Immunopharmacological consequences of immune responses to therapeutic interferon beta

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

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This research was carried out in the

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I dedicate this thesis

to my mum, dad and Shrini.

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Abbreviations

ACN;	acetonitrile
ADA;	anti-drug antibody
ADR;	adverse drug reaction
AE;	adverse events
aHUS;	atypical haemolytic-uremic syndrome
AIDS;	acquired immune deficiency syndrome
AMD;	acute myeloid leukemia
ANOVA;	analysis of variance
APC;	antigen presenting cell
ARMD;	age related macular degeneration
BAb;	binding antibody
BAFF;	B-cell activating factor
BBB;	blood brain barrier
BCIP/NBT;	4-bromo-4-chloro-3'-indoly phosphate/nitro-blue tetrazolium
B-CLL;	B-cell chronic lymphocytic leukemia
BcR;	B-cell receptor
B-LCL;	B-lymphoblastoid cell line
BMP;	bone morphogenetic protein
BSA;	bovine serum albumin
C1q;	complement component 1q
C3a;	complement factor 3a
C5a;	complement factor 5a
CAPS;	cryopyrin associated periodic syndrome
CCRs;	chemokine receptors
CD;	cell surface molecules expressed on various cell types
CD;	Crohn's disease
CF;	cystic fibrosis
CFR;	code of federal regulations
CHO;	Chinese hamster ovary
CI;	class interval
CKD;	chronic kidney disease
CML;	chronic myeloid leukaemia
CNS;	central nervous system
CPE;	cytopathic effect
cpm;	counts per minute
CpG;	deoxy-cytidylate-phosphate-deoxy-guanylate
CRVO;	central retinal vein occlusion
CSA;	cyclosporin A
CTLA;	cytotoxic T-lymphocyte antigen
DAF;	decay accelerating factor
DC;	dendritic cells
DMD;	disease modifying drugs
DME;	diabetic macular edema
DMSO;	dimethyl sulphoxide
DNA;	deoxyribonucleic acid
eIF2;	eukaryotic initiation factor

EBV;	Epstein-Barr virus
E.Coli;	Escherichia coli
EDTA;	ethylenediaminetetraacetic acid
EFNS;	European national neurological societies
e.g;	example
EGFR;	epidermal growth factor receptor
ELISA;	enzyme-linked immunosorbent assay
EMA;	European medicines agency
EMBASE;	excerpta medica database
EpCAM;	epithelial cell adhesion molecule
EU;	European Union
FACS;	fluorescence-activated cell sorter
FBS;	fetal bovine serum
FCAS;	familial cold autoinflammatory syndrome
FCM;	flow cytometer
FDA;	food and drug administration
FITC;	flurescein isothiocyanate-conjugated
Foxp3;	forkhead box P3
GF;	growth factor
GH;	growth hormone
GI;	gastro-intestinal
GITR;	glucocorticoid-induced TNF receptor-related protein
GM-CSF;	granulocyte macrophage colony-stimulating factor
GNRH;	gonadotropin-releasing hormone
GU;	genito-urinary
HBSS;	Hank's Balanced Salt Solution
HER;	human epidermal growth factor receptor
HEPES;	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt
HIV;	human immunodeficiency virus
HLA;	human leukocyte antigens
HPLC;	high-performance liquid chromatography
HRP;	horse radish peroxidase
HSA;	human serum albumin
ICOS;	inducible T-cell COStimulator
IEDB;	immune epitope database
IFN;	interferon
IFNAR;	interferon-α/β receptor
ISG;	interferon-stimulated gene
ISGF;	interferon-stimulated gene factor
ISRE;	interferon-stimulated response element
Ig;	immunoglobulin
IL;	interleukin
IM;	intramuscular
IP;	intellectual property
iTregs;	induced regulatory T-cells
Jak;	Janus kinase

JCV	John Cunnigham virus
JIA;	juvenile idiopathic arthritis
Kcl;	potassium chloride
LC-MS;	liquid chromatography-mass spectrometry
LFA;	lymphocyte function associated antigen
LTT;	lymphocyte transformation test
LPS;	lipopolysaccharide
LVEF;	left ventricular ejection fraction
mAb;	monoclonal antibody
MAC;	membrane attack complex
MACS;	magnetic-activated cell sorting
MALT;	mucosa-associated lymphoid tissues
MASP;	MBL-associated serine proteases
MBP;	myelin basic protein
MBL;	mannose-binding lectin
mCRC;	metastatic colorectal carcinoma
2ME;	2-mercaptoethanol
MEDLINE;	medical literature analysis and retrieval system online
MFI;	mean fluorescence intensity
MHC;	major histocompatibility complex
MI;	myocardial infarction
MIP;	macrophage inflammation protein
MMP;	matrix metalloproteinase
MOPS;	3-(N-morpholino) propanesulfonic acid
MPS;	mucopolysaccharidosis
MRI;	magnetic resonance imaging
mRCC;	metastatic renal carcinoma
mRNA;	messenger RNA
MS;	multiple sclerosis
Mx;	myxovirus
MWS;	muckle-wells syndrome
NaCl;	sodium chloride
NAb;	neutralising antibody
N-NAb;	non-neutralising antibody
NA;	not applicable
NCT;	national clinical trial
NHL;	non-Hodgkin lymphoma
NIBSC;	national institute of biological standards
NK;	natural killer
NKLAM;	natural killer lytic-associated molecule
NLR;	NOD-like receptors
nTregs;	natural regulatory T-cells
NO;	nitrous oxide
NOD;	nucleotide oligomerization domain
NRS;	non-randomised controlled studies
NU;	neutralising units

NuPAGE;	novex bis-tris polyacrylamide gels
OD;	optical density
OR;	odds ratio
PAMP;	pathogen-associated molecular patterns
PBMC;	peripheral blood mononuclear cells
PBS;	phosphate buffer solution
PD;	programmed cell death protein
PDGF;	platelet-derived growth factor
PE;	phycoerythrin
PEG;	polyethylene glycol
PHA;	phytohemagglutinin
PKR;	protein kinase receptor
PML;	progressive multifocal leukoencephalopathy
PNH;	paroxysmal nocturnal hemoglobinuria
Poly I:C;	polyinosinic: polycytidylic acid
PRR;	pattern recognition receptors
PRISMA;	preferred reporting items for systematic reviews and meta-analysis
PSA;	prostate-specific antigen
PsA;	psoriatic arthritis
PVDF;	polyvinylidene fluoride
QW;	once a week
RA;	rheumatoid arthritis
RANKL;	receptor activator of nuclear factor kappa B ligand
RANTES;	regulated upon activation normal T-cell expressed and secreted
RCTs;	randomised controlled trials
RIPA;	radio immunoprecipitation assay
Rh;	rhesus factor
rh;	recombinant human
r-methu G-CSF;	recombinant methionyl human granulocyte colony-stimulating factor
RNA;	ribonucleic acid
ROA;	route of administration
RPMI;	Roswell park memorial institute
RRMS;	relapsing remitting multiple sclerosis
RSV;	respiratory syncytical virus
RTI;	respiratory tract infections
RVO;	retinal vein occlusion
SC;	subcutaneous
SD;	standard deviation
SDS;	sodium dodecyl sulfate
SDS-PAGE;	SDS-polyacrylamide electrophoresis
SJIA;	systemic juvenile idiopathic arthritis
siRNA;	small interfering RNA
SPMS;	secondary progressive multiple sclerosis
STAT;	signal transduction and activators of transcription

TAG;	tumor-associated glycoprotein
TBS;	tris-buffered saline
TcR;	T-cell receptor
Td;	T-cell dependent
TCC;	T-cell clone
TCL;	T-cell line
TEMED;	tetramethylethylenediamine
TFA;	trifluoroacetic acid
Th;	T helper cells
THP-1;	human monocytic cell line
Ti;	T-cell independent
TIM;	T-cell immunoglobulin and mucin domain
TIW;	three times a week
TGF;	transforming growth factor
TLR;	toll-like receptors
TLS;	tumor lysis sundrome
TNF;	tumor necrosis factor
TNFR;	tumor necrosis factor receptor
TNTC;	too numerous to count
TPO;	thrombopoietin
tPA;	tissue plasminogen activator
Tregs;	regulatory T-cells
Tr1;	T regulatory type 1 cells
Tr35;	T regulatory type 35 cells
TT;	tetanus Toxoid
Tyk;	tyrosine kinase
UBC;	University of British Columbia
UK;	United Kingdom
US;	United States
VEGF;	vascular endothelial growth factor
WD;	withdrawal
WHO;	World health organisation

Publications

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Sathish, J. G., S. Sethu, M.C.Beilsky, L.D.Haan, N.S.French, K.Govindappa, et al. (2013). "Challenges and approaches for the development of safer immunomodulatory biologics." Nature Review Drug Discovery 12(4): 306-324.

Sethu, S*., K. Govindappa*, et al. (2013). "Immunoglobulin G1 and Immunoglobulin G4 antibodies in multiple sclerosis patients treated with IFN-β interact with the endogenous cytokine and activate complement." Clinical Immunology.

Hamdam, J., S. Sethu, T. Smith, A. Alfirevic, M. Alhaidari, J. Atkinson, M. Ayala, H. Box, M. Cross, A. Delaunois, A. Dermody, K. Govindappa, et al. (2013). "Safety pharmacology – Current and emerging concepts." Toxicology and Applied Pharmacology.

Conference Publications

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Abstract

Protein therapeutics or biologics represent 30 % of current licensed pharmaceutical products. In general, biologics offer superior safety profiles compared to small molecules. However, significant clinical concerns have emerged in terms of development of anti-drug antibodies (ADAs), a phenomenon that is covered under the term, immunogenicity. Anti-drug antibodies can alter pharmacokinetics, reduce efficacy of the therapeutic and also can in some cases induce allergic reactions. Human recombinant interferon beta (IFN-β) is a biologic used for the treatment of multiple sclerosis (MS) – a chronic, inflammatory and demyelinating disease of the central nervous system. Long-term treatment with IFN-β has been shown to lead to the development of anti-IFN-β antibodies that can cause total loss or reduced efficacy. Anti-drug antibodies can be non-neutralising (N-NAb) and neutralising (NAb) depending on the site to which they bind.

This study aimed to conduct a systematic review to determine factors affecting the formation of neutralising antibodies against three different formulations of IFN-β Avonex™, Rebif™ and Betaseron/Betaferon™. Findings from the systematic review highlight the relative differences in immunogenicity risk of different IFN-β formulations Avonex™, Rebif ™ and Betaseron/Betaferon™ with Avonex™ having the lowest risk, Rebif™ has moderate risk and Betaseron/Betaferon™ has high risk. Characterising the immunoglobulin profile of the IFN-β ADAs from the plasma of ADA positive MS patients revealed that IFN-β ADAs are predominantly of the IgG1 and IgG4 subclass. We also characterised the neutralising potential of the major ADA IgG4 subclass using a IFN-β bioactivity assay and show that IgG4 antibodies may likely contribute to the neutralisation activity. The potential of the neutralising ADAs to cross-react with endogenous IFN-β was investigated using an in vitro bioactivity assay. Findings from this set of experiments revealed varying degrees of neutralisation of endogenous IFN-β. We next explored the potential immunological consequence of ADA with regards to formation of immune complexes and activation of complement. The interaction of ADAs with the biologic can result in formation of immune complex. Immune complexes can activate the complement system. The data revealed IFN-β ADAs can form immune complexes with IFN-β and therefore activate complement. We also attempted to identify IFN-β linear epitopes that the ADAs had the ability to bind. However, a combination of multipin peptide technology and in vitro peptide competitive binding assay failed to reveal a definitive linear epitope although there was some evidence for the existence of potential linear epitopes. We also examined the involvement of T helper cells and T regulatory cells (Tregs) in ADA development. The data revealed no significant differences in the frequency of Tregs among IFN-β ADAs positive, negative and healthy donors. Attempts were also made to identify T helper epitopes within IFN-β that could potentially drive immunogenicity. Using T-cell epitope prediction tools (IEDB-AR and ProPred) and T-cell functional assays we identified an immunogenic sequence of 36 amino acids within IFN-β (position 130-166). Our data revealed that one IFN-β peptide within this sequence is a potential T-cell immunogenic epitope. In addition we identified a possible association of one IFN-β derived peptide with the DRB1*1501HLA haplotype.

In summary, the results presented in this thesis have provided essential information on subclass profile of IFN-β ADAs, the possible involvement of T helper cells and potential antibody epitopes within IFN-β. Future studies should be aimed at providing greater detail on the evolution of the ADA response and test strategies to remove immunogenic determinants from IFN-β.

Chapter 1. General Introduction

1.1 Adverse drug reactions

1.1.1 Definition

The World Health Organisation (WHO) states, 'an adverse drug reaction is any response to a drug which is noxious, unintended and undesirable effect of the drug, which occurs at doses used in humans for prophylaxis, diagnosis or therapy'. Adverse drug reactions (ADR) are a major clinical problem and account for 2-6 % of all hospital admissions (Pirmohamed et al. 1998). From a clinical perspective, ADR can be classified into five main groups Type A, B, C, D and E (Park, Pirmohamed & Kitteringham 1998). Type A reactions are predictable from the known pharmacology of the drug and represent an exaggeration of the pharmacological effect and are usually dose-dependent (for example excessive bleeding is associated with the use of anti-coagulants). Type B reactions are idiosyncratic and not predictable from the known pharmacology of the drug, have no simple dose-response and is host-dependent and (for example hepatitis associated with drug halothane). Type C reactions are toxicological and can be predicted from the chemistry of the medicine (for example hepatotoxicity with paracetamol). Type D reactions are delayed type reactions and include teratogenic effects such as congenital malformation syndromes (for example phocomelia, a birth defect is associated with drug thalidomide). Type E reactions are those that occur upon drug withdrawal (for example withdrawal seizures upon stopping phenytoin) (Park, Pirmohamed & Kitteringham 1998).

The mechanisms of ADR can be due to its pharmacological (on-target) or non-pharmacological (off-target) effects (Clarke 2010; Park 2009). On-target reactions are predictable from the known primary pharmacology (action of drug on its therapeutic target) and secondary pharmacology (action of drug on therapeutically unrelated target) (Valentin et al. 2009). In comparison, the off-target reactions are idiosyncratic and therefore cannot be predicted from the known pharmacology of the drug (Uetrecht 2008). Several off-target reactions are immune-mediated and represent a significant clinical problem (Kola & Landis 2004; Lasser et al. 2002). Development of immune-mediated adverse drug reactions is clinically heterogeneous and accounts for 16 % of all ADRs (Hoigne et al. 1993). These reactions are common to both small molecule drugs and biologics. Small molecule drugs are chemically-derived and usually have molecular weight below 500 Daltons. Biologics are

biotechnology-derived proteins, peptides, antibodies, viruses and vaccines (a detailed description of biologics is given in Section 1.4).

1.2 Overview of the human immune system

This section provides a general overview of the human immune system.

1.2.1 Introduction to human immune system

The immune system is a complex network of cells, tissues and organs. This complex network works to protect an individual from external harmful microorganisms such as bacteria, parasites and fungi. It distinguishes self from non-self and can also have the potential to detect abnormally growing host cells and destroy them.

The immune system comprises of primary lymphoid organs, which are the sites of lymphocytes birth and or maturation, and the secondary lymphoid organs, in which further differentiation of lymphocytes occurs. T lymphocytes or T-cells mature in the thymus whereas B lymphocytes or B-cells mature in the bone marrow. These two tissues constitute the primary lymphoid organs. The cervical, axillary, mesenteric, inguinal lymph nodes and spleen constitutes the secondary lymphoid organs. Mature, naive lymphocytes migrate throughout the body using the blood vessels, and encounter antigens in the secondary lymphoid organs. The antigen is a molecule that interacts with high affinity immunological receptors present on cells of the adaptive immune system (discussed later). Antigens can be classified into three groups, autoantigens, for example are a person's own self antigens, alloantigens which are found in different members of the same species, for example red blood cell antigens and heterophile antigens are identical antigens which are found in the cells of different species.

1.2.2 Functional elements of the human immune system

The human immune responses are the result of the interdependent function of both innate and adaptive immune compartments. The salient features of innate and adaptive immune compartments are listed in Table 1.1 (Janeway & Medzhitov 2002).

	Innate (natural)	Adaptive (acquired)
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal	Clonal
Recognition	Conserved molecular patterns such as LPS, LTA, mannans, glycans	Proteins, peptides, carbohydrates
Self-non self discrimination	Perfect and selected over evolutionary time	Imperfect and selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokines IL-1β, IL-6 Chemokines IL-8	Clonal expansion or anergy IL-2 Effector cytokines IL-4, IFN-γ

Table 1.1: Illustrates the salient features of immune system compartments (adapted from (Janeway & Medzhitov 2002).

1.2.2.1 Innate immune system

Innate immunity is an antigen-non specific defence mechanism and can be divided into immediate and early induced innate immunity. Immediate innate immunity is activated 0-4 hours after exposure to an infection and involves the action of antimicrobial enzymes, complement proteins, antimicrobial peptides, mechanical removal of microbes, anatomical barrier to infection, and bacterial antagonism by normal flora bacteria. Early induced innate immunity is activated 4-96 hours after exposure to an infection and involves recruitment of innate immune cells as a result of PAMP binding to PRR that are expressed on these cells (Janeway 1989). The cells of innate immune system include phagocytic cells (dendritic cells, neutrophils, eosinophils, macrophages and monocytes), cells that release inflammatory mediators (macrophages, mast cells, basophils and eosionophil) and NK cells (Akira & Takeda 2004; Cella et al. 1999; Medzhitov & Janeway 2002; Medzhitov, Preston-Hurlburt & Janeway 1997; Poltorak et al. 1998).

1.2.2.1.1 Complement system

Complement system comprises of 30 soluble proteins circulating in the blood in inactive forms. Complement proteins are activated sequentially in response to recognition of molecular components associated with for example microorganisms. There are three complement pathways: the classical pathway, the lectin pathway and the alternative pathway (Sjoberg, Trouw & Blom 2009). The ultimate product of these pathways is production of an enzyme C3 convertase (refer Figure 1.1). The classical complement pathway is activated by antibody bound to particulate antigen. However, many other substances, such as components of damaged cells, LPS and nucleic acids can initiate this pathway in antibody-dependent manner. The classical complement pathway is initiated by activation of C1 (Morgan 2000). The classical complement pathway is activated when C1 (complement protein complex) interacts with Fc portion of the antibody molecules immunoglobulin G (IgG) or immunoglobulin M (IgM). The C1 protein complex consists of 3 complement proteins C1q, C1r and C1s. The C1q, portion of C1 complex binds to antibodies or the microbes. The binding activates C1r portion of C1 which in turn activates C1s. The activation of C1s cleaves complement protein C4 into C4a and C4b. The formation of C3 convertase cleaves C3 into C3a and C3b. C3b binds to C3 convertase that cleaves C5 into C5a and C5b. The lectin complement pathway is activated by interaction of lectins (microbial carbohydrates) with MBL found in body fluids. The alternative complement pathway is activated by C3b binding to microbial surfaces and antibody molecules.

