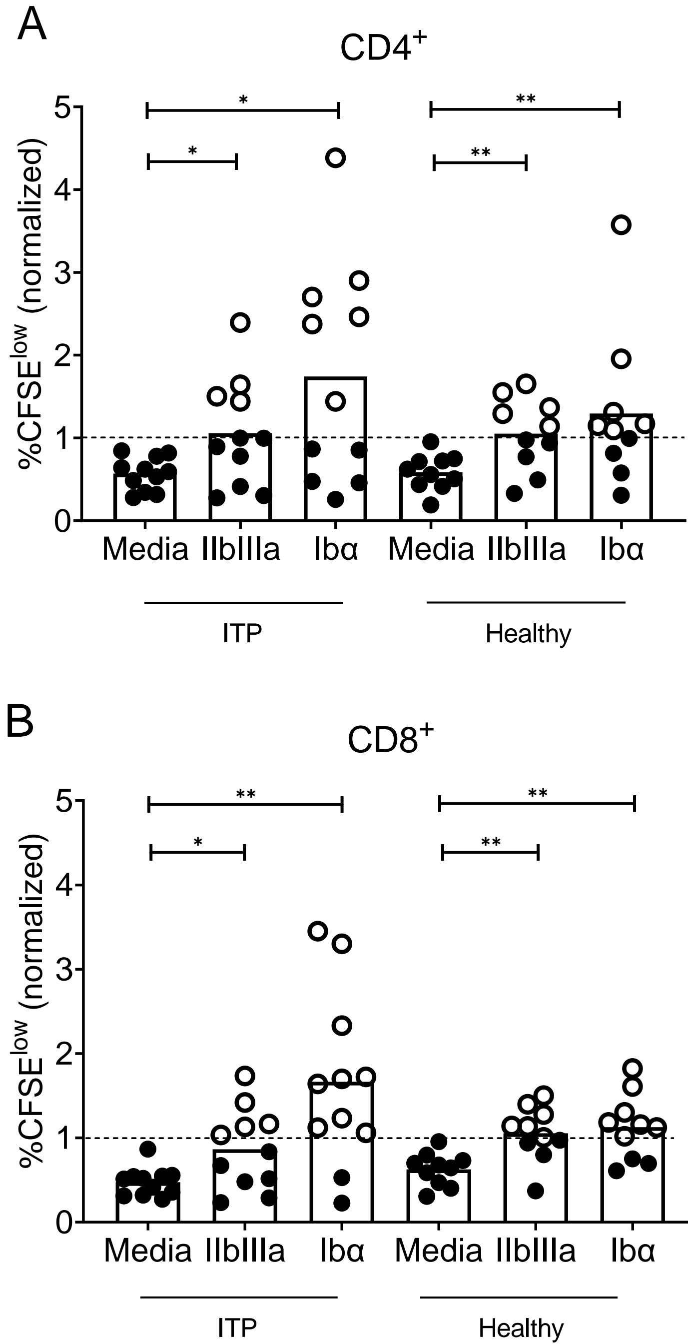
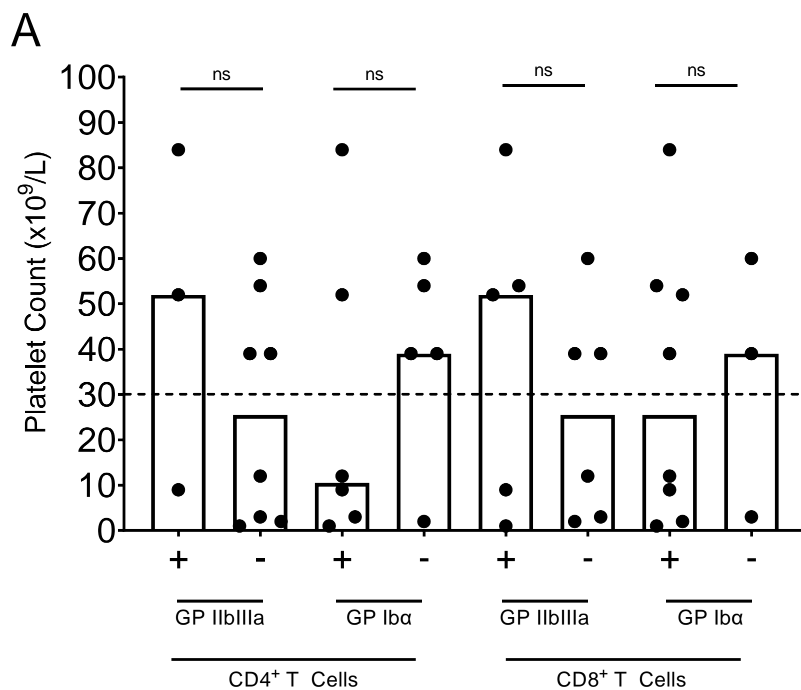
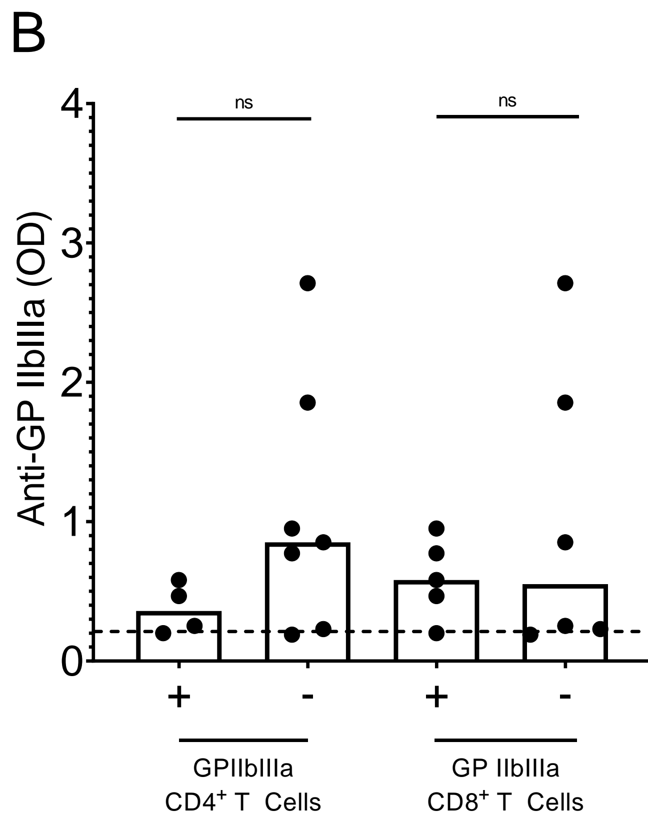