The three complement activation pathways converge on the activation of C3. This activation leads to the concluding common pathway with the assembly of C5-C9 forming transmembrane pore called membrane attack complex (MAC) on the cell surface and cause cell death by osmotic lysis (Parkin & Cohen 2001). The proteins produced by the complement pathways trigger inflammation, promote attachment of antigens to phagocytes, attract phagocytes to the infection site, cause lysis of gram-negative bacteria, serve second signal (discussed later) in activation of B-cells and remove harmful immune complexes.

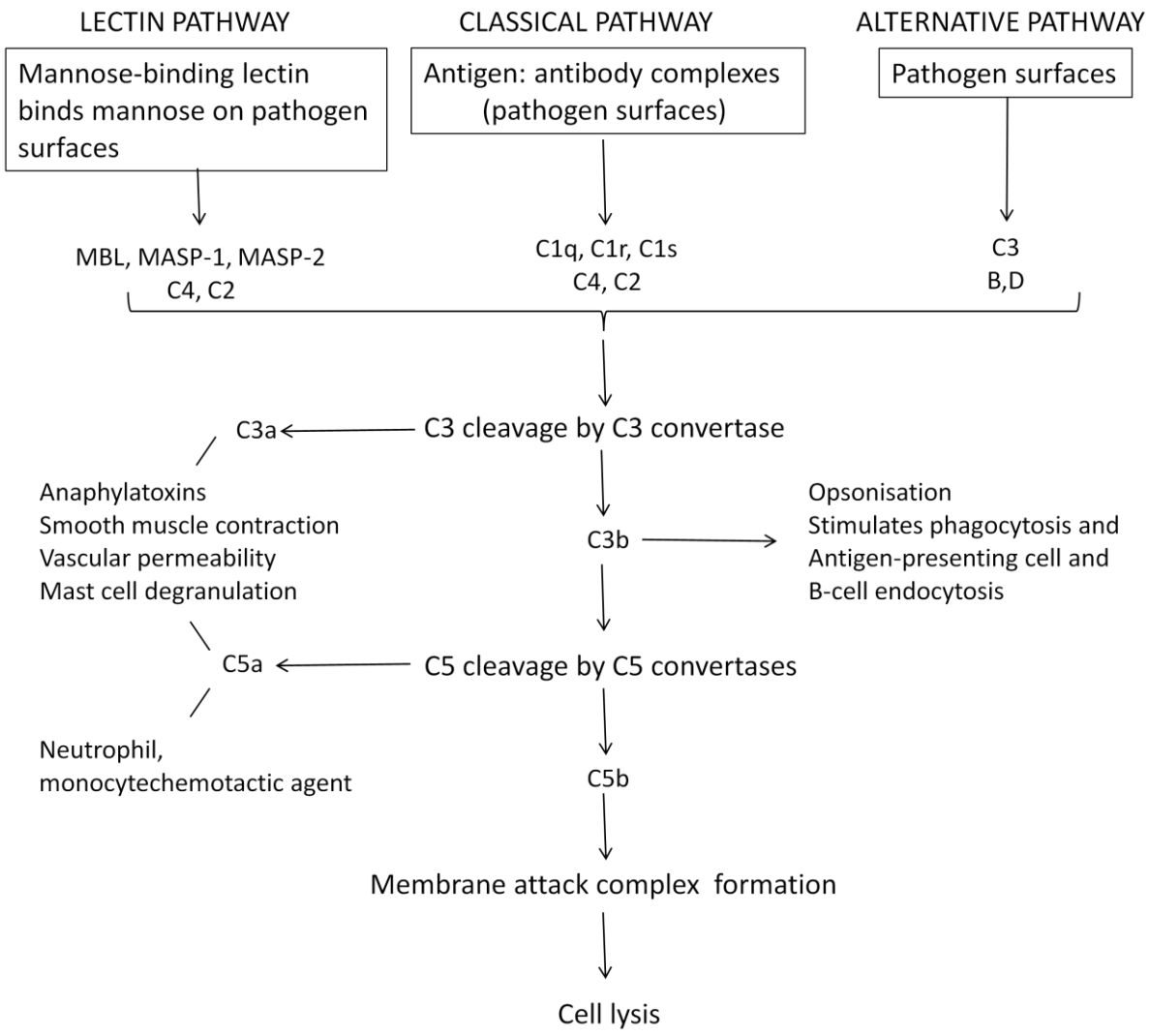


Figure 1.1: Schematic representation of three pathways of complement activation modified from (Parkin & Cohen 2001)**.**

1.2.2.2 Antigens

An antigen can be defined as a substance that reacts with antibody molecules and antigen specific receptors on lymphocytes. An immunogen is an antigen that stimulates an adaptive immune response. In principle, antigens can include a broad spectrum covering different classes of organic molecules ranging from simple chemical compounds to complex macromolecules (Rao 2001). The actual portion of an antigen that can react with antigenic receptors on B-cells and T-cells are called epitopes. The size of an epitope is generally thought to be equivalent to 5-15 amino acids and 3-4 sugar residues. Protein antigens usually have several epitopes because proteins are composed of hundreds of amino acids and can form three-dimensional (3-D) shapes. The humoral response to protein antigens can comprise

of antibodies directed against the linear segments of sequence of amino acids presented as extended conformation or any possible secondary structural arrangements at the surface of the antigen (Colman 1988; Novotny 1987).

1.2.2.3 Adaptive immune system

The adaptive immunity is an antigen-specific defence mechanism and can be divided into humoral immunity and cell-mediated immunity. Humoral immunity involves production of antibodies in response to an antigen and is mediated by B-cells. Cell-mediated immunity involves production of cytotoxic T-cells, activated NK cells, activated macrophages and cytokines in response to an antigen and is mediated by T-cells. The process of adaptive immunity includes transportation of antigens by antigen presenting cells to lymphoid organs where the antigens are recognised by naive B-cells and T-cells (Romani & Schuler 1989). The activated B-cells and T-cells subsequently proliferate and differentiate into effector and memory cells.

1.2.2.3.1 Cells and cell surface molecules of the adaptive immune system

The cells and surface molecules important for adaptive immune responses include major histocompatibility complex (MHC) molecules (MHC class I, II and III), antigen presenting cells (APC) such as dendritic cells (DC), macrophages and B-cells and lymphocytes which include $CD4^+$ and $CD8^+$ T-cells.

- **Major histocompatibility comples (MHC)**

  The human version of MHC molecules is called as human leukocyte antigen (HLA) complex coded by the HLA genes. The MHC consists of more than 200 genes located on chromosome 6. The genes are classified into three groups: MHC class I, MHC class II and MHC class III (Shiina, Inoko & Kulski 2004).

  The human MHC class I genes include HLA-A, HLA-B and HLA-C. The proteins are found on the surface of the cell and these proteins are bound to peptides and have been exported from within the cell. The MHC class I proteins exhibit these peptides to cytotoxic $CD8^+$ T-cells. The MHC class II proteins include HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA1 and HLA-DRB1. The MHC class II presents antigenic peptides to T-helper $CD4^+$ T-cells. T-cell receptors can recognise epitopes when bound to MHC molecules. The HLA genes have several hundred variations referred to as

an allele. The HLA allele can be defined as a variant of a single genetic locus and each of which is given a number for example HLA-B27. The HLA-B27 consists of 40 different alleles and is designated as HLA-B*2701 to HLA-B*2740. Studies have established genetic associations between the susceptibility of disease and particular alleles of HLA genes. For example, HLA-B27 allele increases the risk of ankylosing spondylitis (Khan et al. 2007).

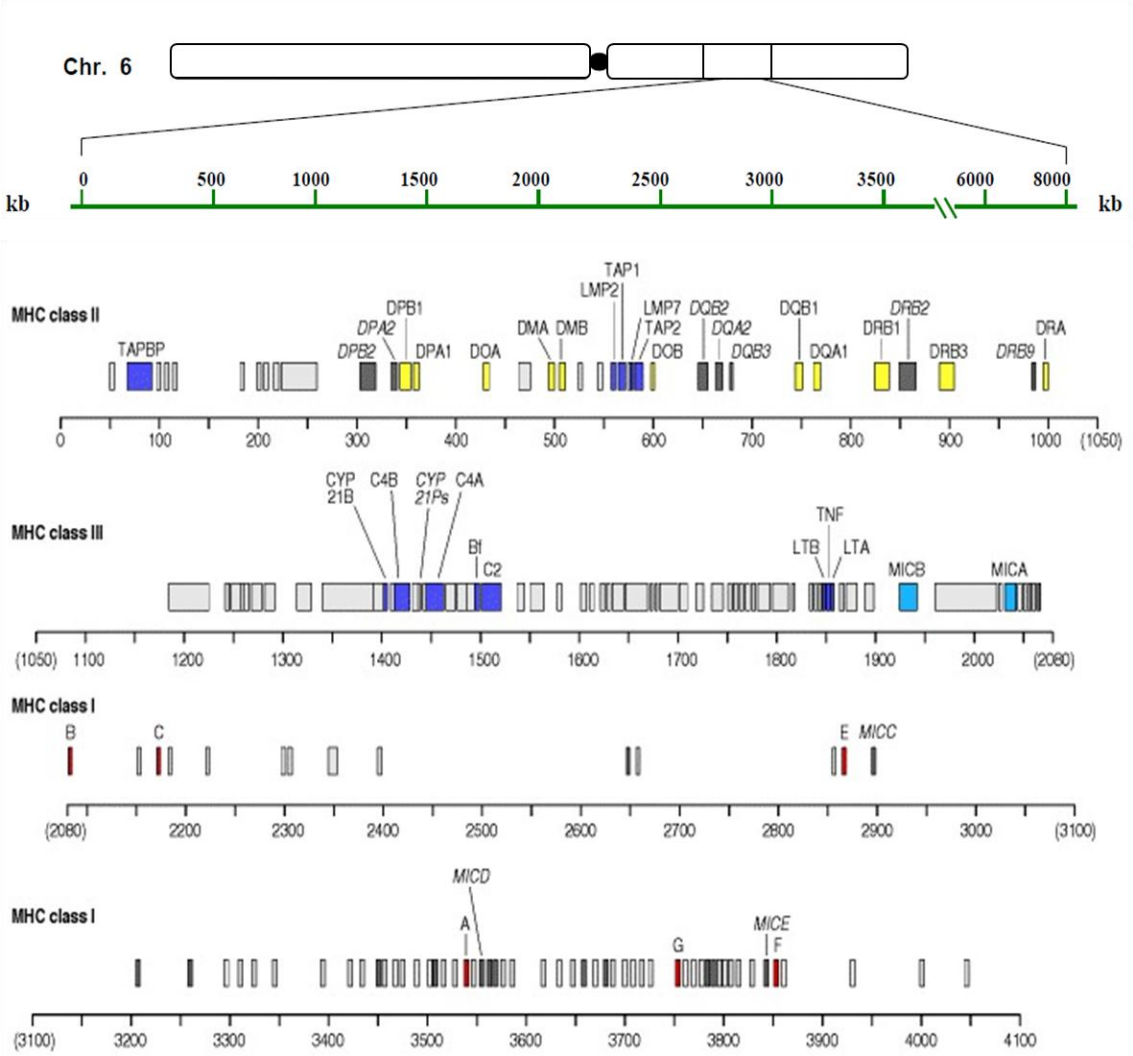


Figure 1.2: Map of human major histocompatilibility complex (MHC). The establishment of MHC class I, II and III regions of human MHC are shown with estimated genetic distances specified in base pairs (kb). The additional genes are designated for MHC class I region E, F and G and are also referred to MHC class-I like genes or MHC class IB molecules, the additional MHC class II genes are referred to as pseudogenes. The genes also shown in MHC class III region code for C2, C4 (C4a and C4b) and factor B (Bf) complement proteins, tumour necrosis factors (TNF) and lympotoxins (LTA and LTB). The gene encoding 21-hydroxylase CYP 21Ban enzyme implicated in

steroid synthesis is also shown. Genes in grey colour and in italics are pseudogenes. The MHC class I genes are shown in red, the MHC class I related genes (MIC) shown in blue and are distinct from MHC class I like genes, the MHC class II genes are shown yellow. The genes which have immune functions but not related to MHC either class I or II are shown in purple. The figure was adapted and modified from (Janeway CA Jr 2001).

- **Antigen presenting cells (APC)**

  The cells include dendritic cells (DC), macrophages and B-cells. The main function of antigen presenting cells (APC) is to engulf and degrade the protein antigens into peptide epitopes, bind those epitopes to MHC molecules and place them on their surface where they can be recognised by the T-cell receptors of T-cells. Antigen presenting cells (APC) express both MHC class I and MHC class II molecules and can perform two major functions. B-cells are involved in presentation of soluble antigens. The peptide fragments are exposed onto the MHC class II molecules of the B-cell surface immunoglobulin receptors (Lanzavecchia 1990). Upon up-regulation the antigen presenting B-cells activate the proliferation and differentiation of $CD4^+$T-cells (Parker 1993).

- **Lymphocytes** – Lymphocytes include B-cells which mediate humoral immunity and produce antibodies against specific antigen (discussed later), and T-cells which are responsible for cell-mediated immunity and regulate adaptive immune responses. Based on the cell surface markers, T-cells can be classified into $CD4^+$ T-cells and $CD8^+$ T-cells. The $CD4^+$ T-cells have CD4 molecules and T-cell receptor for protein antigen recognition and regulate the adaptive immune responses through cytokine production. The activated $CD4^+$ T-cells can differentiate into specific subtypes depending on the cytokine milieu of the microenvironment. The T-cells subsets include T helper 1, T helper 2, T helper 17, Tregs, follicular helper T-cell and T helper 9. The effector functions of T-cells subset are mediated by the cytokines secreted by the differentiated cells. The $CD8^+$ T-cells have CD8 molecules and T-cell receptor for protein antigen recognition and upon activation the cells differentiate into cytotoxic T-cells.

  - $CD4^+$ T-cells – The main functions of $CD4^+$ T-cells include elimination of intracellular pathogens, mount immune response to extracellular parasites including helminthes, bacteria and fungi. These cells are also essential in maintenance of

immunologic tolerance to self and foreign antigens and mediate humoral immunity through interaction with B-cells.

The antigen recognition receptors of T-cells are termed as T-cell antigen receptor (TcR). T-cell receptors are analogues to the B-cell receptor (BcR) and are composed of two highly variable glycoproteins either with αβ subunits or γδ subunits associated with invariable molecules δ, μ, π and Σ) which constitutes the CD3 complex (Rudolph, Stanfield & Wilson 2006). Nearly 95 % of the conventional T-cells exhibit a TcR composed of αβ subunits. The remaining 5 % of T-cells exhibit γδ subunits.The TcR α chain is located on chromosome 14 and comprise of V (variable) and J (joining) genes. The β chain gene is located on the chromosome 7 with V, J and D (diversity) genes. The γ and δ chain gene are located on the chromosome 7 and 14 respectively. The repertoire of TcR αβ is generated by gene rearrangement between V-J segments and V-D-J segments (Starr, Jameson & Hogquist 2003). The junctional diversity is expressed in complementary determining regions (CDR) and will form antigen-recognition site of the TcR (Cabaniols et al. 2001; Rock et al. 1994). The TcR recognises antigen in the form of peptide complexes bound to MHC molecules. The T-cells respond to short contiguous amino acid sequences in proteins.

The TcR and CD4 molecule of naive T-cells detects the corresponding MHC class II peptide complex on mature DC and will send first signal for the activation of naive T-cells. The second signal promotes survival of those T-cells through co-stimulatory molecules such as CD40, CD80 and CD86 on the DC which bind to CD40L and CD28 molecules on the T-cell. The DC produces cytokines such as IL-6, IL-2 and IL-12 which contributes to proliferation and differentiation into effector of T-cells. The activated T-cells synthesise the cytokine IL-2 and high affinity IL-2 receptor. The binding of IL-2 to its high affinity receptor allows T-cell proliferation and formation of T-cell clones. The effector T-cells are classified based on the cytokines they produce and dominated by Th1, Th2, Th17, Tregs and T-follicular cells. The Th1 cells coordinate immunity against intracellular bacteria and promote opsonisation. The opsonisation can be defined as a process of alteration to the surface of a pathogen or some particles so it can be ingested by phagocytosis. The antibody and complement opsonise extracellular bacteria for destruction by macrophages and neutrophils (Janeway CA Jr 2001). They also produce cytokines such as IFN-γ, IL-2, tumour necrosis factor-alpha (TNF-α). The T helper 2 cells coordinate immunity against

helminths and microbes. They also produce B-cell growth factors (IL-4, IL-5, IL-9 and IL-13) and IL-22. The T helper 17 cells promote local inflammatory response and stimulate neutrophils and produce cytokines IL-6 and IL-17. T-follicular cells promote humoral immunity stimulating antibody production and antibody isotype switching by B-cells. They produce cytokines that are characteristics of both T helper 1 and T helper 2 cells. Regulatory T-cells suppress immune responses and produce inhibitory cytokines IL-10 and TGF-β that help limit immune responses and also prevent autoimmunity.

- Regulatory T-cells – are distinct subset of T-cells which plays an important role in maintaining peripheral control of T-cell responses. Regulatory T-cells contribute to immunological tolerance and immune homeostasis and can suppress both innate and adaptive immune cells (Zhang et al. 2009). Sakaguchi et al described CD4+ T-cells expressing IL-2 receptor CD25 which is critical for maintaining peripheral immunological tolerance and it represents 5-10 % of all peripheral CD4+ T-cells. In vitro experiments suggest Tregs are anergic but such cells can expand in vivo through lymphopenia or antigen-driven proliferation (Hong et al. 2005; Wing & Sakaguchi 2010). The immunological tolerance occurs when an immunocompetent host fails to respond to an immunogenic challenge with a specific antigen. The human immune system implements this tolerance mechanism in two different routes – central and peripheral. Central tolerance occurs during the lymphocyte development whereas the peripheral tolerance occurs after the lymphocytes leave the primary lymphoid organs. The tolerance can exist in T-cells, B-cells or both. In several experimental models the state of tolerance to variety of T-dependent and T-independent antigens has been demonstrated. Based on these studies it is clear that a number of factors such as physical form of antigen, route of administration, dosage, age of the recipient and others will contribute to determine whether an antigen will stimulate an immune response or induce tolerance.

  Naturally occurring CD4+ Tregs (nTreg) specifically express a transcriptional factor Foxp3 that plays critical role in their development and function. Foxp3 is an important biological marker and is currently the most specific and reliable molecular marker for nTregs. Evidence suggests that the Tregs can suppress the activation and expansion of autoreactive T-cells. Regulatory T-cells are divided into Foxp3 negative Tregs that is CD4+IL-10 producing T-cells or T regulatory type 1 cells (Tr1 cells) and Foxp3

positive Tregs that is CD4+IL-35 producing T-cells or T regulatory type 35 cells (Tr35 cells). Najafian et al reports Foxp3+Tregs are a subset of CD8+ T-cells (Najafian 2003). Young et al describes Foxp3+Tregs as CD3+CD4-CD8- double negative T-cells (Young & Zhang 2002). Natural killer T-cells are also potent suppressor T-cells in selected models. Foxp3+ Tregs include both CD4+ and CD8+ T-cells. CD4+Foxp3+ Tregs have been extensively studied when compared to CD8+Foxp3+ Tregs. CD4+Foxp3+ Tregs plays important role in immune tolerance and therefore this subset has attracted more attention in translational research. CD4+Foxp3+ Tregs can express other surface markers such as CD25, CTLA-4, GITR, OX40, CD103, CD39 and CD73 (Kang, Tang & Bluestone 2007). Understanding the phenotypical and functional plasticity of such surface markers can provide the means for harnessing the cells into therapeutics. Futhermore, Tregs can also be divided into natural Tregs (nTregs) and induced Tregs (iTregs). Both of these Tregs express Foxp3 as a functional marker and express TcRs. TGF-β and IL-2 are the essential cytokines for induction of Tregs (Chen et al. 2003). CD4+ nTregs are dependent on IL-2 for their function and maintains a fine balance in suppressing autoreactive T-cells, normal intestinal immunity and also dampens anti-pathogen effector mechanisms. Retinoid acid (Benson et al. 2007), leukemia inhibitory factor (Gao et al. 2009), CTLA-4 and PD-1 enhance the induction of iTregs. But IL-4, IL-6, IFN-γ, OX-40, Tim-1 suppress the induction of iTregs (Degauque et al. 2007). Epigenetic differences between nTregs and iTregs might also contribute for Foxp3 expression (Lal & Bromberg 2009). Epigenetics can be defined as mitotically or meiotically heritable changes in cellular phenotype or gene expression resulting in changes at a chromosome without altering the DNA sequence. Many of these factors might heavily contribute for the therapeutic manipulations of Tregs.

T regulatory Type I cells produce large amounts of IL-10. It exhibits no Foxp3 expression and the proliferation is poor in vitro (Groux et al. 1997). IL-10 is the effector cytokine for Tr1 cells (Levings, Sangregorio & Roncarolo 2001). IL-27 can induce aryl hydrocarbon receptor expression in T-cells which binds to c-Maf and then activate IL-10 gene in activated CD4+ T-cells to induce Tr1 differentiation (Apetoh et al. 2010). Tr1 cells can inhibit macrophage activation and therefore Tr1 cells are critical in suppressing innate immunity (Battaglia et al. 2006; Lindsay et al. 2001). It can also inhibit activation of CD4+ T-cells (Groux et al. 1997). Collison et al

described a subset of CD4+ T-cells expressing IL-35 and they do not express Foxp3 transcriptional factor (Collison et al. 2007). But when compared to Foxp3+ Tregs, these IL-35 producing CD4+Foxp3- cells can also suppress proliferation in vitro and blocks the development of experimental autoimmune encephalomyelitis (EAE) in animal models. TGF-β, IL-10 and IL-35 are the key players in Treg suppressive mechanisms (Castellani et al. 2010; Collison et al. 2010).

- $CD8^+$ T-cells – The main function of $CD8^+$ T-cells is to kill infected cells and tumour cells via apoptosis. Once activated the $CD8^+$ T-cells proliferate and differentiate into cytotoxic T-cells. The $CD8^+$ T-cells display surface molecule called CD8 and TcR. The TcR in cooperation with CD8 bind peptides from the endogenous antigens bound to MHC class I molecules.

- **B-cells** – are responsible for production of antibody molecules during adaptive immunity. Naive B-cells are activated by both T-dependent antigens and T-independent antigens. In order for B-cells to proliferate, differentiate and mount an antibody response against T-dependent antigens (for example proteins) the B-cells must interact with effector T-cells. The B-cells and T-cells encounter antigens in secondary lymphoid organs (lymph nodes and spleen). Here the antigens or microbes can bind to BcR on naive B-cells. The naive B-cells are activated and remain in the lymph nodes to proliferate and differentiate.

  The B-cell receptor (BcR) is a membrane bound, signal transducing protein that can bind directly to epitopes of peptides, proteins, polysaccharides, nucleic acids and lipid antigens (Cambier et al. 2007). The BcR are composed of membrane bound immunoglobulin (Ig) that functions as their binding substructure and a heterodimer of CD79a (Ig-α) and b (Ig-β) that function as their transducer substructure. Post activation by antigen, B-cells secretes a soluble form of their receptors termed as antibody (Rao 2001). The antibody or immunoglobulin molecules are Y-shaped macromolecules composed of two different types of polypeptide chains. One is termed as heavy or H chain (50 kDa in size) and the other is termed as light or L chain (25 kDa in size). The two heavy chains are linked to each other by disulphide bonds and each heavy chain is linked to a light chain by a disulphide bond. In an immunoglobulin molecule the two heavy and two light chains are identical and therefore an antibody molecule has two identical antigen-binding sites (Janeway CA Jr 2001).

The activation of B-cells can occur in association with T-cell help (Parker 1993). Following binding of antigen to the BcR triggers signal-transduction cascade for B-cell activation (Benschop & Cambier 1999). This leads to transcriptional activation of genes and so the BcR along with bound antigen is internalised and either degraded or trafficked to an intracellular compartment. In this compartment the MHC class II molecules and antigenic peptides bound to BcR form complexes. The antigen processing occurs and peptide-MHC complexes are transported to the cell surface. The T-helper cell recognises the complexes through the TcR leading to T-cell activation. In this stage, the activated T-cells provide help to B-cells leading to antibody production. Once activated, the B-cells differentiates into plasma cells to produce antibodies (Reth & Wienands 1997). During the process of differentiation, rearrangement, recombination and mutation of Immunoglobulin V, D and J regions occurs to form functional VJ (light chain) and VDJ (heavy chain) genes. At this stage mature B-cells will produce IgD and some membrane IgM that will migrate to the cell surface that can act as antigen receptor. Once the antigenic stimulation happens, the B-cells will differentiate into plasma cell secreting IgM. The class switch happens in some cells due to the rearrangement of DNA placing VDJ gene next to IgG, IgE or IgA constant regions. In the process of repeating antigenic stimulation, these B-cells will differentiate into new plasma cells expressing new isotypes (Pierce 2002). In normal circumstances this results in switch from IgM to IgG.

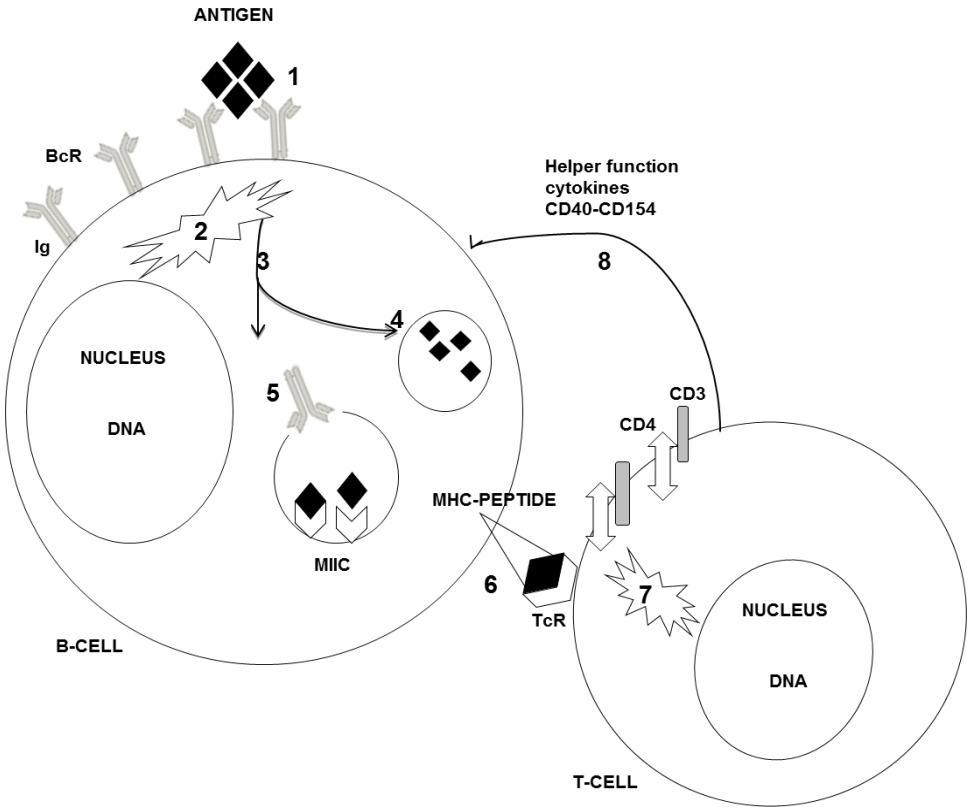


Figure 1.3: Schematic representation of B-cell activation. The antigen binding cause (1) BcR to trigger signal transduction events (2) leads to transcriptional activation of genes (3) BcR internalised (4) or either degraded (5) and trafficked to intracellular compartment and form complexes in association with MHC that can recognise TcR (6) T-cell activation occur (7) the activated T-cells help B-cell initiating B-cell activation (8) using cytokines and cell-cell interactions.

B-cells also mount an antibody response to T-independent antigens (for e.g. carbohydrates and lipids) without the requirement of interaction with effector T-cells. The resulting antibody responses will be of IgM isotype and do not give rise to memory response. There are two different types of T-independent antigens. TI-1 antigens or PAMPs (for e.g. LPS) can elicit B-cell activation binding to toll-like receptors. TI-2 antigens (for e.g. capsular carbohydrates) with multiple, repeating subunits activate B-cells by simultaneously cross-linking a number of BcRs. Several reasons contribute for T-cell independent antibody responses. These include large molecular weight, repeated antigenic sequence and ability to activate the complement cascade. In animal models the TI-2 antigens elicits robust and long lasting primary antibody responses (Garcia de Vinuesa et al. 1999). However, these antigens in general do not elicit a recall response (Hosokawa 1979; Hosokawa, Amagai & Muramatsu 1979).

1.2.2.3.2 Humoral immunity

Humoral immunity refers to production of antibody molecules in response to an antigen. Antibodies, also referred to as immunoglobulins (Ig) are specific glycoprotein molecules produced from B-cells and plasma cells in response to specific antigen and capable of reacting to the antigen. Antibodies are of five classes or isotypes IgG, IgM, IgA, IgD and IgE (Alzari, Lascombe & Poljak 1988; Davies & Metzger 1983).

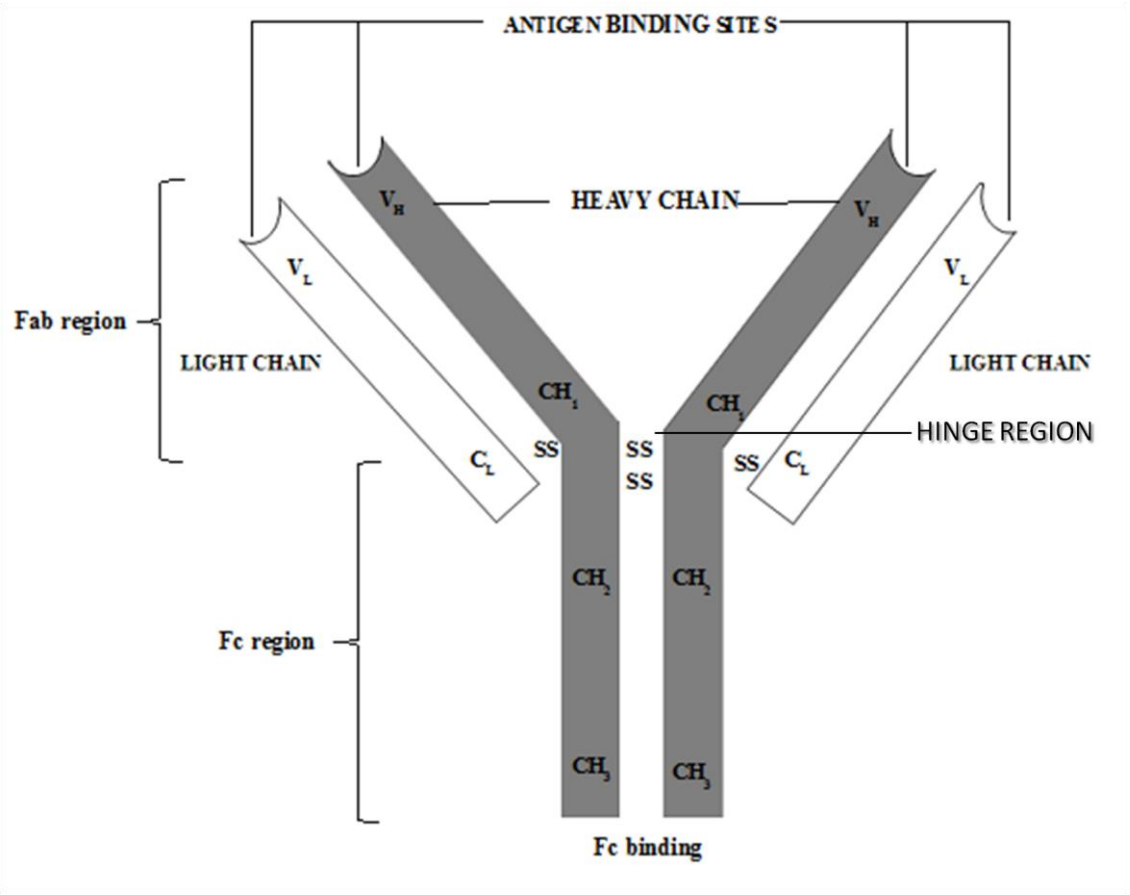


Figure 1.4: Structure of an antibody molecule. It consists of two identical heavy and two identical light chains and both are attached to disulphide bonds. The light chain interacts with heavy chain to form Fab portion and Fc domain. The antigen binding sites are located at the tip of arms, which are tethered by a flexible hinge region. In the amino acid sequence of an antigen binding sites there are three complement determining regions (CDR) CDR1, CDR2 and CDR3.

The immunoglobulin monomer is composed of 4 glycoprotein chains – 2 identical heavy chains and 2 identical light chains. The two tips of Y-monomer are called as the antigen-binding fragments or Fab portions. The bottom part of the Y, C terminal region and is called

as Fc portion. The Fc portion is responsible for the biological activities (for e.g. activating the complements). The IgM is a pentamer and consist of five linked antibody like molecules. The IgA is a dimer and consists of two antibodies like molecules.

Immunoglobulin G is one of the most abundant proteins in the blood. It accounts for approx. 75 % of the total immunoglobulin in the plasma and made up of 4 glycoprotein chains with the molecular weight of 150 kD (Alzari, Lascombe & Poljak 1988). The human IgG subclass is composed of immunoglobulin G1 (IgG1), immunoglobulin G2 (IgG2), immunoglobulin G3 (IgG3) and immunoglobulin G4 (IgG4). The relative concentrations are IgG1>IgG2>IgG3=IgG4 (French 1986). The stimulation of antibody responses towards certain antigens might result in selective increase in IgG subclasses antibodies. The distribution of IgG subclass depends in part on the nature of antigen. Protein antigens elicit IgG1 and IgG3 subclass antibodies and polysaccharide antigens elicits IgG2 subclass antibody (Ferrante, Beard & Feldman 1990; Siber et al. 1980). Long term antigenic stimulation may lead to increased IgG4 levels (Gibbs & Oger 2007). IgG antibodies exerts two important functions complement activation and opsonisation. IgG4 with IgG1 can amplify immune complex mediated complement activation. Deficiencies of IgG subclasses can lead to disturbed immune responses and are associated with prolonged or severe infections, autoimmunity and haemolytic diseases of the new born (Aucouturier et al. 1992). The physiochemical, biological properties of IgG subclasses and clinical outcome of IgG subclasses deficiency are illustrated in Table 1.2 (Grey & Kunkel 1964; Huizinga et al. 1989; Jefferis 1986; Schur 1987; Vlug et al. 1994).

Properties	**IgG1**	**IgG2**	**IgG3**	**IgG4**
Heavy chain type	gamma 1	gamma 2	gamma 3	gamma 4
Molecular mass (Taylor et al.)	146	146	170	146
Average serum concentration mg/ml	4.9-11.4	1.5-6.4	0.2-1.1	0.08-1.4
Proportion of total IgG %	43-75	16-48	1.7-7.5	0.8-11.7
Half life (days)	21-23	20-23	7-8	21-23
Antibody responses:				
Proteins	++	+/-	++	+/-
Carbohydrates	+	++	-	-
Allergens	+	-	-	++
Complement activation: C1q binding	++	+	+++	-

IgG subclass	**Clinical outcome of IgG subclass deficiency**
IgG1	Recurrent infections
	IgG3 deficiency
	Chronic fatigue syndrome
IgG2	Recurrent RTI
	IgA and IgG4 deficiency
IgG3	Recurrent infections
	Chronic lung disease
IgG4	Recurrent RTI
	Autoimmune conditions
	HIV infected patients

Table 1.2: Illustrates the properties of IgG subclass and the clinical outcomes of IgG subclass deficiency. Key: (-) indicates nil, **(**+) indicates low, (++) indicates moderate and (+++) indicates high.

Generation of antibody diversity – Antibody diversity refers to the total variety of antibodies in the body of an individual and the mechanisms that generate this are described. The Fab portion is composed of a heavy and light protein chains. The variable heavy chain is coded for by a combination of 3 genes called VH (variable), DH (Diversity) and JH (joining). The variable light chain consists of either kappa or a lambda chain coded for a combination of two genes called VL (variable) and JL (joining). The DNA of each B-cell consists of multiple forms of each one of these variable determinant genes. The exact number of each genes varies from person and while a person inherits alleles for the various V(D)J genes from each parent an individual B-cell will only express an inherited allele set from one parent. Hence, this increases diversity of antibodies in that individual. Random gene translocations give rise to different combinations of multiple forms of each gene resulting in thousands of possible gene combination.

Affinity maturation – is defined as the gradual modification of the shape of the epitope binding site for BcR as a result of somatic hypermutation.

Somatic hypermutation is a process which happens when B-cells respond to antigen along with activated T-cells signals. During this process, the rearranged VH and VL genes are

mutated and the pattern of nucleotide base changes in V region genes resulting in somatic hypermutation. The mutations alter the amino acid sequences in the V region of an antibody causing enhanced binding to antigen. In conclusion, somatic hypermutation cause point mutations into the rearranged V region genes of activated B-cells, creating diversity and enhanced antigen binding. The longer and more tightly the antigen binds to BcR, the greater the chance the B-cell has of surviving and replicating.