Figure 3




B
4
3
2
1
0
Anti-GP IIbIIIa (OD)
ns
ns
+
-
+
-
GPIIbIIIa
CD4+ T Cells
GP IIbIIIa
CD8+ T Cells

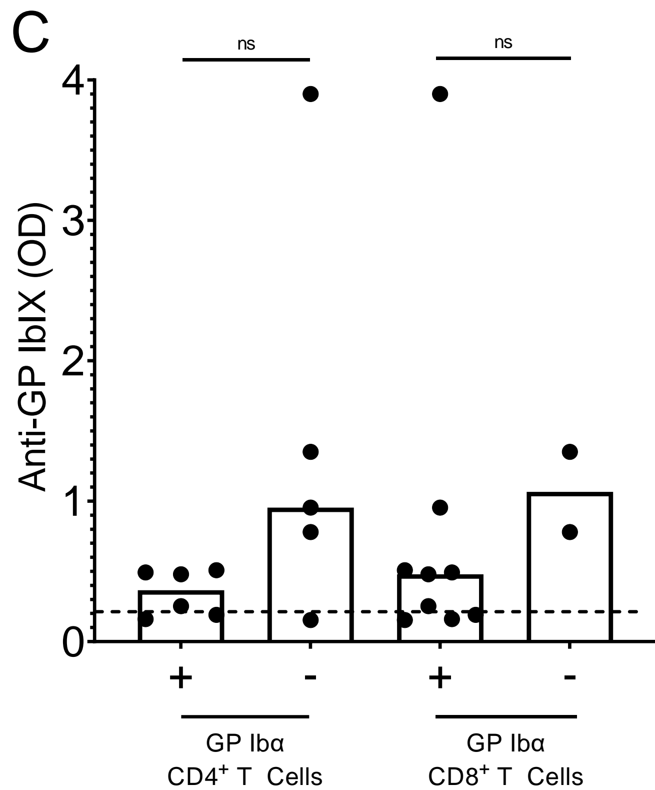
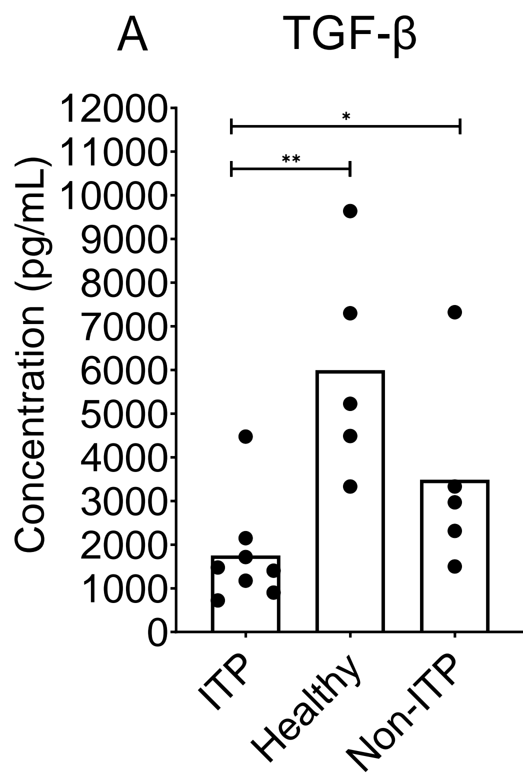
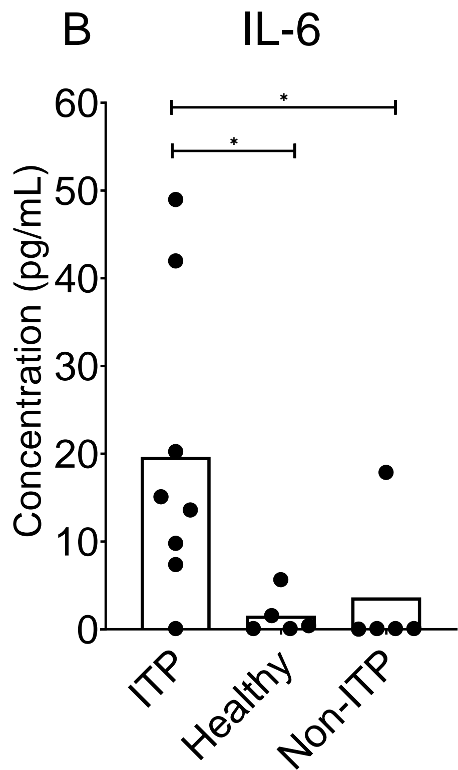

C
ns
ns
Anti-GP IbIX (OD)
0
1
2
3
4
+
-
+
-
GP Ibα
CD4+ T Cells
GP Ibα
CD8+ T Cells