Clonal selection and expansion – The cytokines produced from effector T-cells enable activated B-cells to proliferate producing clones of identical B-cells and this is referred to as clonal expansion. A single activated B-cell produces approximately 4000 antibody-secreting cells within seven days and B-memory cell also have these high affinity antibodies on their surface.

The effector action of an antibody is influenced by the isotype of heavy chain C region. The heavy chain V region can become associated with C region of any isotype through the process of *isotype switching*. The first antibody to be produced is IgM which is referred as primary antibody response. However, the activated B-cells subsequently undergo isotype switching secreting antibodies of different isotypes IgG, IgA and IgE which is referred as secondary antibody response. The secondary response is characterised in the first few days by the production of small amounts of IgM and large amounts of IgG, with some IgA and IgE antibodies. *Isotype switching* occurs by recombination involving deletion of DNA between the rearranged V region and the selected C region. In animal models IL-4 has shown to induce switching to IgG1 and IgE, TGF-β induces switching to IgG2 and IgA. IL-5 induces switching to IgA.

Memory response – During humoral immunity several circulating B-memory cells and T-memory cells develop. The exposure to the same antigen results in rapid production of antibodies. The primary response to a new antigen generally peaks 5-10 days. IgM is made first and is later replaced by IgG. The secondary response peaks in 1-3 days where there is increase in amount of IgG. B-memory cells have a long life and replicate and produce antibodies periodically when they are exposed to persisting epitope remaining on the surface of follicular dendritic cells.

1.2.2.3.3 Cell-mediated immunity

Cell-mediated immune response does not involve antibodies but rather involves activation of macrophages and NK-cells, production of antigen-specific T-cells and release of various cytokines. Cell-mediated immunity is mediated though T-cells. T-cells mature in the thymus into the naïve T-cells expressing T-cell receptors (TcRs) allowing them to interact with their cognate antigen.

T-cell receptors are complex protein molecules consisting of a heterodimer of two glycoprotein chains αβ T-cell linked together by a disulphide bond which represents 95 % of T-cells. It is responsible for recognising the antigens bound to MHC molecules. The amino terminus of this TcR is extracellular with a hypervariable region which also represents the polymorphic antigen-interacting molecule. The CD4+ T-cells recognise the antigens bound to MHC class II molecules which are sourced from the extracellular environment through endocytic or lysosomal pathway. The CD8+ T-cells recognise the antigens bound to MHC class I molecules originating from the intracellular environment through proteasome degradation pathway. Two signals are required to stimulate T-cells.

Signal 1 – antigen binding to MHC molecule

The T-cells recognise the peptide fragments which are bound to MHC class I or class II molecules that display antigenic peptides on the surface of the APC (Germain 1994).

Signal 2 – co-stimulation

T-cell receptors interact with co-stimulatory molecules on the surface of APC (Janeway & Bottomly 1994). Important molecules include B7 molecules such as CD80 and CD86, ICOS inducible co-stimulator ligand and CD40 recognised through CD28, CTLA-4 cytotoxic T lymphocyte antigen, ICOS and CD40-ligand on T-cells. These signals are critical and contribute to the priming and differentiation of effector T-cells. It should also be noted that differences in these signals and TcR recognition of peptides lead to cell death which contributes for the tolerance towards self-antigens (Miller & Basten 1996; Ross et al. 2000a).

T-cell activation causes release of cytokines and the activated T-cells rapidly proliferate to produce large clones of thousands of identical T-cells, a process called clonal expansion. These cells differentiate into effector T-cells.

1.2.2.4 Mechanisms underlying immunogenic responses to biologics

The formation of anti-drug antibodies can be through T-cell dependent (Td) and T-cell independent (Ti) mechanisms (De Groot & Scott 2007).

In the Td pathway the T-cells recognise the antigenic peptides which are derived from the biologic and are presented by APC via MHC class II molecules. The activated T-cells stimulate B-cells to generate antibodies against the biologic. It causes proliferation and expansion of epitope specific B-cells (Bachmann et al. 1994). It results in a robust antibody production which is long lasting, high affinity antibody titre response and is predominantly of the IgG isotype. The T-cell subset polarisation determines the profile of anti-drug antibodies generated. The Th1 mounts an IgG1 and IgG2 specific anti-drug antibodies. However, the Th2 response drives neutralising IgG4 specific anti-drug antibodies (Baker et al. 2010; Reding 2006; Reding et al. 2002).

The T-cell independent pathway involves polyvalent antigens that bind to the B-cell receptor (BcR) and induce receptor clustering (Vos et al. 2000). The aggregated biologic displaying repeating epitopes can cluster BcRs and cause B-cell activation generating antibodies without the assistance of T-cells (Batista & Harwood 2009)(Batista and Harwood 2009). The generated anti-drug antibodies will be of IgM isotype or low-affinity IgG. While the immunological mechanisms of Td and Ti pathways are distinct, cross talk between Td and Ti pathways exists (Sethu et al. 2012). In Ti pathway the antigen laden marginal zone B-cells can function as APC which presents biologic to T-cells (Fehr et al. 1998). The other mechanism which represents interaction between Td and Ti pathways is B-cell epitope spreading. Epitope spreading perhaps plays an important role in evolution of anti-drug antibodies specificities.

1.3 Immune-mediated adverse drug reactions

The Food drug administration (FDA) defines immune-mediated adverse drug reactions as ‘any adverse effect on the structure and function of the immune system or on other systems as a result of immune mediated dysfunction'. Based on underlying mechanisms, immune mediated adverse drug reactions to biologics (Pichler 2006) have been classified as;

- Type α – high cytokine and cytokine release syndrome wherein immunostimulation leads to excessive cytokine release. For example, muromonab-CD3 used for the treatment of organ transplant rejections, can provoke release of several cytokines such as tumor

necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukins (IL) 2, 3 and 6 (Vasquez, Fabrega & Pollak 1995).

- Type β – immunogenicity, anti-drug antibodies (ADA) formation and subsequent hypersensitivity reactions. Unwanted immune reactions with ADA production may neutralise the biological activity of the therapeutic protein and can also cross-react with endogenous protein counterpart (for example beta interferons in the treatment of multiple sclerosis) (Baert et al. 2003).

- Type γ – immune or cytokine imbalance syndrome due to immune deviation. It is further classified into;

    - Impaired immune functions as in the case of the immunosuppressive effects of TNF inhibitors (used in treatment of rheumatoid arthritis) (Chatenoud 1993) leading to reactivation of tuberculosis.

    - Immune or cytokine imbalance syndrome that leads to autoimmune reactions, inflammatory or allergic reactions as seen with autoimmunity associated with interferon alpha treatment (Rifkin et al. 2005).

- Type δ – cross reactivity reactions may occur due to expression of same antigen on different cell types, for example cetuximab (epidermal growth factor receptor EFGR) antagonist which is used for the treatment of colorectal cancer) causing acneiforme eruptions due to cross-reactivity with EGFR on skin cells (Perez-Soler & Saltz 2005).

- Type ε – non immunological mediated reactions with unclear/unknown mechanisms, example etanercept, an anti-tumour necrosis factor drug used in the treatment of autoimmune diseases which may cause heart insufficiency (Kwon et al. 2003).

1.4 Biologics

1.4.1 Definitions

The FDA definition of biological products or biologics ‘is any virus, therapeutic serum, toxin, antitoxin or analogous product applicable to the prevention, treatment or cure of diseases or injuries in man’. This can include vaccines, blood and blood products, cells, tissues, genes, recombinant proteins and allergenics. The European medicines agency (EMA) has extended the breadth of biologics by defining it as ‘a medicinal product whose active substances is made by or derived from a living organism’.

Biologics consist of hormones, growth factors, cytokines, vaccines, enzymes, antibodies and fusion proteins (Giezen et al. 2008). Biologics are made using biological methods with or without recombinant DNA technology (Leach 2013). They differ from small molecule drugs in a number of features that are listed in Table 1.3. Biologics are derived from well-characterised cells using various types of expression systems which includes bacteria, yeast, insect, plants and mammalian cells and can be produced using cell cultures, animals or plants (ICH S6 1997). Biologics are made up of carbohydrates, proteins or nucleic acids and sometimes complex combinations of these substances (FDA 2012a).

Since 1982 over 334 (according to FDA and EMA biologic product approvals) biologics have been approved in the United States (US) or European Union (EU) for the treatment of serious life threatening diseases and other disease conditions. The diseases include diabetes, cancer, autoimmune diseases, viral infections and hereditary deficiencies (Figure 1.5) (Giezen et al. 2008; Schellekens 2008). Biologics are classified into four different groups (I, II, III and IV) based on their function and therapeutic application (Leader, Baca & Golan 2008). The examples for each of this classification are provided in Table 1.4. A comprehensive list of approved biologics excluding blood and blood derived products has been obtained from FDA (listed in drugs@FDA page on the FDA website and tabulated in Appendix 1)

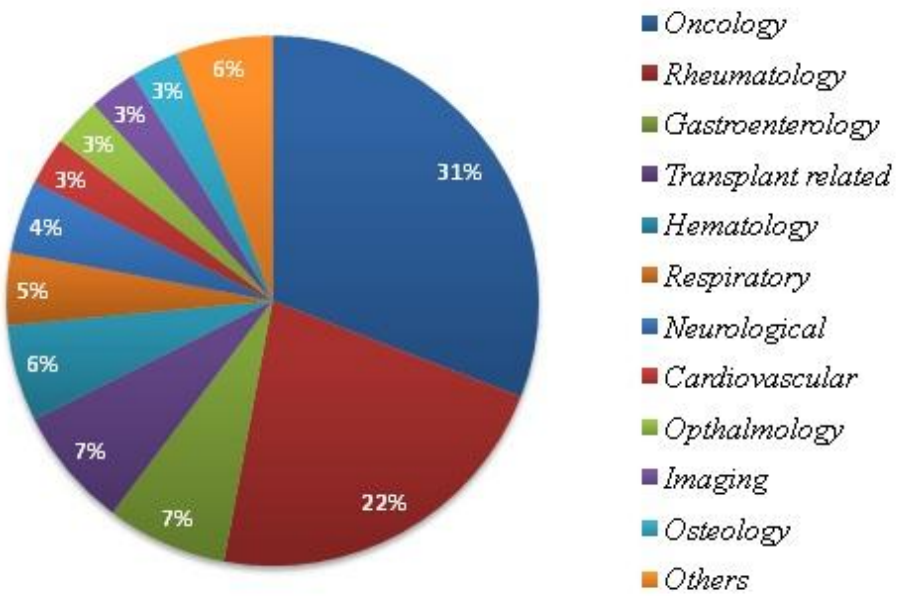


Figure 1.5: Food and drug administration-approved biologics excluding blood and blood derived products by therapeutic areas (source Drugs@FDA page on the FDA website). Oncology related biologics contribute 31 % and rheumatology related biologics contribute 22 % of the global share in the biologics market. 'Others' include biologics for the treatment of renal, skin, ulcer and palpable cord disorders.

Small-molecule drugs

- Low molecular weight (typically < 500 Daltons).
- Manufactured with high consistency and using standard chemical processes.
- The majority of these drugs are manufactured from chemical precursors.
- The contents and purity of drug active agents can be analysed quickly and accurately.
- The absorption of drug is determined by the drug's physio-chemical properties, the formulation and mode of administration. Small molecules drugs tend to diffuse across the cell membrane from a region of high concentration (for e.g. GI) to one of low concentration (for e.g. blood) more rapidly.

Biologics

- Derived or synthesized from biological sources.
- The molecular mass is typically >20,000 Daltons and has a complex structure.
- They require rigorous biochemical analysis to assess quality and purity.
- The characteristics are highly dependent on their method of manufacture.

Table 1.3: The table illustrates characteristics of small-molecule drugs over biologics (Bernton 2007; Leach 2013; Vugmeyster et al. 2012). Abbreviations: GI, Gastrointestinal; e.g, example

Group I **Biologics with enzymatic or regulatory activity**	Group II **Biologics with special targeting activity**	Group III **Protein vaccines**	Group IV **Protein diagnostics**
• **Group Ia**: Replacing a protein that is deficient or abnormal. • Examples: Insulin, GH, somatotropin, Factor VIII, Factor IX, Protein C, β-glucocerbrosidase, α-1-protienase, adenosine deaminase, human albumin. • **Group Ib**: Augmenting an existing pathway. • Examples: Erythropoietin, IL-11, Interferons, Factor VIIa, BMP7, GnRH, PDGF. • **Group Ic**: Providing a novel function or activity • Examples: Botulinum toxin, Collgenase, Hyaluroindase, L-asparaginase, Streptokinase.	• **Group IIa**: Interfering with a molecule or organism. • Examples: monoclonal antibodies targeting VEGF, EGFR, CD3, CD52, TNF-α, IL1R, C5 and fusion proteins. • **Group IIb**: Delivering other compounds or proteins. • Examples: Diptheria toxin conjugated with IL-2, anti-CD33 conjugated to calicheamicin.	• **Group IIIa**: Protecting against a deleterious foreign agent. • Examples: Hepatitis B, HPV vaccines. • **Group IIIb**: Treating an autoimmune disease. • Example: anti Rh IgG. • **Group IIIc**: Treating cancer. • Examples: Vaccines for B cell non-Hodgins lymphoma.	• Examples: Glucagon, GHRH, Secretin, Imaging agent labeled anti-PSA, anti-TAG-72, GPIIb/IIIa receptors, antibodies against HIV and Hepatitis C virus.

Table 1.4: Biologics are classified into 4 groups. Group I – Biologics with enzymatic or regulatory activity; Group II – Biologics with special targeting activity; Group III – Protein vaccines and Group IV – Protein diagnostics. BMP – Bone morphogenetic protein; CD – Cluster of differentiation; EGFR – Epidermal growth factor receptor; GH – Growth hormone; GnRH – Gonadotropin-releasing hormone; GP – Glucoprotein; HIV – Human immunodeficiency virus; IL – Interleukin; PDGF – Platelet-derived growth factor; PSA – Prostate specific antigen; TAG – Tumor-associated glycoprotein; TNF – Tumor necrosis factor; VEGF – Vascular endothelial growth factor

1.4.2 Adverse drug reactions associated with biologics

Biologics in general, offer superior safety profiles when compared to small molecule drugs due to the lower risk of off-target reactions. However, in some patients unwanted immune responses to biologics may cause immunologically based adverse events such as infections, malignancy, CRS, anaphylaxis, serious skin reactions and immunogenicity (refer Appendix 1) (Giezen et al. 2008; Leader, Baca & Golan 2008). The adverse drug reactions can be broadly classified into on-target or off-target effects. The adverse reactions due to on-target reactions (or exaggerated pharmacology) include malignancies, serious infections, CRS, autoimmunity and tumour lysis syndrome. Off-target adverse drug reactions can be further classified based on immune response-mediated (for example immunogenicity of interferon beta) and non-immune response mediated (for example Fc-mediated acute phase reaction associated with golimumab (anti-TNF drug) treatment). In this section the adverse drug reactions associated with biologics is discussed in detail.

- Immune reactions

Immune reactions include infusion reactions, immunogenicity, cytokine release syndrome (CRS) and hypersensitivity reactions (Presta 2006). An example of this is the acute anaphylactic (IgE mediated) and anaphylactoid reactions reported with rituximab (a chimeric CD20 specific mAb used for treatment of non-Hodgkin lymphoma and CLL) (Chung et al. 2008; Coiffier 2002) (refer Appendix 1 for more examples). Immunogenicity of the biologic can result in formation of ADA and can lead to loss of therapeutic efficacy (discussed in detail in Section 1.5). Cytokine release syndrome often referred as cytokine storm is uncontrolled hypercytokinaemia that results in multiple organ damage (Carter 2001; Loertscher & Lavery 2002). Muromomab-CD3, a mouse mAb against human CD3 used in the treatment of renal transplantations can cause cytokine release syndrome (CRS) (Cohen et al. 2008; Ransohoff & Zamvil 2007).

- Serious Infections

Serious infections are major adverse drug reactions associated with biologics. Out of all the biologics mentioned in Appendix 1, twenty biologics have been associated with infections including reactivation of bacterial (such as mycobacterial, streptococcal, listerial or meningococcal), viral (such as hepatitis B or C virus), fungal (such as histoplasmosis) or sometimes opportunistic infections (such as JCV). An increased risk of tuberculosis has been reported with infliximab – TNF specific chimeric mAbs – for treatment of inflammatory

bowel disease(Theis & Rhodes 2008). PML, a fatal demyelinating disease of CNS is due to reactivation of latent John Cunnigham virus (JCV) infection and has been reported with using natalizumab (used for the treatment of RRMS) (Carson et al. 2009; Major 2010). Natalizumab causes reduced T-cell trafficking to the CNS and may compromise the immune surveillance and control of JCV in susceptible patients increasing the risk of PML (Bloomgren et al. 2012).

- Platelet and thrombotic disorders

Drug induced immune thrombocytopenia, characterised by decrease in the number of circulatory platelets has been reported with biologics such as alemtuzumab, a humanised mAb against CD52 (used for treatment of graft-versus-host disease). The mechanism behind this adverse event is due to direct cytolysis of blood cells (which is a mechanism of action of alemtuzumab) that can lead to severe multi-lineage haematopoietic cell depletion resulting in lymphopenia, neutropenia and thrombocytopenia (Cox et al. 2005; Lorenzi et al. 2008).

- Autoimmunity

The immunomodulatory functions of biologics can lead to immunosuppression and can cause various autoimmune diseases (Mongey & Hess 2008). The use of anti-TNF mAbs for treatment of rheumatic diseases has been linked with development of anti-nuclear antibodies and antibodies to double-stranded DNA and cause Lupus-like syndromes – a musculoskeletal disorder (Ramos-Casals et al. 2007). The potent immunosuppressive mAb alemtuzumab when used in treating multiple sclerosis developed autoantibodies to thyrotropin receptor due to potent immunosuppressive action causing antibody-mediated thyroid autoimmunity and developed autoimmune hyperthyroidism (Coles et al. 2006).

- Cancer

A review of 3,400 plus RA patients who received TNF-specific mAb demonstrated a dose-dependent increase in risk of malignancy (Askling et al. 2005). However, the evidence for anti-TNF therapy as a cause for malignancy has continued to be questioned (Askling et al. 2009; Dixon & Silman 2006; Scott 2006).

The IL-12/23 specific mAbs are associated with theoretical concerns of potential tumorigenicity in patients suffering from moderate-to-severe plaque psoriasis and in CD.

Interleukin-12 has important role in anti-tumour immunity through its effects on tumour infiltrating cytotoxic T-cells (Longowski, J.L et al 2006).

- Dermatitis

Cetuximab, an EGFR specific chimeric mAb, and panitumumab, an EGFR specific humanised mAb is used for the treatment of mCRC (Jean & Shah 2008). Unfortunately these mAbs can cause skin rash on the face and upper torso, pruritus, dermatitis and erythema (Perez-Soler et al. 2005). The dermatitis is considered to be due to pharmacodynamic action as EGFR is widely expressed on epithelial cells (Bianchini et al. 2008).

- Cardiotoxicity

Trastuzumab, a humanised mAb against human HER2/neu, used in the treatment of HER2/neu positive metastatic breast cancer is associated with cardiotoxicity (Hudis 2007). Cardiac dysfunction was seen in 4 % of treated patients and this is characterised by impaired LVEF and tends to be reversible (Force, Krause & Van Etten 2007). The mechanism behind trastuzumab cardiotoxicity is due to blocking HER2/neu downstream signalling causing programmed cell death of heart muscle cells with impaired contractility and LV function (Kuramochi et al. 2006).

1.5 Immunogenicity to Biologics

Unwanted immunogenic responses have been described with the majority of biologics and is characterised by formation of anti-drug antibodies (refer Appendix 1). The development of ADA is one of the causes of clinical non-response due to neutralisation of the biological activity and also cross-react with endogenous protein (Koren 2002; Koren, Zuckerman & Mire-Sluis 2002). Patient specific factors, treatment specific factors and biologic specific factors may contribute to the formation of anti-drug antibodies. Immunogenicity to biologics is a major safety concern for manufacturers, regulatory bodies, healthcare professionals and the patients.

1.5.1 Clinical consequences

The consequences of immunogenicity to biologics range from transient appearances of antibodies with or without any clinical significance to potentially severe life threatening conditions. Potential consequences are severe hypersensitive reactions for e.g. basiliximab (Han et al 2004), anaphylaxis for e.g. abciximab and alglucosidase alfa (Shopnick, Kazemi et al. 1996; Corona, Leon et al. 1999), a decrease in efficacy for e.g. interferon beta, induction of autoimmunity for e.g. alemtuzumab and interferon alpha (Li, Yang et al. 2001; Sorensen, Ross et al. 2003; Aarskog, Maroy et al. 2009; (Perez-Alvarez et al. 2013) and cross reactivity to endogenous proteins for e.g. interferon beta (Baker et al. 2010). The formation of ADA will vary among different biologics, the manufacturing processes, indication and the target patient population. Anti-drug antibodies can be binding antibodies (BAb) or neutralising antibodies (NAb) and results in clinically meaningful antibody induced alteration.

1.5.1.1 Effects of anti-drug antibodies on therapeutic efficacy

The formation of anti-drug antibodies (both binding and neutralising) limits the efficacy of the biologic. Binding antibodies alter the pharmacokinetics for example in the cases of bevacizumab and adalimumab (Wang 2005). High titre BAb can also mistarget the drug to Fc receptor (FcR) expressing cells and therefore reduces efficacy for e.g. as in the case of replacement lysosomal enzymes such as alglucerase and imiglucerase (Wang et al. 2008). Binding antibodies are also referred to as clearance antibodies. Neutralising antibodies can block the biologic efficacy and may cross-react with endogenous protein counterpart for e.g. interferon beta (van de Stadt et al. 2013).

1.5.1.2 Effects of anti-drug antibodies on patient safety

The major safety problems associated with immunogenicity include anaphylaxis for e.g. abciximab and alglucosidase alfa (Shopnick, Kazemi et al. 1996; Corona, Leon et al. 1999), infusion reactions for e.g. with alemtuzumab and denileukin diftitox (Baert et al. 2003), non-acute reactions for example delayed hypersensitivity with rituximab (Han et al. 2003), immune responses secondary to immune complex formation for e.g. with alpha-glucosidase (Rojas et al. 2005) and cross-reactivity to endogenous proteins for e.g. with interferon beta (Rossert, Casadevall & Eckardt 2004).

1.5.2 Properties, functional effects and factors affecting formation of anti-drug antibodies

The anti-drug antibodies formed against biologics can result in significant changes in the pharmacokinetics and efficacy. The formation of ADA can be due to overt immune response to an exogenous version of endogenous human proteins or failure of immune tolerance to self-antigens (Goodnow 2001; Schellekens 2003; Sethu et al. 2012). The generation of ADAs is influenced by the biologic structure, manufacturing process, formulation and route of administration. Biologics are complex macromolecules and so a small change in the particular region may result in major change in overall properties. For instance oxidation of recombinant protein IFN-α may lead to form aggregates (Hochuli 1997). The changes in primary amino acid sequence causes increased immune response for example insulin derived non-human sources (Schernthaner 1993). The potential for immunogenicity is also impacted by the manufacturing process. Changes in the manufacturing processes are likely to affect the biologic product quality and therefore can result in immunogenicity (Chirino & Mire-Sluis 2004). The administration of biologics at high dosage or through a mucosal route can reduce immunogenicity through peripheral tolerance induction and is mediated by inducing tolerogenic DC and Tregs (Meritet, Maury & Tovey 2001).

1.5.2.1 Binding antibodies (non-neutralising antibodies)

Binding antibodies are also referred to as non-neutralising antibodies (Giannelli et al. 1994). They bind to epitopes that do not participate in the interaction between the biologic and its respective receptor or in other words these antibodies are capable of binding to a biologic but may or may not neutralise its biological effects (Wadhwa & Thorpe 2010). Binding antibodies can either expedite the clearance, called clearing antibodies or can prolong the bioavailability, called sustaining antibodies (Ponce et al. 2009). It is also been reported that the BAb titres can be higher than NAb and the BAb are produced much earlier during the course of treatment than NAb (Scagnolari et al. 2002b). Binding antibodies can also reside much longer than the NAb (Bellomi et al. 2003).

1.5.2.2 Neutralising antibodies

Neutralising antibodies neutralise the biological activity of the drug and could also alter the function of endogenous protein counterparts (Ponce et al. 2009). Significant correlations have been made between the NAb titres and the loss of clinical responses and this has been reported in several biologics such as interferon beta and natalizumab (Casato et al. 1991; Itri

et al. 1987; Lok, Lai & Leung 1990; Milella et al. 1993; Porres et al. 1989; von Wussow et al. 1988; Wussow et al. 1991). It is also reported that the percentage of NAb positive patients varies depending upon the source of the product, the route and frquency of administration and also the type of assay used to detect NAb. The non-human origin of biologics, the degree of humanisation, the presence of impurities, the dosage and different routes of administration contribute to the formation of antibodies. Disis et al 2004 reported HER-2/neu peptide based vaccine BAb promote the generation of NAb through mechanism of epitope spreading (Disis et al. 2004). *Epitope spreading* can be defined as, 'the development of immune responses to endogenous epitopes secondary to the release of self-antigens during a chronic autoimmune or inflammatory response' (Vanderlugt & Miller 1996). The full details of factors contributing to immunogenicity are described in Section 1.5.5. The cross reactivity of NAb with their endogenous proteins have also been reported (Antonelli et al. 1999; Bertolotto et al. 2000; Khan & Dhib-Jalbut 1998). In terms of timeline of NAb development, using a typical example of NAb formation to interferon beta (IFN-β), approximately 45 % (mean ~ 25 %) of the patients develops or become NAb positive within 6-12 months of treatment. The NAb can also persists for a long time irrespective of the withdrawal of the drug (Gneiss et al. 2004b; Malucchi et al. 2005; Petersen et al. 2006; Sorensen et al. 2005b).

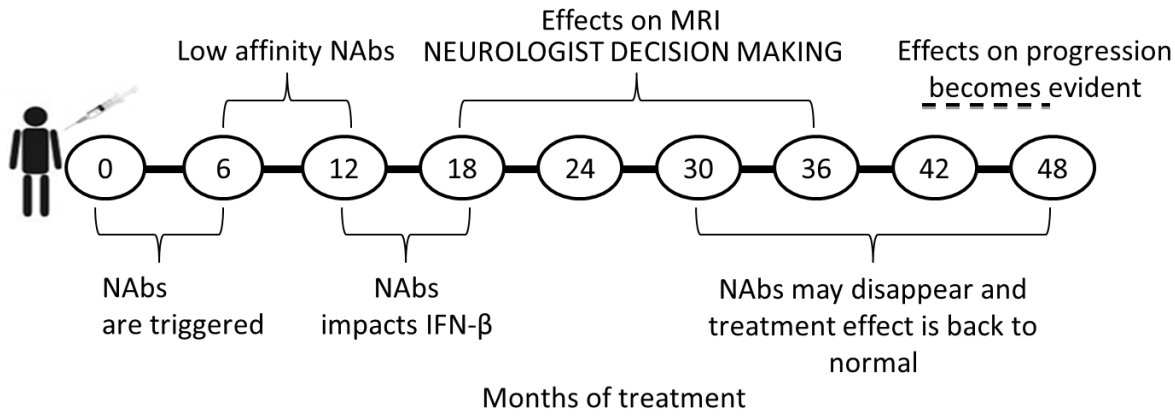


Figure 1.6: Dynamics and clinical consequences of NAb by time during treatment with IFN-β. The schematic represents the longitudinal clinical impact of NAb. The formation of NAb impacts IFN-β treatment and this reflects on routine magnetic resonance imaging (MRI) scans of the patient post treatment particularly during 12-36 months (Ross et al. 2006). The figure was adapted and modified from (Sorensen 2008).

1.5.3 Assays for immunogenicity

A number of immunogenicity testing assays have already been or being developed that has reasonable predictivity. Some currently used assays for quantifying binding and neutralising antibodies are described in the following sections

1.5.3.1 Binding antibody assays

Several assays are available for measurement of binding antibodies BAb (Chirmule, Jawa & Meibohm 2012; Krieckaert, Rispens & Wolbink 2012) including Enzyme-linked immuno sorbent assay (ELISA), radioimmunoassay or affinity chromatography. The ELISA methods most commonly used are direct ELISA or capture ELISA. In order to measure anti-IFN-β antibodies in plasma samples; the direct ELISA involves coating the wells with IFN-β whereas in capture ELISA, the plate are coated with monoclonal capture antibody specific for human IFN-β. Radioimmunoassays are also used for anti-drug antibody measurements and in the case of ADAs to anti-TNF radiolabelled therapeutic anti-TNF antibodies are used to bind to free anti-drug antibodies in the plasma samples (Baert et al. 2003).

1.5.3.2 Neutralising antibody assays

Biologics in general can act on cellular targets in vivo and alter the expression of several genes within the target cells. The NAb assays quantify these changes in gene expression where in there is inhibition of gene changes in presence of NAb. Interferon beta, a recombinant protein, binds to the interferon alpha or beta receptor (IFNAR) receptor activating intracellular signalling casade leading to synthesis of gene products such as myxovirus A (MxA), neopterin, oligoadenylate synthetase (OAS), tumour necrosis factor apoptosis inducing ligand (TRAIL) and β_2 microglobulin. The presence of NAb in the samples fails to bind and activate the receptors and thus these downstream gene expression effects are lost. Hence, IFN-β induced gene products can be used in assays to measure its bioactivity. The World Health Organisation (WHO) expert committee on biological standardisation 1985 recommends use of Cytopathic Effect assay (CPE) for measurement of IFN-β induced NAb. Cytopathic Effect assays use virus responsive cells and the ability of IFN-β to inhibit viral replication is measured.

The guidelines issued from EFNS recommend cell based assays for measuring IFN-β induced MxA mRNA or protein (Sorensen et al. 2005a). Sandwich type ELISA, western blots (Kob et al. 2003), fluorescence activated cell sorting (FACS) (Pachner 2003; Pachner, Bertolotto &

Deisenhammer 2003; Pachner, Oger & Palace 2003) and polymerase chain reactions (PCR) can also be used for measurements of MxA mRNA or protein. The recent EMA guidelines on immunogenicity states use of competitive ligand binding (CLB) assays for assessing the neutralising capacity of antibodies induced against therapeutic mAb such as CD40.

A potential source of artefact is from the ability of NAb to cause decrease or prevent binding of drug to reagents in the capture or detection methods. Several new assay formats are under research for example acid disassociation to detect anti-drug antibodies and drug (Patton et al. 2005) and affinity capture elution assay to detect anti-drug antibodies to therapeutic mAb in presence of high levels of drug (Kelley et al. 2013).

1.5.4 Immunoglobulin isotypes involved in immunogenicity

Studying immunoglobulin isotypes involved in immune response against biologics may provide further insights into evolution of immune responses. Several of biologics specific anti-drug antibodies are mainly of IgG isotype, although some IgA, IgM and IgE isotype have been reported (Candon et al. 2006; Kosmac et al. 2011; Vultaggio et al. 2010). The presence of high-affinity IgG and formation of IgG1 and IgG4 anti-drug antibodies indicates the presence of T-cell help (Baker et al. 2010). Infliximab and adalimumab IgG anti-drug antibodies are predominantly of IgG1 and IgG4 subclasses. Interferon beta anti-drug antibodies are predominantly of IgG1 and IgG3 within the first 6 months while IgG4 antibodies peaked later after 24 months (Gibbs & Oger 2007). The formation of IgG4 anti-drug antibodies are suggestive of repeated antigenic exposure (Aalberse, van der Gaag & van Leeuwen 1983).

1.5.5 Factors contributing to immunogenicity to biologics

Several different factors are thought to contribute the immunogenicity of biologics. The factors could be patient specific, treatment specific or biologic specific.

1.5.5.1 Patient specific factors

- Patient age

  The immune response against a biologic can be an age-related phenomenon. For example children might have different immune response compared to adults and therefore age factor is considered crucial for immunogenicity (according to the guidelines issued from European and US Federal agencies). Ageing is associated with a decline in the immune system function and increases the risk of infections (Dorshkind & Swain 2009).

- Genetic status

  Genetic factors such as human leukocyte antigen (HLA) haplotypes can modulate immune responses to biologic. Definite HLA types have been discovered in the anti-drug antibodies response to several biologics (Sethu et al. 2012). HLA-DRB1*0401, HLA-DRB1*1601, HLA-DRB1*0408 (Buck et al. 2011; Hoffmann et al. 2008), HLA-DRB1*0701 (Barbosa et al. 2006), HLA-DR2, HLA-DQ6, DQB1*0602, HLA-DR15 (Stickler et al. 2004) for interferon beta, HLA-DQA1*0102, HLA-DR4.1, DQ4, DQA1*0301 (Ohta et al. 1999; Simonney et al. 1985), HLA-DRA-DRB1*1104 (Ettinger et al. 2010) for factor VIII and HLA-DRB1*09-DQB1*0309 for erythropoietin (Praditpornsilpa et al. 2009). Human leukocyte antigen mapping studies may define subsets of populations at risk.

- Immunological status and competence of the patient

  Patients suffering from immune suppression may have lower risk of mounting immune response to biologics when compared to healthy donors with intact immune system. Neutralising antibodies was detected in 95 % of immune-competent cancer patients treated using recombinant GM-CSF compared to only 10 % of immune-compromised cancer patients (Ragnhammar et al. 1994).

1.5.5.2 Treatment specific factors

- Treatment regime (route of administration, dosage and frequency of administration)

  Route of administration can influence the risk of sensitisation. Intradermal, SC and inhalational route of administration are more immunogenic when compared to intravenous route of administration. Research suggests SC administration is relatively hostile to an already immunogenic biologic however SC route of administration by itself cannot render non-immunogenic biologics, immunogenic (Schellekens 2005). Hence, the strength of immune response to an antigen can be subcutaneous > intramuscular > intravenous. For obvious reasons SC route of administration is the most convenient route for at least when self-administration is made by patients. In conjunction to route of administration, the dose, frequency and duration also contribute to immunogenicity (Rosenberg and Worobec 2004). For instance long term treatment with high dose factor VIII induced tolerance, while intravenous abatacept (a fusion protein) has shows only a mild increase in immunogenicity. High dose infliximab and pre-treatment with high doses of non-antigen-binding variant of the therapeutic antibody with alemtuzumab have shown

decreased immunogenicity and induce immunological tolerance using the mechanism of exhaustion of the immune response (Maini et al. 1998; Sathish et al. 2013).

- Combination therapy

  Immunomodulators when given in combination with biologics have been described to inhibit formation of anti-drug antibodies (Rojas et al. 2005). For example co-administration of anti-TNF agents with azathioprine, mercaptopurine, steroids or methotrexate is associated with decreased frequency of anti-drug antibodies formation (Jani M et al 2013).

1.5.5.3 Biologic specific factors

- Target binding capability

  The influence of target binding capacity on ADA formation is exemplified in a study where 20 patients initially received nonbinding form and binding form of alemtuzumab followed by therapeutic alemtuzumab. The percentage of anti-drug antibodies patients were found to be lower in the group who were treated with target-binding form of alemtuzumab (Khono, T et al 2007).

- Molecular structure or amino acid sequence differences between native and therapeutic protein

  Different degrees of humanisation of the biologic can lead to different degrees/types of immunogenicity for e.g. a fully humanised IgG1 monoclonal antibody which is derived from by CDR grafting can elicits a human anti-human antibody response (Nechansky 2010). Dalum et al have shown non-human epitopes can elicit T-cell response and therefore facilitate epitope spreading to generate ADAs (Dalum et al. 1997).

- T-cell epitopes

  When the biologic is taken up by antigen presenting cells, the protein is digested into peptides and these can be loaded on to MHC molecules. The MHC-antigen complexes are transported to the surface where they bind to T-cell receptors (TcR) and induce proliferation of antigen specific T-cells. T-cells can recognise linear epitopes from proteins that are processed by antigen presenting cells. The antigenic proteins are broken down by proteolytic enzymes in the antigen presenting cells. During this process large numbers of peptides are formed. Nearly, 2 % of the peptides generated contain right amino acid sequence that allow them to bind in the MHC grove and are presented on the surface of antigen presenting cells. One of the important aspects to consider in terms of immunogenicity is the binding strength of T-cell receptor to MHC molecules (Lazarski et

al. 2005). The nature of T-cell, TcR and the MHC-peptide complexes determine the overall effect of T-cell responses. Various methods such as in silico prediction tools and in vitro proliferation assays have been used to investigate the role of T-cell epitopes in immunogenicity. In order to aid the identification of T-cells epitopes computer algorithms such as Immune Epitope Database and ProPred have developed to map the locations for MHC class I and II restricted T-cell epitopes within proteins. Insilico prediction tools have already been applied to design vaccines and also in selection of epitopes in autoimmunity studies (De Groot & Scott 2007). MHC class II predictions methods can be useful for evaluation of T-cell dependent immunogenicity. MHC class II restricted T-cells provide help to B-cells leading to formation of anti-drug antibodies.

- B-cell epitopes

  Some biologics contain multivalent antigenic determinants and hence can induce T-cell independent anti-drug antibodies formation. Mapping of binding regions of anti-drug antibodies on biologics may enable to identify B-cell epitopes involved in immunogenicity. B-cell epitopes are important component of anti-drug antibody response. The B-cell epitopes of recombinant factor VIII has been extensively studied. The precise amino acids which are required for antibody binding have been identified on various factor VIII domains (Fulcher et al. 1985; Scandella et al. 1995). This identification had led to development of factor VIII variants with reduced immunogenicity (Parker et al. 2004).