Figure 4




B
IL-6
Concentration (pg/mL)
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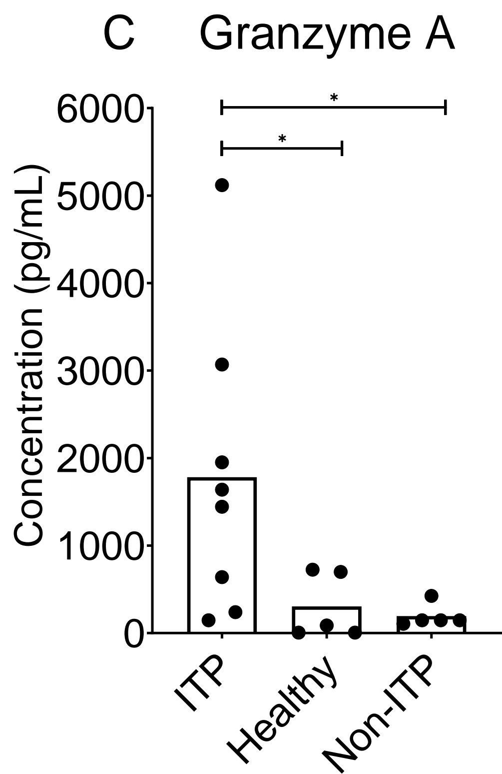
50
40
30
20
10
0
*
*
ITP
Healthy
Non-ITP


C
Granzyme A
Concentration (pg/mL)
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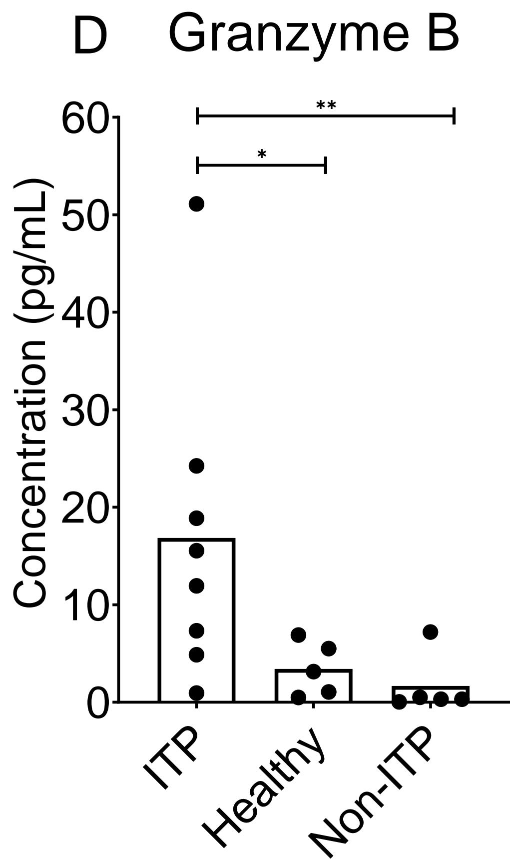
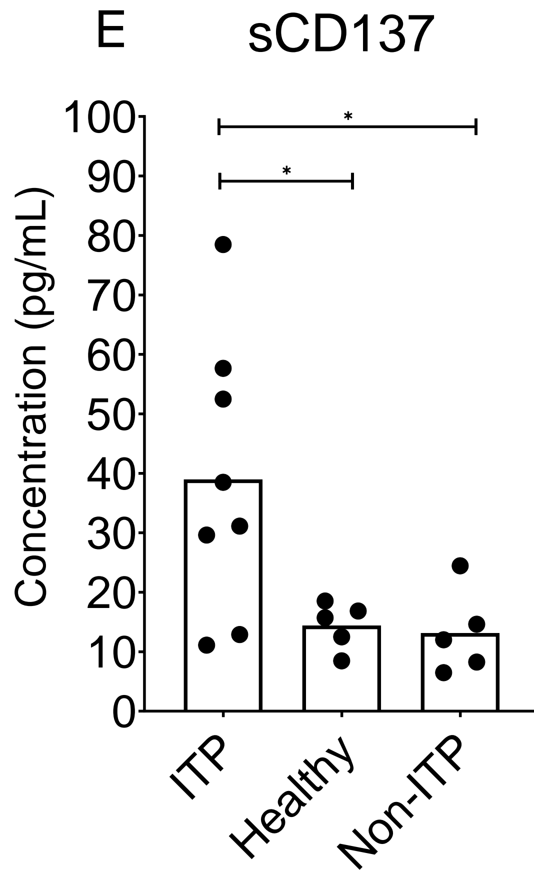
5000
4000
3000
2000
1000
0
*
*
ITP
Healthy
Non-ITP


D
Granzyme B
Concentration (pg/mL)
60
50
40
30
20
10
0
**
*
ITP
Healthy
Non-ITP


E
sCD137
Concentration (pg/mL)
100
90
80
70
60
50
40
30
20
10
0
*
*
ITP
Healthy
Non-ITP

Discussion

The pathogenesis of ITP is typically attributed to the effects of anti-platelet autoantibodies. However, half of all ITP patients do not have detectable anti-platelet autoantibodies[6]. $CD8^+$ T cells represent another possible mechanism of disease in ITP[7], however platelet-specific $CD8^+$ T cells have not been described to date. In this study, we examined the ability of $CD8^+$ T cells to proliferate in response to the platelet autoantigens GP IIbIIIa and GP Ibα. Of the 11 ITP patients in this study, 9 (82%) elicited a $CD8^+$ T cell proliferative response to platelet autoantigens; of which 5 (55%) responded to GP IIbIIIa and 9 (100%) responded to GP Ibα. A similar $CD8^+$ T cell response to GP IIbIIIa and GP Ibα was produced in healthy individuals with normal platelet counts, so this observation is not specific to ITP. The proliferative response to platelet autoantigens suggests that ITP patients and even healthy individuals harbour populations of $CD8^+$ T cells with T cell receptors (TCRs) specific to platelet peptide-MHC I complexes. In a prior study, $CD8^+$ T cells from ITP patients proliferated in response to incubation with antigen presenting cells (APCs) and autologous platelets[8]. Li et al found that $CD8^+$ T cell proliferation was greater in ITP patients compared to healthy individuals, which may be due to elevated T cell responsiveness to platelet autoantigens or enhanced antigen presentation by APCs[17]. However, the specificity of the $CD8^+$ T cell response was uncertain since platelets can internalize, process, and present exogenous antigens in the context of MHC class I[10,18,19]. Our study provides direct evidence for the existence of $CD8^+$

T cells specific to platelets since proliferation occurred in response to the presentation of platelet autoantigens.

In the absence of anti-platelet autoantibodies, ITP patients were not more likely to have platelet-specific $CD8^+$ T cells than patients with autoantibodies, which suggests that autoantibodies and $CD8^+$ T cells are not mutually exclusive disease mechanisms in ITP. In order to better understand the relationship between autoantibodies and autoreactive T cells in ITP, we also measured $CD4^+$ T cell proliferation in response to platelet autoantigens. We found that ITP patients and healthy individuals had $CD4^+$ T cells capable of proliferating in response to GP IIbIIIa and GP Ibα. These results suggest that the responding $CD4^+$ T cells have TCRs specific to platelet peptide-MHC II complexes. It will be important to determine which $CD4^+$ T cell subsets (T_H1/T_H2, etc.) are represented within the group of platelet-specific $CD4^+$ T cells in order to better understand their function. Our results are consistent with previous work which demonstrated $CD4^+$ T cell specificity to platelet autoantigens such as GP IIbIIIa in ITP patients[20,21] and in healthy individuals[22]. We expected an association between platelet-specific $CD4^+$ T cell proliferation and direct anti-platelet autoantibody results, since $CD4^+$ T cell help is central to the production of class switched antibodies[23]. However, there were no associations between autoantibody results and platelet-specific $CD4^+$ T cell proliferation. In the group of ITP patients we studied, most had detectable anti-GP IIbIIIa or anti-GP IbIX autoantibodies, which might explain the lack of association between T cell proliferation results and autoantibody testing since

only 2 patients did not have autoantibodies. The absence of an association could also be due to the T cell source (peripheral blood), since most T cells reside and function in tissues[24], and since lymphoid organs are the major site of interaction between $CD4^+$ T cells and B cells[23].

There were no associations between platelet counts in ITP patients and T cell proliferation in response to platelet autoantigens. This may be partially explained by the disease stage and treatment status of most ITP patients included in the study. Of 11 ITP patients included in this study, 10 had chronic ITP and 1 had persistent ITP, and all ITP patients were receiving treatment except for one patient. In a study that searched for autoreactive T cells in patients with T1D, $CD8^+$ T cells specific to islet amyloid polypeptide were more readily detected in patients with recently diagnosed T1D[25]. A follow-up study designed to detect platelet-specific T cells in newly diagnosed and untreated ITP patients would be better suited to uncover potential associations between platelet-specific T cells and disease severity.

We measured plasma cytokine levels using a multiplex assay. TGF-β and IL-6 were assessed as surrogate markers of regulation. TGF-β is an immunoregulatory cytokine that promotes the differentiation of T regulatory (T_{reg}) cells and is produced by T_{regs}[26]. TGF-β suppresses the expansion and cytolytic activity of $CD8^+$ T cells and promotes tolerance in autoreactive T cells that have escaped negative selection[27]. IL-6 is a proinflammatory cytokine with pleiotropic effects, and we included it in our assessment of regulation because it inhibits T_{reg}

generation[28,29]. We observed low TGF-β and elevated IL-6 in ITP patients compared to healthy individuals and thrombocytopenic non-ITP patients, which suggests that T cell regulation is impaired in ITP. These results are consistent with the impaired T_{reg} activity previously described in ITP[30]. Our data is also consistent with previous reports of reduced levels of TGF-β in ITP[31,32], but not with data which demonstrated IL-6 levels were lower[33] or normal[34] in ITP.

In order to determine the extent of $CD8^+$ T cell activity in ITP, we assessed plasma granzyme A and granzyme B levels. Granzymes are serine proteases in cytotoxic granules which are released from $CD8^+$ T cells and natural killer (NK) cells to induce apoptosis in target cells through caspase-dependent or -independent pathways[35]. Our results indicated that plasma granzyme A and granzyme B were elevated in ITP patients compared to healthy individuals and thrombocytopenic non-ITP patients, suggesting that there is enhanced $CD8^+$ T cell activity in ITP. These results are consistent with previous studies which showed that plasma granzyme levels were elevated in ITP patients compared to healthy individuals[36], and that $CD8^+$ T cells from ITP patients have a high cytotoxic potential[16]. Whether platelet-specific $CD8^+$ T cells are the source of elevated granzymes A and B in ITP, and whether these platelet-specific $CD8^+$ T cells can directly destroy platelets or megakaryocytes remains to be determined.