- Protein aggregates

  Studies have also shown that the aggregated forms of biologics can stimulate humoral immune responses (Braun et al. 1997). Protein aggregates are made up of either intact native protein or degraded or denatured protein. Subvisible particulates of protein aggregates in the size 0.1-10 microns have strong potential to elicit an immune response (Berkowitz 2006; Gamble 1966; Gross & Zeppezauer 2010; Roda et al. 2009). The mechanisms of aggregate induced ADA generation include the following – cross linking of B-cell receptors and causing B-cell activation (Bachmann et al. 1993; Dintzis et al. 1989), increased antigen uptake, processing and presentation (Seong & Matzinger 2004), followed by recruiting T-cells and B-cells to produce high-affinity IgG antibody (Bachmann & Zinkernagel 1997).

- Post translational modifications (glycosylation or pegylation)

  Glycosylation in general enhances solubility and diminishes aggregation and antibodies formation. Glycosylation can alter protein immunogenicity by shielding immunogenic

epitopes from immune system (Cole et al. 2004; Wei et al. 2003). Pegylation can also diminish antibodies formation via similar mechanisms (Harris, Martin & Modi 2001; Inada et al. 1995). However, immune response to polyethylene glycol (PEG) itself has been recently identified (Liu et al. 2011).

- Impurities with adjuvant activity

  Microbial impurities in the biologic formulations can potentially contribute towards immunogenicity. These impurities exert immune enhancing activity and prompts antigen presenting cells to stimulate B-cells to produce antibodies (Verthelyi & Wang 2010).

- Formulation

  Large protein excipients in the formulation for example HSA may form protein adducts under abnormal storage conditions and can predispose ADA generation (Braun & Alsenz 1997).

- Manufacturing process

  Proteins can denature and form aggregates during the manufacturing process wherein they may be exposed to heat, light and mechanical agitation.

1.6 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS). Multiple sclerosis is documented all over the world, with approximately 2.5 million people affected worldwide, around 400,000 people in the US and 100,000 people in the UK (Coles 2009; Noonan, Kathman & White 2002; Rosati 2001). The highest incidence is found in Caucasian woman, people of Northern European descent and those living in northern latitudes (International et al. 2009). Diagnosis is established between the ages of 20 and 40 and it affects women almost twice as much as men (Alonso et al. 2007; Koch-Henriksen 1999). The overall life expectancy is lowered by about seven years (Ragonese et al. 2008).

1.6.1 Causes and molecular pathology

The cause of this disease includes environmental exposure and genetic susceptibility.

Environmental factors – The disease is common in regions populated by northern Europeans. Epstein-Barr virus infection in young adults increases the risk of developing multiple sclerosis (Levin et al. 2003).The patients suffering from this disease also exhibit infections such as measles, mumps and rubella (Martyn, Cruddas & Compston 1993).

Two different mechanisms have been proposed

- Molecular mimicry – activation of autoreactive cells by cross-reactivity between self-antigens and antigens from microbes. It involves reactivity of T-cell and B-cells with peptides or antigenic determinants shared by infectious agents and self-antigens. It leads to activation and migration of cells across the blood brain barrier (BBB), CNS infiltration, tissue damage and disease onset.

- Bystander activation – The autoreactive cells are activated due to nonspecific inflammatory events during infections. It involves TcR independent bystander activation of autoreactive T-cells through inflammatory cytokines, superantigens and Toll-like receptor (TLR) activation.

Genetic factors – The familial re-occurrence rate of multiple sclerosis is about 20 %, a genetic association exists between this disease and MHC alleles DRB1*1501, DRB5*0101, DQA1*0102 and DQB2*0602 (Olerup & Hillert 1991). The association was again strongest in northern Europeans but is seen in all populations with DR4 (DRB1*0405 – DQA1*301 – DQB1*0302) MHC alleles (Marrosu et al. 1992).

Disease mechanisms –

The hallmark of this disease is formation of sclerotic plague in the CNS, which are characterised by inflammation, demyelination and remyelination, oligodendrocyte depletion and astrocytosis, and neuronal and axon degeneration (Hohlfeld & Wekerle 2004). The lesions can develop in any part of the CNS; however tendency exists to accumulate near periventricular and exterior surfaces of brain and spinal cord. The lesions are round to oval shaped and are centered to one or numerous medium sized vessels (Hohlfeld et al. 2006).

The pathogenesis of this disease begins with increased migration of autoreactive $CD4^+$ T-cells across the BBB. The activated T-cells produce a range of pro-inflammatory cytokines which stimulate other T-cells, B-cells, macrophages, natural killer cells and microglia to augment and perpetuate inflammatory process. The cytokines cause increased permeability of the BBB, alter adhesion molecule expression, produce antibodies and recruit other immune cells into the CNS. The advent of T helper 17 cells has led to further understanding of T-cell regulation. The evidence suggests these cells secrete IL-17 with IL-6 and TNF-α and plays a crucial role in inflammation (Bowman, Chackerian & Cua 2006). Damage to axons can be mediated by cytotoxic T-cells, macrophages, antibodies, loss of trophic support and oxidative stress (Sospedra & Martin 2005). Loss of trophic support for axons and exposure to lethal

metabolites also leads to degeneration of axons. The redistribution of ion channels, mitochondrial failure and calcium mediated toxicities all contribute to axonal degeneration (Waxman 2006a, 2006b).

1.6.2 Clinical picture

The clinical manifestations of multiple sclerosis can include impairment in the variety of cognitive functions, loss of vision or abnormalities of eye movements, fatigue, spasticity, imbalance, in-coordination and sensory loss. There is no cure for multiple sclerosis once this condition is detected and it remains rest of the life. There are treatment options that can relieve symptoms and relapses. These can be divided into three main categories.

Treatment for acute relapses – corticosteroids are thought to suppress the immune system and hence reduce the inflammation and increase the duration between relapses but have no impact on the progression of the disease. The mechanism of action of corticosteroids in the treatment of relapses is still unclear. But, the potential mechanism of action includes reducing oedema, stabilising blood-brain barrier, diminishing pro-inflammatory cytokines and causing T-cell apoptosis (Gold, Buttgereit & Toyka 2001). The complications associated with corticosteroids such as avascular bone necrosis, diabetes, exacerbation of infections, hypertension, reduced bone density, weight gain has to be considered before the treatment (Pozzilli, Romano & Cannoni 2002).

Treatment for specific symptoms – is a critical component of general management of multiple sclerosis disease. Symptomatic treatment is intended at elimination or reduction of symptoms impairing the functional abilities associated with the disease. The Multiple Sclerosis Therapy Consensus Group (MSTCG) and the German Multiple Sclerosis Society consensus publication contain suggestions for the treatment of common symptoms (Henze et al. 2006).

Treatment to slow the progression of multiple sclerosis using disease modifying drugs (DMD) – this includes immunomodulatory drugs such as azathioprine, interferon beta, cyclophosphamide, glatiramer acetate, intravenous immunolgobulin, methotrexate, mitoxantrone and natalizumab (Clegg, Bryant & Milne 2000). Research into long term effects of these drugs are limited but they can reduce the number and severity of relapses and may also help to slow the progression of the disease.

1.7 Interferon beta IFN-β

Interferons are naturally occurring proteins which stimulate intracellular and intercellular responses that regulate resistance to viral infections, enhance immune responses and modulate cell survival and death (De Maeyer & De Maeyer-Guignard 1982). In 1975 Isaacs and Lindenmann introduced interferons as antiviral agents (Isaacs & Lindenmann 1957)

Three types of interferons have been identified with Type I IFN being composed of 16 members which includes 12 IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. Interferon alpha and beta share 32-40 % of the amino acid sequence. Both are encoded in chromosome 9 and act on a common cell surface receptor (Lin 1998). Interferon beta exists in humans and is produced primarily by virus-infected fibroblasts. It is also produced in the glial cells in the CNS. Human IFN-β is a 23-kD glycoprotein and consists of 166 amino acids and 20 % carbohydrates.

Type II IFN includes IFN-γ which exhibits antiviral activities and is produced from immune T-cells.

Type III IFN or IFN-λ family includes IFN-λ1 (also known as IL-29), IFN-λ2 (also known as IL-28A) and IFN-λ3 (also known as IL-28B). Recent evidence suggests type III IFN is similar to members of interleukin-10 (IL-10) family. These interferons also exhibit antiviral responses and activate IFN-stimulated genes (Gad et al. 2009).

Type I IFN proteins were developed as therapeutic proteins for the treatment of multiple sclerosis because of its anti-viral properties as the patients suffering from this disease exhibited viral infections (Borden et al. 2007). Type I IFN proteins also exhibit numerous immunomodulatory functions and alter T helper 1 and T helper 2 balance (Hussien et al. 2001), antagonise proinflammatory cytokines such as IFN-γ, IL-12 and TNF-α, down regulate MHC class II expression which affects antigen presentation (Yong 2002), possess anti-proliferative properties on T-cells and also can cause T-cell apoptosis (Sharief et al. 2001; Yong 2002). The evidence also suggests that Type I IFN inhibits transmigration of immune cells across the BBB (Leppert et al. 1996).

Interferon alpha and beta exert their biological effect, by binding to the same high affinity cell surface receptor (Type I IFN receptor) and initiating a signalling cascade. The Type I IFN receptor consists of two subunits (interferon alpha or beta receptor) IFNAR1 and IFNAR2 which are associated with two cytoplasmic tyrosine kinases Janus Kinase 1 (Jak1)

and Tyk2 (Muller et al. 1993; Novick, Cohen & Rubinstein 1994; Uze, Lutfalla & Gresser 1990). The interferon molecule binds to IFNAR2 which recruits IFNAR1 and induces phosphorylation of Jak1 and signal transduction and activators of transcription (STAT). The further processes include transactivation from interferon stimulated response elements (ISRE) contained in the promoter regions of several genes (Platanias 2005).

Interferon beta triggers the synthesis of host cell proteins contributing to its several immunomodulatory properties. The induction of host cell proteins such as 2', 5'-oligoadenylate synthetase, myxovirus (Mx) protein, double-stranded RNA-dependent protein kinases and inducible nitrous oxide (NO) can inhibit viral replication (Baron et al. 1991; Borden et al. 1988). Interferon beta exhibits potent T-cell anti-proliferative properties (Bekisz et al. 2010). It might also affect T-cell function via inhibition of T helper 1 cytokines such as IFN-γ, IL-12 and TNF, and promotes T helper 2 cytokine secretion such as IL-10 (Dayal et al. 1995; Noronha, Toscas & Jensen 1993; Rudick et al. 1996). Prolongation of cell cycle and depletion of metabolites might cause anti-proliferative effects in tumor cells (Borden 1992; Shearer & Taylor-Papadimitriou 1987). Interferon beta also increases the expression of HLA class I molecules and this also might contribute to antiviral effects (Spear et al. 1987). It inhibits HLA class II molecules induced from IFN-γ and so inhibits the antigen presentation (Jiang et al. 1995; Johnson & Panitch 1989; Joseph et al. 1988; Ling, Warren & Vogel 1985; Noronha, Toscas & Jensen 1992; Ransohoff et al. 1991).

Jacobs et al found the beneficial effect of IFN-β in patients suffering from multiple sclerosis (Jacobs & Johnson 1994; Jacobs et al. 1982a; Jacobs et al. 1982b). Interferon beta treatment dampens the plaque formation, decreases relapses and eventually improves disease prognosis. In 1993 IFN-β-1b was approved for the treatment of RRMS (Hoyer 1993; Paty & Li 1993). Since then, two recombinant forms of IFN-β products IFN-β-1a and IFN-β-1b have been approved for the treatment of multiple sclerosis. Four different preparations are currently available: IFN-β-1a (Avonex™ from Biogen), IFN-β-1a (Rebif™ from Merck Serona), IFN-β-1b (Betaseron™ from Bayer) and IFN-β-1b (Extavia™ from Novartis). IFN-β-1a (Avonex™ and Rebif™) is a 166 amino acid glycosylated protein produced in Chinese hamster ovary (CHO) cells (Revel 2003). IFN-β-1b (Betaseron™ and Extavia™) is a 165 amino acid non-glycosylated protein produced in Escherichia Coli (E Coli) and lacks glycosylation and contains serine residue substitution for cysteine at position 17 (Lin 1998). The majority of multiple sclerosis patients exhibit improvement to this treatment (PRISMS et

al 1998). It reduces relapses and also slows down the disease progression (Killestein & Polman 2011).

1.7.1 Structure of interferon beta

In 1992, the first structure of murine IFN-β was elucidated (Senda et al. 1992). In 1997, the crystal structure of human IFN-β was elucidated (Karpusas et al. 1997). Interferon beta is roughly cylindrical in shape and belongs to family of long-chain helical cytokines. It contains 166 amino acid residues and consists of five α-helices designated as helix A (amino acid residues 2-22), helix B (amino acid residues 51-71), helix C (amino acid 80-107), helix D (amino acid 118-136) and helix E (amino acid 139-162). The helices are connected by a long AB loop and 3 short loops BC, CD and DE. The AB loop is further divided into 3 segments AB1 (amino acid residues 23-35), AB2 (amino acid residues 36-40) and AB3 (amino acid residues 36-40). The structure also consists of several hydrophobic residues Phe-70, Phe-154, Trp-79 and Trp-143 which are involved in interaction with each other that stabilise the core of the molecule.

A glycosylation site exists at residue Asn-80. The studies suggest that the glycosylation of IFN-β plays a critical role in protein solubility and stability. Non-glycosylated forms of IFN-β are more susceptible to form aggregates and lead to immunogenicity. The limited number of studies suggests similar effects of both IFN-α and IFN-β on the relapse rates (Durelli et al. 1996; Hughes 1997; Myhr et al. 1999).

The subunit of IFN receptor IFN-α-R1 interacts with exposed helix C residues and exposed residues of helix A and helix C side (residues Lys83 and Tyr89) of IFN-β. IFN-α-R2 interacts with AB loop, helix D, DE loop and helix E. Residues Arg33/35, Arg144/147, Glul 46/149 and Arg 149/151 are highly conserved and are involved in direct binding (Radhakrishnan et al. 1996).

The structure-function analysis of the IFN-β can provide critical information on the INFAR binding receptors, neutralising antibody binding sites, identification of T-cell and B-cell antigenic determinants. The potential overlapping between sequences of amino acids can be identified.

1.7.2 Side effects of interferon beta

Nearly 80 % of the patients experience flu-like symptoms within 24 hours of IFN-β injections. The increase in temperatures can be due to up-regulation of inflammatory

cytokines such as IL-6 and IFN-γ. Skin reactions occur in 50 % of patients. Skin necrosis has been reported in 5 % of patients (Lublin et al. 1996; Walther & Hohlfeld 1999). Formation of anti-drug antibodies have been reported with all the 4 different IFN-β preparations ('Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group' 1998; 'PRISMS-4: Long-term efficacy of interferon-beta-1a in relapsing MS' 2001; Rudick et al. 1998; UBC 1996). The formation of neutralising antibodies impacts clinical safety and efficacy (the details of this aspect will be discussed in more detail in the systematic review described in chapter 2).

1.8 Thesis aim and hypotheses

The aim of the research presented in this thesis was to understand immunogenicity, anti-drug antibody formation and the immunological consequence of NAb against therapeutic protein IFN-β-1a Rebif™.

This thesis set about addressing the following hypotheses:

- IFN-β-1a Rebif™ IgG subclass antibodies exhibit differential neutralising capabilities and neutralise endogenous IFN-β.
- IFN-β-1a Rebif™ IgG antibodies form immune complex with IFN-β-1a Rebif™ and activate complement.
- IFN-β-1a Rebif™ IgG antibodies are associated with specific B-cell epitopes.
- IFN-β-1a Rebif™ IgG antibodies are associated with specific T–cell epitopes.
- Defect in the regulatory T-cells leads to IFN-β-1a Rebif™ immunogenicity.

Chapter 2: Influence and clinical significance of interferon beta neutralising antibodies in multiple sclerosis – a systematic review and meta-analysis

2.1 Introduction

Multiple sclerosis is a chronic inflammatory and neurodegenerative immune-mediated disorder of the CNS and is characterised by inflammation, demyelination and primary or secondary axonal degeneration (Compston & Coles 2008).

Type I interferons which include IFN-β are used in the treatment of relapsing forms of multiple sclerosis and shown to be beneficial ('The IFNB Multiple Sclerosis Study Group' 1993; Paty & Li 1993; Yong et al. 1998). There are two forms of IFN-β, namely IFN-β-1a and IFN-β-1b, which are prescribed for the treatment of multiple sclerosis (refer to Table 2.1). IFN-β-1a is produced in CHO cells and is identical to the human form of IFN-β. Interferon beta-1b is produced in E.coli and consists of 165 amino acids due to lack of N terminal methionine and cysteine is substituted with serine at position 17. There are four different formulations of IFN-β: IM IFN-β-1a Avonex™ from Biogen-Idec, SC IFN-β-1a Rebif™ from Merck-Serono, SC IFN-β-1b Betaferon/Betaseron™ from Bayer-Schering and SC IFN-β-1b Extavia™ from Novartis (refer Figure 2.1 for amino acid sequences). Studies indicate IFN-β decreases the annualised relapse rate by approximately one-third (Napier 1997). Overall IFN-β is welltolerated in the management of multiple sclerosis.

A

```
MTNKCLLQI   ALLLCFSTTA  LS
MSYNLLGFLQ  RSSNFQCQKL  LWQLNGRLEY  CLKDRMNFDI  PEEIKQLQQF  QKEDAALTIY
EMLQNIFAIF  RQDSSSTGWN  ETIVENLLAN  VYHQINHLKT  VLEEKLEKED   FTRGKLMSSL
HLKRYYGRIL  HYLKAKEYSH  CAWTIVRVEI  LRNFYFINRL  TGYLRN
```

B

```
MSYNLLGFLQ  RSSNFQCQKL  LWQLNGRLEY  CLKDRMNFDI  PEEIKQLQQF  QKEDAALTIY
EMLQNIFAIF  RQDSSSTGWN  ETIVENLLAN  VYHQINHLKT  VLEEKLEKED  FTRGKLMSSL
HLKRYYGRIL  HYLKAKEYSH  CAWTIVRVEI  LRNFYFINRL  TGYLRN
```

C

```
 SYNLLGFLQ  RSSNFQSQKL  LWQLNGRLEY  CLKDRMNFDI  PEEIKQLQQF  QKEDAALTIY
EMLQNIFAIF  RQDSSSTGWN  ETIVENLLAN  VYHQINHLKT  VLEEKLEKED  FTRGKLMSSL
HLKRYYGRIL  HYLKAKEYSH  CAWTIVRVEI  LRNFYFINRL  TGYLRN
```

Figure 2.1: The amino acid sequences. The Figure A represents the amino acid sequence of human endogenous IFN-β molecule with 187 amino acids which includes 1-21 signal peptide and 22-187 interferon beta molecule. The Figure B represents the amino acid sequence of IFN-β-1a molecule with 166 amino acids and Figure C represents the amino acid sequence of IFN-β-1b molecule with 165 amino acids.