We assessed sCD137, which is generated through alternative splicing in $CD137^+$ cells[37,38]. $CD4^+$ and $CD8^+$ T cells transiently express CD137 upon activation and T_{regs} constitutively express CD137[39]. Bidirectional signalling

between CD137 on T cells and CD137L on APCs provides co-stimulation to T cells and activates APCs. sCD137 competes with membrane bound CD137 (mCDC137) for CD137L binding on APCs, which acts as a negative feedback mechanism by inhibiting T cell co-stimulation[40]. We found that sCD137 was elevated in ITP patients compared to healthy individuals and thrombocytopenic non-ITP patients, which is consistent with elevated sCD137 in patients with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus[38,40,41]. Our observation might suggest elevated T_{reg} numbers in ITP patients since T_{regs} are a major source of sCD137[39,42]. However, this explanation is not aligned with previous findings which demonstrated normal[30] or reduced[43] T_{reg} numbers and reduced T_{reg} activity[30] in ITP. A more likely explanation for the elevated plasma sCD137 in ITP patients is the activation and clonal expansion of autoreactive $CD4^+$ or $CD8^+$ T cells responding to repeated platelet autoantigen exposure, which is consistent with our observations of decreased T_{reg} activity and enhanced effector $CD8^+$ T cell activity in ITP.

A limitation of this study is that platelet-specific $CD8^+$ T cells were not characterized for their ability to directly destroy platelets or megakaryocytes. Assessing platelet and megakaryocyte interactions with platelet-specific $CD8^+$ T cells will be an essential future step in interpreting the role that autoreactive $CD8^+$ T cells play in ITP. A strength of this study is that purified platelet autoantigens were used as a stimulus, which provides direct evidence of a $CD8^+$ T cell response towards platelets. Another strength is that the T cell responses to

platelet autoantigens and tetanus toxoid were repeatable. The percentage of responding cells, as well as the percentage of responding individuals to tetanus toxoid were consistent with previous reports[44,45]. Furthermore, the extent of autoantigen-specific T cell responses from ITP patients were comparable to other autoimmune diseases[44].

Summary

Herein, we provide direct evidence of $CD8^+$ T cells specific to platelet glycoproteins IIbIIIa and Ibα in ITP patients. These platelet-specific $CD8^+$ T cells can also be found in healthy individuals with normal platelet counts, so they are not a unique feature of ITP. However, the lack of T cell regulation in ITP relative to healthy individuals and thrombocytopenic non-ITP patients suggests that platelet-specific $CD8^+$ T cells might escape peripheral tolerance mechanisms and actively participate in the pathogenesis of ITP. Furthermore, the elevated levels of granzyme A, granzyme B, and sCD137 in ITP patients relative to healthy individuals and thrombocytopenic non-ITP patients suggests that $CD8^+$ T cells are highly active in ITP. Whether or not platelet-specific $CD8^+$ T cells can directly reduce the circulating platelet count will require an assessment of their ability to destroy platelets or megakaryocytes. Particular attention should be focused on the interactions between platelet-specific $CD8^+$ T cells and megakaryocytes since each megakaryocyte can produce thousands of platelets.

Conflicts of Interest Disclosures

None of the authors have conflicts of interest to declare.

Acknowledgements

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Authorship Contributions

JRV takes primary responsibility for the paper. JRV designed the research. JRV performed T cell experiments. HB, MR, and VR performed and analyzed anti-platelet autoantibody tests. JRV collected and analyzed data and performed statistical analysis. JRV, DMA, JGK, ML, RC, and IN interpreted data. JRV wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Supplementary Material

Table S1: Clinical Characteristics of Healthy Individuals

ID	Age	Sex	Platelet Count ($x10^9/L$)
HC 1*	58	F	234
HC 2*	58	M	246
HC 3	24	F	301
HC 4*	32	F	251
HC 5*	27	F	324
HC 6	63	M	235
HC 7	28	M	298
HC 8*	64	M	226
HC 9	26	M	249
HC 10	32	F	349

*: plasma cytokine analysis was conducted for these patients. HC: healthy control. F: female, M: male.

Table S2: Clinical Characteristics of Thrombocytopenic Non-ITP Patients and ITP Patients Tested for Plasma Cytokines Only

ID	Age	Sex	Platelet Count ($x10^9/L$)	Diagnosis
Non-ITP 1*	63	F	40	Splenomegaly/hypersplenism
Non-ITP 2*	32	F	72	Familial thrombocytopenia
Non-ITP 3*	68	M	60	Splenomegaly/hypersplenism
Non-ITP 4*	52	F	19	Splenomegaly/hypersplenism
Non-ITP 5*	72	F	47	Liver disease
ITP 12*	85	F	90	Primary ITP
ITP 13*	64	F	17	Primary ITP
ITP 14*	44	F	84	Primary ITP

*: plasma cytokine analysis was conducted for these patients, and T cell proliferation was not tested. Non-ITP: thrombocytopenic control patient. ITP: immune thrombocytopenia. F: female, M: male.