IFN-β is the most commonly prescribed treatment for multiple sclerosis. However, IFN-β induces formation of neutralising antibodies which may abrogate the efficacy of the treatment. Immunogenicity of IFN-β is the major complication associated with treatment. The formation of neutralising antibodies against biologics including IFN-β is due to several factors (Schellekens 2005) –

- Patient specific factors for example patient age, genetic status, immunological status and competence of the patient
- Treatment specific factors for examples treatment regime, route of administration, dosage and frequency of administration
- Biologic specific factors for examples molecular structure or differences in the amino acid sequence, degree of humanisation, T-cell epitopes, B-cell epitopes, protein aggregates, post-translational modifications, impurities, formulation, manufacturing process.

Up to 45 % (mean approx. 25 %) of patients develop neutralising antibodies to different formulations of IFN-β (Creeke & Farrell 2013). Neutralising antibodies are more frequently produced with IFN-β-1b than with IFN-β-1a (Bertolotto et al. 2003; Cook et al. 2001; Malucchi et al. 2004; Panitch et al. 2002). Research also suggests that SC IFN-β-1a is more immunogenic than with the IM route of administration (Giovannoni, Munschauer & Deisenhammer 2002). The causes for the formation of neutralising antibodies are unclear. Patients with neutralising antibodies exhibit higher relapse rates, prominent lesion activity on MRI and increased rate of disease progression (Sorensen 2008).

This chapter describes a systematic review which aimed to determine factors affecting the formation of neutralising antibodies against different formulations of IFN-β in the treatment of multiple sclerosis. The review aimed to define the reasons for variability in the reported incidences of neutralising antibodies, and furthermore assess the methodological recording and reporting of clinical risk factors which contribute to the formation of neutralising antibodies against IFN-β.

Interferon beta formulations			Produced in	Protein modification	Federal approval	Molecular target	Molecular structure	Licensed for	Route	Frequency	Distributor
Avonex™	Interferon beta-1a	Recombinant form of native protein	CHO cells	Glycosylated	1996	Type 1 interferon receptors	Recombinant human physiologic protein molecular weight 22.5 kDa	RRMS	IM	QW	Biogen Idec
Rebif™	Interferon beta-1a	Recombinant form of native protein	CHO cells	Glycosylated	2002	Type 1 interferon receptors	Recombinant human physiologic protein molecular weight 22.5 kDa	RRMS	SC	TIW	Merck Serono, Pfizer
Betaseron/ Betaferon™	Interferon beta-1b	Recombinant form	E.coli	Not glycosylated	1993	Type 1 interferon receptors	Recombinant human physiologic protein molecular weight 18.5 kDa	RRMS	SC	EOD	Bayer
Extavia™					2009						Novartis

Table 2.1: Characteristics of different interferon beta formulations (source Drugs@FDA page on the FDA website). Abbreviations: CHO, Chinese Hamster Ovary; E.coli, Escherichia coli; kDa, Kilodalton; RRMS, relapsing remitting multiple sclerosis; IM, intramuscular; SC, subcutaneous; QW, once a week; QIW, 3 times a week; EOD, every other day.

2.2 Methods

2.2.1 Search strategy

The databases used in the review included MEDLINE OVID (1950 to February Week 4 2013), EMBASE OVID (1980 to February Week 4 2013), CENTRAL on The Cochrane Library (2011, Issue 4) and the Clinicaltrials.gov (1997 to February Week 4 2013). Search strategies were developed specifically for each database and are presented in Appendix 2. The reference lists of identified review articles and of all included trials were searched to find other potentially eligible studies.

2.2.2 Criteria for considering studies in this review

Randomised controlled trials (RCTs) and non-randomised controlled studies (NRS) comparing the efficacy of different formulations of IFN-β such as IFN-β-1a Avonex™, IFN-β-1a Rebif™, IFN-β-1b Betaferon/Betaseron™, and IFN-β-1b Extavia™ in patients with multiple sclerosis were included. Men and women (aged 18 or more) were eligible. Trials in which IFN-β were used to treat or control other disease conditions were excluded. Studies were only considered for inclusion if they were published in English.

2.2.3 Types of outcome measure

The primary outcome of interest extracted in each study was the frequency of IFN-β neutralising antibodies.

2.2.4 Study selection

2.2.4.1 Inspection of citations

After duplicate citations were removed, all titles and abstracts were independently reviewed by two reviewers with reference to the inclusion or exclusion criteria (refer appendix 3 - Study Eligibility Screening Form) and a decision was made about whether to retrieve the full report of the study. The number of titles or abstracts identified, selected and rejected was recorded.

2.2.4.2 Inspection of retrieved reports

Once the full reports were retrieved, they were inspected for relevance to the review and the inclusion and exclusion criteria were applied. Studies not meeting the pre-determined criteria were excluded. If there was any disagreement about whether to include any of the studies, a third reviewer assessed them and, together with the other reviewers made a consensus decision about whether to include or exclude. A record was made of the number of full reports retrieved and the number excluded.

2.2.5 Data collection

A formal data extraction form was designed, piloted independently on a small selection of studies and adjusted as necessary (refer appendices 3 and 4). For each study, information regarding the participants, comparison groups, treatments and outcomes were tabulated. Where they were recorded or provided by the author, the following data were extracted for each randomised study:

1. Study characteristics: number of participants, number of participants in each treatment group, year completed, setting, inclusion criteria, definition of neutralising antibody positivity and length of follow up.
2. Participant characteristics: age, gender, underlying disease and indication for IFN-β treatment.
3. Treatments: number of treatment groups, treatment details (Avonex™ and/or Rebif™ and/or Betaferon/Betaseron™ and/or Extavia™), dosage, route of administration and frequency of treatment.
4. Outcomes reported: status of neutralising antibodies and the method used to detect neutralising antibodies.

Where they were recorded or provided by the author, the following data were extracted for each non-randomised study:

1. Study characteristics: number of participants, number of participants in each intervention group, year completed, setting, inclusion criteria, definition of neutralising antibody positivity and length of follow up.
2. Participant characteristics: age, gender, underlying disease and indication for IFN-β treatment.

3. Treatment characteristics: Avonex™ and/or Rebif™ and/or Betaferon/Betaseron™ and/or Extavia™, dosage, route of administration and frequency of treatment.
4. Status of neutralising antibodies and the method used to detect neutralising antibodies.

2.2.6 Quality assessment

The methodological quality of frequency of neutralising antibodies recording and reporting was assessed for both randomised and non-randomised studies using selected elements of the McMaster Quality Assessment Scale of Harms for primary studies (the McHarm scale) http://hiru.mcmaster.ca/epc/mcharm.pdf. The elements used were selected based on an evaluation of their relevance to our research question and what they aimed to evaluate: the quality and appropriateness of study design and reporting, the applicability of the study findings to the population and measures taken to reduce bias (refer appendix 6).

Both data collection and quality assessment of studies were undertaken from one reviewer (Karthik Govindappa), with three randomised and three non-randomised studies assessed by a second reviewer (Dr Jamie Kirkham) to check for consistency.

2.2.7 Statistical analysis and synthesis

Statistical analyses were performed using RevMan (version 5.2 software). As the frequency of IFN-β neutralising antibodies data is dichotomous (i.e. the data from outcomes have two possible values for example yes or no), the data were analysed by calculating the Odds Ratio (OR) with corresponding 95% confidence intervals using the method of Mantel-Haenszel. Data from randomised controlled trials were analysed separately from the non-randomised study data.

2.2.7.1 Meta-analysis

We aimed to conduct two primary meta-analyses. For RCTs, the frequency of neutralising antibodies against IFN-β-1a Avonex™, IFN-β-1a Rebif™, IFN-β-1b Betaferon/Betaseron™ or IFN-β-1b Extavia™ in patients with multiple sclerosis was determined. For NRS, the frequency of neutralising antibodies against IFN-β-1a Avonex™, IFN-β-1a Rebif™, IFN-β-1b Betaferon/Betaseron™ or IFN-β-1b Extavia™ in patients with multiple sclerosis was determined.

2.2.7.2 Heterogeneity and sub group analyses

If clinical heterogeneity was too great, studies were not pooled in a meta-analysis and narrative synthesis was used to compare studies. A chi-squared test for statistical heterogeneity was undertaken and the I^2 statistic was calculated. Where the necessary data were available, the following analyses were also planned:

- Comparison of neutralising antibodies against IFN-β-1a Avonex™ 30 μg and 60 μg;
- Comparison of neutralising antibodies against IFN-β-1a Rebif™ 22 μg and 44 μg and
- Comparison of neutralising antibodies against IFN-β-1a and IFN-β-1b

2.2.7.3 Publication bias

Publication bias for the randomised trials included in the meta-analyses was assessed by visual inspection of a funnel plot providing a sufficient number of studies were included in the analysis. All study authors were contacted where ever possible for missing outcome data.

2.3 Results

2.3.1 Search results

The database searches undertaken in February Week 4 2013, identified 249 abstracts for screening. After review of abstracts, 80 full text articles were reviewed. Of these, 40 articles were found to be not relevant, duplicates, one article was in Spanish language (Fernandez et al. 1999), two articles did not meet set inclusion criteria >20 patients (Chiu et al. 2007; Reske et al. 2004) and another two studies focused on neutralising antibody positive patients (Malucchi et al. 2005; Petersen et al. 2006). The remaining 35 articles were found to be eligible for this review (see Figure 2.2).

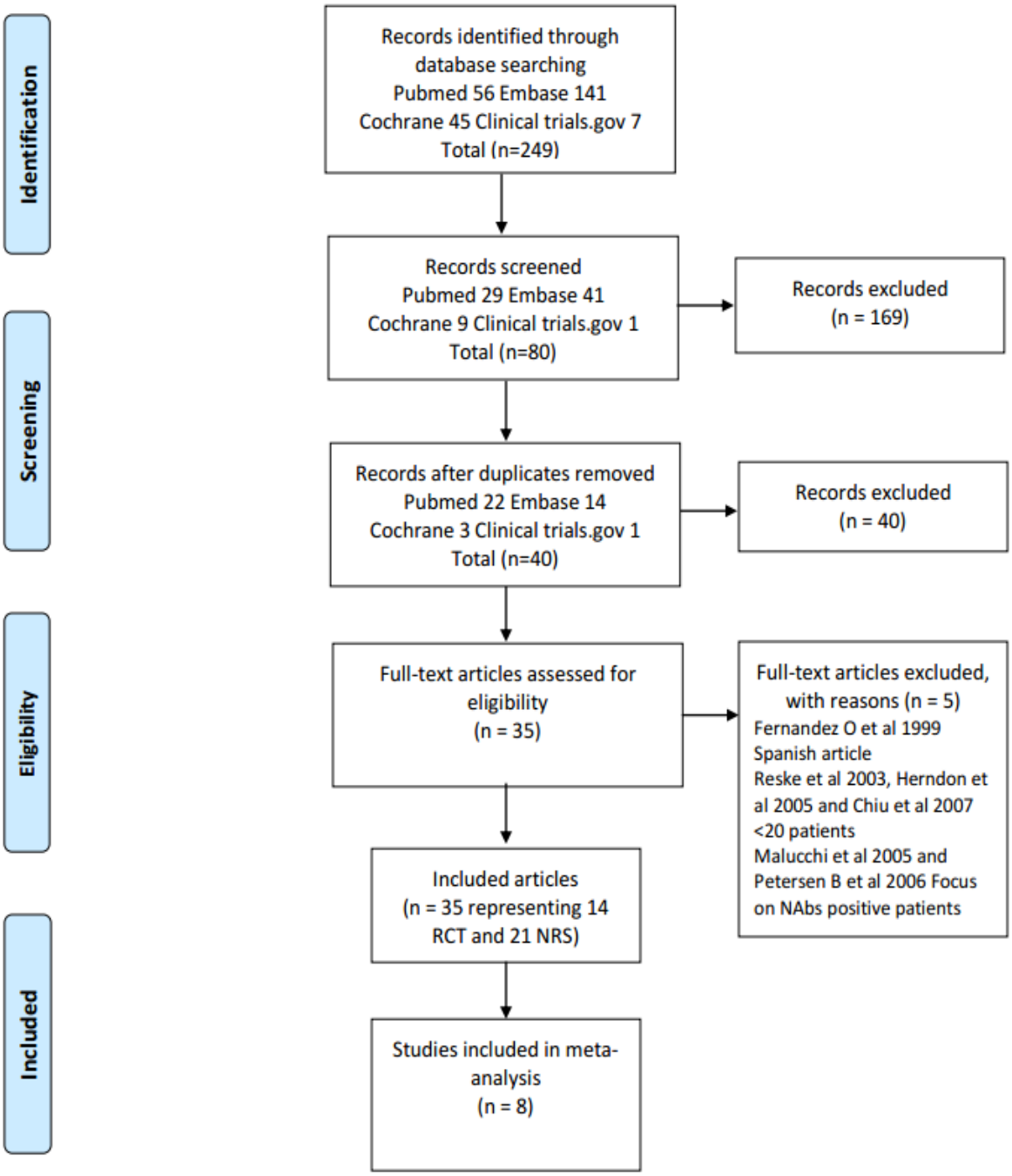


Figure 2.2: PRISMA flow diagram illustrating search strategy. Abbreviations: PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses; NAbs – neutralising antibodies; RCT – randomised control trials; NRS – non randomised studies.

2.3.2 Included studies

The characteristics of the studies included in this review such as the intervention, formulation, dosage, route of administration, frequency, number of patients, neutralising antibodies definition, method used for detection of neutralising antibodies and the follow up

duration in months of all the RCTs and NRS are shown in Tables 2.2, 2.3 and 2.4. The Table 2.2 list the characteristics of RCTs (n = 8) included in the meta-analysis (Barbero et al. 2006; Clanet et al. 2002; Francis et al. 2005; Freedman et al. 2005; Kappos et al. 2005; Li et al. 2001a; Schwid & Panitch 2007; Sorensen et al. 2003). Table 2.3 list the characteristics of RCTs (n = 6) not included in the meta-analysis ('North American Study Group' 2004; Goodin et al. 2012; Polman et al. 2003; Rudick et al. 1998; Shahkarami et al. 2013; UBC 1996) and Table 2.4 list the characteristics of NRS (n = 21) (Bellomi et al. 2003; Bertolotto et al. 2002; Durelli et al. 2008; Durelli et al. 2009; Farrell et al. 2008; Frank et al. 2004; Gilli et al. 2007; Giovannoni et al. 2007; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Hegen et al. 2012a; Knobler et al. 1993; Malucchi et al. 2005; Minagar, Murray & Investigators 2008; Pachner et al. 2009a; Petkau et al. 2004; Phillips et al. 2004; Ross et al. 2000b; Scagnolari et al. 2002a; Sorensen et al. 2005d) and Clinical trial NCT00367484 2011.

The meta-analysis included 4146 patients with multiple sclerosis randomised to receive either IFN-β or placebo. Of those 1963 received IFN-β-1a Avonex™, 1694 received IFN-β-1a Rebif™ and 256 received IFN-β-1b Betaseron/Betaferon™. The comparison was established between these studies. The six RCTs where no comparison was able to establish the neutralising antibodies against different IFN-β formulations are described descriptively. However, a total of 3575 patients with multiple sclerosis received either IFN-β or placebo. Of those 408 received IFN-β-1a Avonex™, none received IFN-β-1a Rebif™ and 2489 received IFN-β-1b Betaseron/Betaferon™.

Of the 21 non randomised studies, the comparison was established in seven studies consisting of 1763 patients with multiple sclerosis to receive IFN-β. Of those 497 received IFN-β-1a Avonex™, 768 received IFN-β-1a Rebif™ and 498 received IFN-β-1b Betaseron/Betaferon™. The remaining 14 non randomised studies where no comparison was able to establish the neutralising antibodies against different IFN-β formulations are described descriptively. However, a total of 2409 patients with multiple sclerosis received either IFN-β. Of those 294 received IFN-β-1a Avonex™, 969 received IFN-β-1a Rebif™ and 1146 received IFN-β-1b Betaseron/Betaferon™.

Characteristics of the RCTs included in the meta-analysis

Study	Year	Intervention	Formulation	Dosage	Route	Frequency	Patients	NAbs define	NAbs detection method	Follow up duration (months)
Li	2001	IFN-β-1a	Rebif™	22 µg	SC	TIW	303	≥ 20	CPE	36 months
		IFN-β-1a	Rebif™	44 µg	SC	TIW	297			
Clanet	2002	IFN-β-1a	Avonex™	30 µg	IM	QW	402	≥ 20	CPE	36 months
		IFN-β-1a	Avonex™	60 µg	IM	QW	400			
Sorenson	2003	IFN-β-1a	Rebif™	22 µg	SC	QW	103	≥ 20	CPE	Up to 60 months
		IFN-β-1a	Rebif™	22 µg	SC	TIW	162			
		IFN-β-1a	Avonex™	30 µg	IM	TIW	82			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	194			
			Placebo				233			
Francis	2005	IFN-β-1a	Rebif™	22 ug	SC	TIW	186	≥ 20	CPE	Up to 48 months
		IFN-β-1a	Rebif™	44 ug	SC	TIW	182			
Freedman	2005	IFN-β-1a	Rebif™	22 µg	SC	QW	95	≥ 20	Not reported	Up to 24 months
		IFN-β-1a	Rebif™	44 µg	SC	QW	98			
Kappos	2005	IFN-β-1a	Avonex™	30 µg	IM	QW	400	≥ 20	CPE	Up to 48 months
		IFN-β-1a	Avonex™	60 µg	IM	QW	395			
Barbero	2006	IFN-β-1a	Avonex™	30 µg	IM	QW	65	≥ 20	Not reported	24 months
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	62			
Schwid	2007	IFN-β-1a	Rebif™	44 µg	SC	TIW	268	≥ 20	Not reported	Up to 16 months
		IFN-β-1a	Avonex™	30 µg	IM	QW	219			

Table 2.2: Characteristics of RCTs included in the meta-analysis. Abbreviations: IFN-β, interferon beta; µg, microgram; SC, subcutaneous; IM, intramuscular; NAbs, neutralising antibodies; TIW, 3 times a week; QW, once a week; EOD, every other day; CPE, Cytopathic effect assay; MxA, Myxovirus A protein assay.