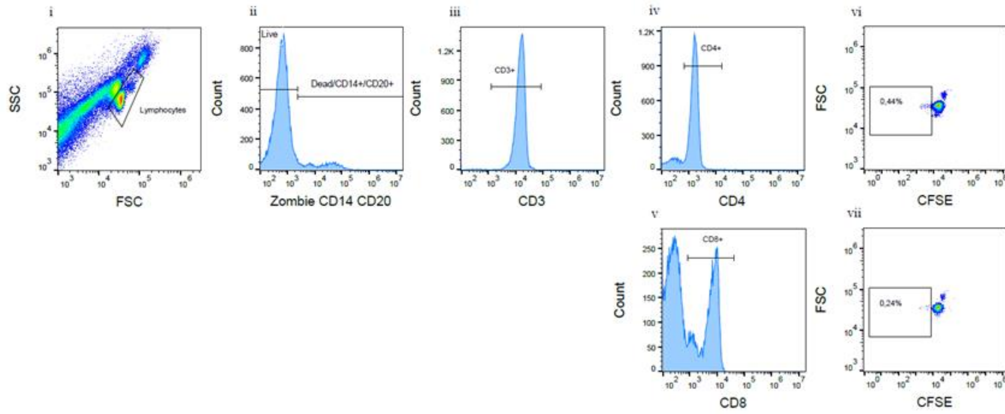

Figure S1: The Gating Strategy to Identify Proliferating T Cells. Gates were created to identify: i) lymphocytes based on forward and side scatter, ii) live cells (based on the exclusion of zombie violet viability dye) that were not monocytes ($CD14^-$) or B cells ($CD20^-$), iii) $CD3^+$ T cells, iv) $CD4^+$ T cells, v) $CD8^+$ T cells, and vi/vii) $CFSE^{low}$ (indicating proliferation). The $CFSE^{low}$ cells in vi ($CD4^+$) and vii ($CD8^+$) are background levels (media + buffer).

List of Reagents and Suppliers

Heparin vacutainer (BD Biosciences, NJ, USA, 367874)

Acid citrate dextrose vacutainer (BD Biosciences, NJ, USA, 1014043)

EDTA vacutainer (BD Biosciences, NJ, USA, 367861)

RPMI (Gibco, NY, USA, 11875-093)

CFSE (Invitrogen, Oregon, USA, C34554)

AB Serum (Sigma, Oakville, CAN, H6914-100mL)

ACK (Gibco, NY, USA, A10492-01)

Penicillin/Streptomycin (Gibco, NY, USA, 15070-063)

L-glutamine (Gibco, NY, USA, 25030-081)

Fetal bovine serum (Wisent, Inc., 080450)

24-well tissue culture plates (Eppendorf, Hamburg, Germany, 0030722019)

Anti-CD3/28 (Stem Cell Technologies, Canada, 10971)

rh-IL-2 (Stem Cell Technologies, Canada, 781451)

Tetanus toxoid (Millipore Sigma, 582231-25UG)

ddH_2O (Sigma, MO, USA, 95289-100 mL)

GP IIbIIIa (R&D Systems, MN, USA, 7148-A2)

GP Ibα (R&D Systems, MN, USA, 4067-GP)

Zombie violet viability dye (Biolegend, San Diego, USA, 423113)

Anti-CD3 PE (BD Biosciences, MD, USA)

Anti-CD4 APC Cy7 (BD Biosciences, MD, USA)

Anti-CD8 APC (BD Biosciences, MD, USA)

Anti-CD14 BV421 (BD Biosciences, MD, USA)

Anti-CD20 BV421 (BD Biosciences, MD, USA)

Trypan Blue (Bio Rad, UK, 1450013)

Histopaque 1077 (Sigma, Oakville, CAN, 10771-100 mL)

Goat-anti mouse IgG (Jackson Immunoresearch, PA, USA, 115-005-071)

Alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immunoresearch, PA, USA, 109-056-098)

p-nitrophenyl phosphate 5 mg tablets (Sigma Aldrich, Canada, 333338-18-4 N9389)

96-well flat bottom plates (Thermo Fisher Scientific, Canada, 3855)

Anti-GP IIbIIIa (in-house)

Anti-GP IbIX (in-house)

Bicarbonate buffer, pH 9.6 (in-house)

Tris/NaCl wash buffer, pH 7.4 (in-house)

Tris/NaCl/Tween wash buffer (in-house)

2% BSA blocking buffer (in-house)

Diethaloamine buffer, pH 9.7 (in-house)

CHAPTER 5

Conclusions

Central Messages

- Anti-platelet autoantibodies specific to glycoproteins IIbIIIa and IbIX can be detected in only half of all ITP patients, suggesting that other mechanisms of disease should be investigated in ITP.
- There are several $CD8^+$ T cell abnormalities in ITP patients including an elevated cytotoxic potential, which refers to the ability of $CD8^+$ T cells to degranulate and release cytotoxic effector molecules. However, our understanding of the role of $CD8^+$ T cells in ITP is limited by the fact that platelet-specific $CD8^+$ T cells have not been investigated.
- Platelet-specific $CD8^+$ T cells can be detected in ITP patients and in healthy individuals. In ITP patients, the regulation of T cells is diminished and the activity of $CD8^+$ T cells is elevated compared to healthy individuals and thrombocytopenic patients with non-ITP.
- The discovery of platelet-specific $CD8^+$ T cells provides a foundation for our understanding of the role of $CD8^+$ T cells in ITP. Investigating the interaction between platelet-specific $CD8^+$ T cells and megakaryocytes should be a high priority since each megakaryocyte produces thousands of platelets.

Summary of Findings

Chapter 1

This introductory chapter is an up-to-date summary of the evidence for the involvement of $CD8^+$ T cells in ITP. There are several $CD8^+$ T cell abnormalities in ITP patients that have been detailed in the literature. Most notably, several studies have shown that exogenously stimulated $CD8^+$ T cells from ITP patients can destroy autologous platelets in vitro[1-4]. Chapter 1 explains how this observation is not sufficient evidence of a platelet-specific response since the $CD8^+$ T cells were artificially triggered to degranulate. At the end of Chapter 1, better methods to identify platelet-specific $CD8^+$ T cells are described, including the laboratory methods that have been used to detect autoreactive $CD8^+$ T cells in other autoimmune diseases[5].

Chapter 2

This chapter presents the results of a systematic review and meta-analysis of anti-platelet autoantibody testing in ITP[6]. This study provides the rationale for investigating alternate mechanisms of disease in ITP such as $CD8^+$ T cells. The results from Chapter 2 show that anti-platelet autoantibody tests can help ‘rule in’ ITP since they have a high specificity. However, when the test is negative, ITP cannot be ‘ruled out’. Autoantibodies to GP IIbIIIa and GP IbIX directly on the platelet surface can be detected in only 53% of ITP patients. The absence of a detectable anti-platelet autoantibody in approximately half of all ITP patients

suggests that other potential underlying causes of thrombocytopenia require investigation. $CD8^+$ T cells are a plausible mechanism of thrombocytopenia in ITP since platelets and megakaryocytes express MHC I, which is required for the interaction between $CD8^+$ T cell receptors and target cells.

Chapter 3

In this chapter the characteristics of $CD8^+$ T cells are examined, including their frequency in the circulation and their cytotoxic potential. The frequency of $CD8^+$ T cells was normal in ITP patients, while the cytotoxic potential of $CD8^+$ T cells was elevated in a subset of ITP patients, demonstrating their enhanced capacity to destroy target cells through degranulation[7]. These results suggest that $CD8^+$ T cells are highly active in ITP patients, which may explain why several studies discussed in Chapter 1 found that stimulated $CD8^+$ T cells could destroy autologous platelets in vitro. However, the elevated cytotoxic potential of $CD8^+$ T cells observed in ITP patients is not platelet-specific. In order to advance our understanding of $CD8^+$ T cells in ITP, platelet-specific $CD8^+$ T cells should be the focus of investigation.

Chapter 4

This chapter provides evidence of platelet-specific $CD8^+$ T cells in ITP patients. Platelet-specific $CD8^+$ T cells were identified based on their ability to proliferate in response to the presentation of platelet autoantigens (GP IIbIIIa and GP Ibα). Platelet-specific $CD8^+$ T cells can also be detected in healthy

individuals, which indicates that these cells are not specific to ITP. In order to better understand the role of platelet-specific $CD8^+$ T cells in ITP, the regulation and activity of $CD8^+$ T cells were also investigated. Compared to healthy individuals and thrombocytopenic patients with non-ITP, ITP patients have a cytokine profile suggestive of diminished T cell regulation and elevated T cell activity. More specifically, $CD8^+$ T cell activity was elevated as demonstrated by high levels of granzymes A and B in plasma from ITP patients. Altogether, the results from this chapter suggest that platelet-specific $CD8^+$ T cell activity is elevated in ITP patients. The ability of platelet-specific $CD8^+$ T cells to destroy platelets and megakaryocytes will be a central point of future investigations.

Future Directions

$CD8^+$ T Cell-Mediated Platelet Destruction

As discussed in Chapter 1, previous studies have demonstrated that exogenously stimulated $CD8^+$ T cells can destroy platelets, but this is not sufficient evidence of a platelet-specific response[5]. This thesis aims to address this limitation by providing evidence for the existence of platelet-specific $CD8^+$ T cells. In order to better understand the role of platelet-specific $CD8^+$ T cells in ITP, their ability to induce platelet destruction should be investigated. In theory, such an investigation would involve the isolation of platelet-specific $CD8^+$ T cells from ITP patients, followed by a co-culture with autologous platelets. Platelet apoptosis and $CD8^+$ T cell degranulation could be measured simultaneously to

determine if platelet-specific $CD8^+$ T cells can induce platelet destruction. In order to avoid non-specific degranulation, the $CD8^+$ T cells should not be stimulated with anti-CD3. Blocking the interaction between $CD8^+$ T cell receptors and platelets using monoclonal antibodies to MHC class I could further demonstrate the direct cytotoxic activity of platelet-specific $CD8^+$ T cells.

If platelet-specific $CD8^+$ T cells can directly destroy platelets in vitro, it will be important to establish whether this also occurs in vivo. At this time, $CD8^+$ T cell-mediated platelet destruction is an unlikely driving mechanism of severe thrombocytopenia due to the number of platelets each $CD8^+$ T cell would have to destroy each day. Compared to platelets, $CD8^+$ T cells are far less abundant in the circulation (0.2-1 x 10^9 $CD8^+$ T cells/L vs 150-400 x 10^9 platelets/L)[8,9]. Further considering that the $CD8^+$ T cell pool is comprised of many different clonotypes with various TCR specificities (the frequency of autoreactive T cells is typically less than 0.01%)[10], and that 10^{11} platelets are produced each day in healthy individuals[11], it is unlikely that $CD8^+$ T cells could directly destroy enough platelets each day to sustain a chronic state of thrombocytopenia. Therefore, if $CD8^+$ T cells are involved in ITP as a mechanism of disease, it is more likely that they target megakaryocytes, which are the progenitors of platelets.

$CD8^+$ T Cell-Mediated Platelet Underproduction

Since thousands of platelets are produced from each megakaryocyte[12], $CD8^+$ T cell-mediated megakaryocyte destruction has the potential to cause a

severe reduction in the circulating platelet count. As discussed in Chapter 1, a previous study investigating the interaction between $CD8^+$ T cells and megakaryocytes found that $CD8^+$ T cells in ITP patients suppressed megakaryocyte apoptosis and promoted megakaryocytopoiesis while paradoxically preventing platelet production[13]. This is counterintuitive since $CD8^+$ T cells typically induce apoptosis of their target cells, and since a greater number of megakaryocytes should produce more platelets. These results might be explained by the fact that megakaryocytes in the later stages of apoptosis (Annexin V^+ Propidium $Iodide^+$ cells) were not analyzed in this study[13]. Another possible explanation for these results could be that this study did not examine the effects of platelet-specific $CD8^+$ T cells on megakaryocytes and platelet production. The discovery of platelet-specific $CD8^+$ T cells, as described herein, will help inform our future understanding of the relationship between $CD8^+$ T cells, megakaryocyte apoptosis, and platelet production.

In order to investigate the interactions between $CD8^+$ T cells and megakaryocytes, platelet-specific $CD8^+$ T cells could be isolated and co-cultured with autologous megakaryocytes. A method to differentiate peripheral blood $CD34^+$ cells into megakaryocytes was recently developed[14]. Platelet-specific $CD8^+$ T cells can be identified using the proliferation assay from Chapter 4, then isolated by fluorescence-activated cell sorting and expanded in vitro. These methods will be useful for investigating whether platelet-specific $CD8^+$ T cells can inhibit platelet production by directly destroying megakaryocytes. During co-

culture, $CD8^+$ T cell degranulation (CD107a expression), megakaryocyte apoptosis, and platelet production can be measured simultaneously. The results from this experiment would inform us about whether $CD8^+$ T cells have the capacity to promote severe thrombocytopenia in ITP.

The effects of platelet-specific $CD8^+$ T cells in ITP may extend beyond direct cytotoxicity since $CD8^+$ T cell-derived cytokines can impact megakaryocytes. For example, the $CD8^+$ T_c1 and T_c2 cell subsets are capable of target cell cytotoxicity, but they also produce IFN-γ and IL-4, respectively. IFN-γ promotes megakaryocytopoiesis[15], while IL-4 inhibits megakaryocytopoiesis[16]. Therefore, the immunophenotyping of platelet-specific $CD8^+$ T cells will be important to understand how they affect megakaryocytes and thrombopoiesis.

This thesis and other investigations of $CD8^+$ T cells in ITP have primarily focused on circulating $CD8^+$ T cells, while very few studies have focused on $CD8^+$ T cells at the site of platelet production in the bone marrow. Analyzing bone marrow $CD8^+$ T cells in ITP will be crucial to elucidate their effects on megakaryocytes and thrombopoiesis. In type 1 diabetes (T1D), which develops due to autoimmune destruction of β pancreatic cells, autoreactive $CD8^+$ T cells have been detected in pancreatic samples using in situ MHC tetramer staining[17]. In the future, the detection of platelet-specific $CD8^+$ T cells using MHC tetramers in bone marrow samples would provide valuable insights into how $CD8^+$ T cells are involved in platelet underproduction in ITP.

Clinical Implications of Platelet-Specific $CD8^+$ T Cells in ITP Patients

The discovery of platelet-specific $CD8^+$ T cells will lead to clinical insights and better therapeutic outcomes for patients with ITP. In autoimmune diseases in which the role of $CD8^+$ T cells is already well established, such as T1D, monitoring autoreactive $CD8^+$ T cells in patients can provide clinical insights, such as when a patient will continue to require insulin administration after islet transplantation[18]. Monitoring platelet-specific $CD8^+$ T cells may provide similar insights in ITP, such as identifying which patients will respond to platelet transfusion or immunosuppressive agents that target T cells. Insights into the disease-modifying mechanisms of therapies used to treat ITP will be gained by monitoring changes in the frequency and characteristics of platelet-specific $CD8^+$ T cells. First line therapies indicated for the management of ITP such as prednisone or dexamethasone have broadly anti-inflammatory effects, including the suppression of neutrophil migration[19]. These drugs can also have direct effects on T cells; for example, dexamethasone induces cytotoxic T-lymphocyte-associated protein 4 expression, which downregulates T cell activation[20]. Second line therapies used to treat ITP such as cyclosporine can inhibit T cell activity[21], although they impact T cells broadly, and it would be useful to understand how various treatments affect platelet-specific $CD8^+$ T cells. The discovery of platelet-specific $CD8^+$ T cells, as outlined in this thesis, will lead us to a better understanding of the effects of immunosuppressive therapies on $CD8^+$ T cells that are directly involved in the immunopathology of ITP. It will also lead to the

development of treatments that aim to delete or suppress platelet-specific $CD8^+$ T cells. Toxin-coupled MHC class I tetramers have been used to specifically delete autoreactive $CD8^+$ T cells in an experimental animal model of T1D[22], and the peptide-MHC I combination could be modified to delete autoreactive platelet-specific $CD8^+$ T cells in the future.

Overall Implications

Our current understanding of the role of $CD8^+$ T cells in ITP is extremely limited due to the lack of focus on platelet-specific $CD8^+$ T cells. This thesis advances our understanding of disease mechanisms in ITP by demonstrating that platelet-specific $CD8^+$ T cells exist, and by providing the foundation to define their role in platelet destruction and underproduction. As discussed in Chapter 1, the investigations of $CD8^+$ T cells in ITP to date have focused on $CD8^+$ T cells without regard for their TCR specificity. In this thesis, a method to detect platelet-specific $CD8^+$ T cells is described (Chapter 1) and deployed (Chapter 4). The results herein demonstrate that platelet-specific $CD8^+$ T cells are present in ITP patients and in healthy individuals, and that $CD8^+$ T cell activity is enhanced in ITP patients compared to healthy individuals and thrombocytopenic patients with non-ITP. This thesis provides the foundation for the isolation and characterization of platelet-specific $CD8^+$ T cells. Future efforts in characterizing $CD8^+$ T cells in ITP should focus on platelet-specific $CD8^+$ T cells, especially in relation to their ability to destroy platelets and megakaryocytes. The discovery of platelet-specific $CD8^+$ T cells, as described in this thesis, will allow for a better understanding of

the role of $CD8^+$ T cells as a mechanism of disease in ITP, which will lead to the development of better treatment strategies.

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