Characteristics of the RCTs not included in the meta-analysis

Study	Year	Intervention	Formulation	Dosage	Route	Frequency	Patients	NAbs define	NAbs detection method	Follow up duration (months)
UBC	1996	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	91	≥ 20	CPE	60 months
			Placebo				87			
Rudick	1998	IFN-β-1a or 1b	Avonex™ or Betaseron™	30 or 250 µg	IM or SC	QW or EOD	23 103	≥ 20	CPE	Up to 60 months
		IFN-β-1a	Avonex™	30 µg	IM	QW	301			24 months
Polman	2003	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	360	≥ 20	CPE and MxA	36 months
			Placebo				358			
N American	2004	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	238	≥ 20	MxA	36 months
			Placebo				233			
Goodin	2012	IFN-β-1b	Betaseron/ Betaferon™	250 µg 500 ug	SC	EOD	861 836	≥ 20	MxA	Up to 42 months
Shahkarami	2013	IFN-β-1a	Avonex™	30 µg	IM	QW	42	≥ 20	CPE and Luciferase	24 months
			Cinnovex™				42			

Table 2.3: Characteristics of RCTs not included in the meta-analysis. Abbreviations: UBC, University of British Columbia; IFN-β, interferon beta; Cinnovex, is avonex biosimilar; µg, microgram; SC, subcutaneous; IM, intramuscular; NAbs, neutralising antibodies; TIW, 3 times a week; QW, once a week; EOD, every other day; CPE, Cytopathic effect assay; MxA, Myxovirus A protein assay.

Characteristics of non-randomised studies										
Study	**Year**	**Intervention**	**Formulation**	**Dosage**	**Route**	**Frequency**	**Patients**	**NAbs define**	**NAbs detection method**	**Follow up duration (months)**
Knobler	1993	IFN-β-1b	Betaseron/ Betaferon™	Various	SC	TIW	24	≥ 20	CPE	Up to 72 months
Ross	2000	IFN-β-1a	Avonex™	30 µg	IM	QW	140	Not reported	CPE	24 months
		IFN-β-1a	Rebif™	22 µg	SC	QW	143			
				22 µg	SC	TIW	160			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	311			
Bertolotto	2002	IFN-β-1a	Avonex™	30 µg	IM	QW	44	≥ 20	CPE	Up to 18 months
		IFN-β-1a	Rebif™	22 µg	SC	TIW	36			
				22 µg	IM	QW	16			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	29			
Scagnolaric	2002	IFN-β-1a	Rebif™ Avonex™	22 µg	SC	TIW	27	Not reported	CPE	12 months
				30 µg	IM	QW	32			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	32			
Bellomi	2003	IFN-β-1a	Rebif™	33 µg	SC	TIW	13	Not reported	CPE	Up to 72 months
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	TIW	29			
Frank	2004	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	30	≥ 20	MxA	Up to 26 months
Malucchi	2004	IFN-β-1a	Avonex™	30 µg	IM	QW	33	≥ 20	CPE	Up to 36 months
		IFN-β-1a	Rebif™	22 µg	SC	TIW	25			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	20			
Phillips	2004	IFN-β-1a	Avonex™	30 µg	IM	QW	153	≥ 20	CPE	Up to 24 months
Petkau	2004	IFN-β-1b	Betaseron/ Betaferon™	50 µg	SC	EOD	125	≥ 20	CPE and MxA	36 months
				250 µg			124			
Sorenson	2005	IFN-β-1a	Avonex™	30 µg	IM	QW	70	Not reported	CPE	Up to 78 months
		IFN-β-1a	Rebif™	22 or 44 µg	SC	TIW	170			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	TIW	191			
Gottesman	2006	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	20	≥ 20	MxA	6 months
Giovannoni	2007	IFN-β-1a	Rebif™	44 µg	SC	TIW	259	≥ 20	CPE	Up to 22 months

Durelli	2008	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	88	≥ 20	MxA	48 months
Gilli	2007	IFN-β-1a	Avonex™	30 µg	IM	QW	60	≥ 20	MxA	Not reported
		IFN-β-1a	Rebif™	22 µg	SC	TIW	95			
				44 µg			50			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	TIW	51			
Farrell	2008	IFN-β-1a	Avonex™	30 µg	IM	QW	119	≥ 20	Luciferase	36 months
		IFN-β-1a	Rebif™	22 or 44 µg	SC	TIW	149			
		IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	55			
Minager	2008	IFN-β-1a	Avonex™	30 µg	IM	QW	69	Not reported	Not reported	Up to 24 months
		IFN-β-1a	Rebif™	44 µg	SC	TIW	67			
Durelli	2009	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	145	≥ 20	MxA	48 months
				375 µg						
Pachner	2009	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	30	≥ 20	CPE and MxA	Not reported
Hartung	2011	IFN-β-1b	Betaseron/ Betaferon™	250 µg	SC	EOD	277	≥ 20	MxA	60 months
Hegan	2011	IFN-β-1a	Avonex™	Not reported	IM	Not reported	71	≥ 20	MxA	96 months
		IFN-β-1a	Rebif™	22 or 44 µg	SC		69			
		IFN-β-1b	Betaseron/ Betaferon™	Not reported			63			
Clinical trial NCT00367484	2011	IFN-β-1a	Rebif™ Clone 484-39	44 µg	SC	TIW	458	Not reported	Not reported	12 months

Table 2.4: Characteristics of non-randomised studies. Abbreviations: IFN-β, interferon beta; Various, 25, 125, 250, 500 µg; µg, microgram; SC, subcutaneous; IM, intramuscular; NAbs, neutralising antibodies; TIW, 3 times a week; QW, once a week; EOD, every other day; CPE, Cytopathic effect assay; MxA, Myxovirus A protein assay.

2.3.3 Evaluating risk of bias

The results of McHarm scale assessment for both RCTs and NRS were as follows: twenty- eight studies defined neutralising antibodies cut-off titres ≥ 20 NU/mL, all studies actively collected the neutralising antibodies data using a pre-defined lab diagnostic test, fourteen studies specified the baseline, timing and frequency of neutralising antibodies data, thirteen studies mentioned the Kawade method to report neutralising antibodies and fourteen studies clearly mentioned the protocol or methods for detection of neutralising antibodies (refer Table 2.5 and 2.6).

Evaluating risk of bias in RCTs						
Studies	**Elements adopted from McHarm Scale**	**Was NAb positivity pre-defined using standard or precise definitions?**	**Was the mode of NAb data collection specified as active?**	**Did the study specify the baseline, timing and frequency of NAb data collection?**	**Did the authors use standard scales or checklist in NAb data collection?**	**Is there a possibility of selective outcome reporting?**
1	UBC 1996	Yes	Yes	No baseline	Unclear	No
2	Rudick 1998	Yes	Yes	Yes	Yes	Yes
3	Li 2001	Yes	Yes	No baseline	Unclear	No
4	Clanet 2002	Yes	Yes	Yes	Unclear	No
5	Polman 2003	Yes	Yes	No baseline	Unclear	No
6	Sorenson 2003	No	Yes	No baseline	Unclear	Yes
7	N American 2004	Yes	Yes	No baseline	Unclear	No
8	Francis 2005	Yes	Yes	No baseline	Yes	Yes
9	Freedman 2005	Yes	Yes	No baseline	Unclear	No
10	Kappos 2005	Yes	Yes	Yes	Yes	Yes
11	Barbero 2006	Yes	Yes	Yes	Unclear	No
12	Schwid 2007	Yes	Yes	No baseline	Unclear	No
13	Goodin 2012	Yes	Yes	Yes	Unclear	No
14	Shahkarami 2013	Yes	Yes	Yes	Yes	No

Table 2.5: Risk of bias for RCTs using McHarm Scale. The purpose of McHarm scale is to evaluate the harms associated in the studies and it provides the quality assessment checklist to harms in studies included for analysis.

Evaluating risk of bias in NRS						
Studies	**Elements adopted from McHarm Scale**	**Was NAb positivity pre-defined using standard or precise definitions?**	**Was the mode of NAb data collection specified as active?**	**Did the study specify the baseline, timing and frequency of NAb data collection?**	**Did the authors use standard scales or checklist in NAb data collection?**	**Is there a possibility of selective outcome reporting?**
1	Knobler 1993	Yes	Yes	No	Unclear	No
2	Ross 2000	No	Yes	No frequency	Yes	Yes
3	Bertolotto 2002	Yes	Yes	Yes	Yes	Yes
4	Scagnolaric 2002	No	Yes	No baseline and timing	Yes	Yes
5	Bellomi 2003	No	Yes	No baseline and timing	Yes	Yes
6	Frank 2004	Yes	Yes	No baseline	Unclear	No
7	Maluccchi 2004	Yes	Yes	Yes	Yes	Yes
8	Phillips 2004	Yes	Yes	Yes	Yes	Unclear
9	Petkau 2004	Yes	Yes	No baseline	Unclear	No
10	Sorenson 2005	No	Yes	No baseline	Unclear	Yes
11	Gottesman 2006	Yes	Yes	Yes	Unclear	No
12	Giovannoni 2007	Yes	Yes	Yes	Unclear	No
13	Gilli 2007	Yes	Yes	Yes	Yes	Yes
14	Durelli 2008	Yes	Yes	No baseline	Unclear	No
15	Farrell 2008	Yes	Yes	No baseline and timing	Yes	Yes
16	Minager 2008	No	Yes	Yes	Unclear	No
17	Durelli 2009	Yes	Yes	No baseline	Unclear	Yes
18	Pachner 2009	Yes	Yes	Yes	Unclear	No
19	Hartung 2011	Yes	Yes	No baseline and timing	Yes	No
20	Hegan 2011	Yes	Yes	No baseline and timing	Unclear	Yes
21	Clinical trial NCT00367484 2011	No	Yes	No baseline and timing	Unclear	No

Table 2.6: Risk of bias for NRS using McHarm Scale. The purpose of McHarm scale is to evaluate the harms associated in the studies and it provides the quality assessment checklist to harms in studies included for analysis.

2.4 Randomised controlled trials comparing formation of neutralising antibodies against

2.4.1 IFN-β-1a Avonex™ and Rebif™

Two studies involving 834 participants published in 2003 and 2007 (Schwid & Panitch 2007; Sorensen et al. 2003) provided data for this comparison. Sorensen et al followed patients for up to 60 months, while Schwid et al followed patients for up to 24 months. The primary outcome of Sorensen et al was to follow the formation of neutralising antibody against Avonex™ and Rebif™ and Schwid et al was to compare the clinical efficacy of Avonex™ and Rebif™. In Sorenson's study, patients received Avonex™ 30 µg once a week administered IM and Rebif™ 22 µg either once or three times a week administered SC. The neutralising antibody was measured using the CPE (Cytopathic effect) assay using different assay sensitivities and was measured every 12 months. In Schwid's study, patients received Avonex™ 30 µg once a week administered IM and Rebif™ 44 µg either three times a week administered SC. Neutralising antibody formation was assessed every 6 months but no information was provided on the detection method. The neutralising antibody cut-off positive titre was defined at ≥ 20 neutralising units (NU)/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL). The frequency of neutralising antibodies was 22 out of 301 (7.30 %) for Avonex™ and 152 out of 533 (28.51 %) for Rebif™. The pooled estimate significantly favoured Avonex™ (fixed effect, OR 0.22, 95 % CI 0.13, 0.35) and the results showed little heterogeneity, I^2 = 0 % (refer Figure 2.3).

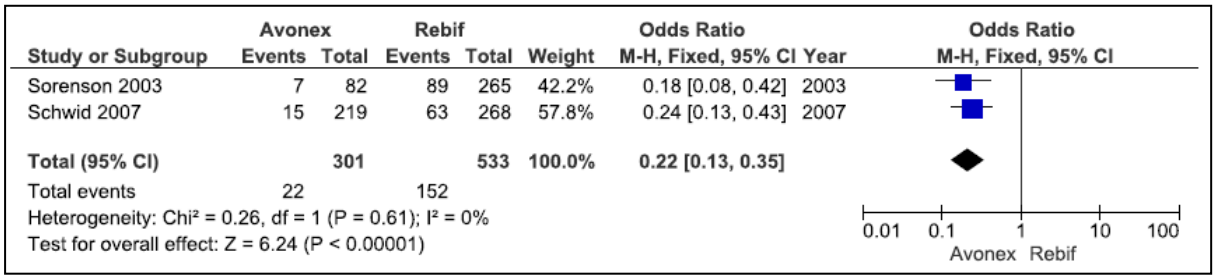


Figure 2.3: Forest plot. Individual and pooled OR for the outcome formation of neutralising antibodies in the studies considering two formulations of IFN-β Avonex™ and Rebif™.

2.4.2 IFN-β-1a Rebif™ and IFN-β-1b Betaseron/Betaferon™

Only one study involving 459 participants provided data for this comparison (Sorensen et al. 2003). Sorensen el al followed patients for up to 60 months and the primary outcome of the study was to follow the formation of neutralising antibodies against Rebif™ and Betaseron/Betaferon™. Patients received Rebif™ 22 µg either once or three times a week administered SC and Betaseron/Betaferon™ 250 µg every other day administered SC. Neutralising antibody was measured using the CPE assay using different assay sensitivities and was measured every 12 months. The frequency of neutralising antibodies in this study was 89 out of 265 (33.58 %) for Rebif™ and 88 out of 194 (45.36 %) for Betaseron/Betaferon™. The pooled estimate was significant favouring Rebif™ (fixed effect, OR 0.61, 95 % CI 0.42, 0.89).

2.4.3 IFN-β-1a Avonex™ and IFN-β-1b Betaseron/Betaferon™

Two studies involving 403 participants provided data for this comparison (Barbero et al. 2006; Sorensen et al. 2003). Sorensen et al followed patients for up to 60 months, while Barbero et al followed patients for 24 months. The primary outcome of Sorenson et al was the formation of neutralising antibody against Avonex™ and Betaseron/Betaferon™ and Barbero et al was the efficacy of Avonex™ and Betaseron/Betaferon™. In Sorenson's study, patients received Avonex™ 30 µg once a week administered IM and Betaseron/Betaferon™ 250 µg every other day administered SC. Neutralising antibody was measured using CPE assay using different assay sensitivities and was measured every 12 months. In Barbero's study, patients received Avonex™ 30 µg once a week administered IM and Betaseron/Betaferon™ 250 µg every other day administered SC. No information was provided on the neutralising antibody detection method but this was measured every 6 months. The neutralising antibody cut-off positive for titre was defined a ≥ 20 neutralising units (NU)/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL). The frequency of neutralising antibodies in these studies was 11 out of 147 (7.48 %) for Avonex™ and 110 out of 256 (42.96 %) for Betaseron/Betaferon™. The pooled estimate significantly favoured Avonex™ (fixed effect, OR 0.11, 95 % CI 0.06, 0.22) and the results showed little heterogeneity, $I^2 = 0$ % (refer Figure 2.4).

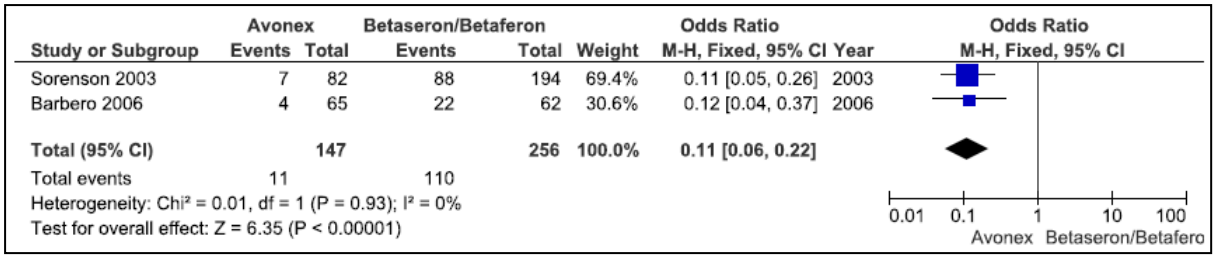


Figure 2.4: Forest plot. Individual and pooled OR for the outcome formation of neutralising antibodies in the studies considering two formulations of IFN-β Avonex™ and Betaseron/Betaferon™.

2.5 Subgroup meta-analysis

2.5.1 IFN-β-1a Avonex™ 30 µg and 60 µg

Two studies involving 1597 participants provided data for this comparison (Clanet et al. 2002; Kappos et al. 2005). Clanet et al followed patients for 36 months, while Kappos et al followed patients for up to 48 months. The primary outcome for Clanet et al was to compare the clinical efficacy of IFN-β-1a Avonex™ 30 µg IM and 60 µg IM decreasing disease progression and Kappos et al was to determine the incidence and significance of neutralising antibodies against IFN-β-1a Avonex™ 30 µg IM and 60 µg IM. Both studies used the CPE assay to detect neutralising antibody every 3 months (Kappos et al. 2005) and 6 months (Clanet et al. 2002) respectively. The neutralising antibody cut-off positive titre was defined at ≥ 20 neutralising units (NU)/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL).The frequency of neutralising antibodies in these studies was 16 out of 802 (1.99 %) for Avonex™ 30 µg IM and 42 out of 795 (5.49 %) for Avonex™ 60 µg IM. The pooled estimate was significant favouring Avonex™ 30 µg IM (fixed effect, OR 0.36, 95 % 0.20, 0.65) and the results showed little heterogeneity, $I^2 = 0$ % (refer Figure 2.5).

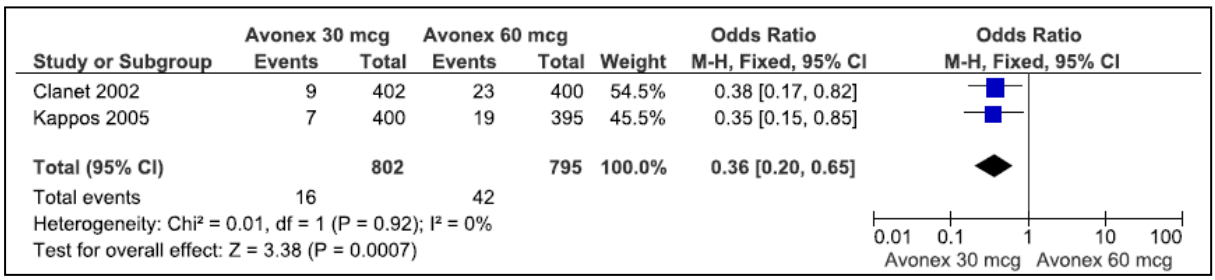


Figure 2.5: Forest plot. Individual and pooled OR for the outcome of neutralising antibodies in the studies considering Avonex™ 30 µg and 60 µg.

2.5.2 IFN-β-1a Rebif™ 22 µg and 44 µg

These three studies involving 1161 participants were published in 2001 and 2005 (Francis et al. 2005; Freedman et al. 2005; Li et al. 2001a). Li et al followed patients for 36 months; Francis et al followed patients for up to 48 months and Freedman et al followed patients for up to 24 months. The primary outcome of Li et al was to investigate the change in the MRI during IFN-β-1a Rebif™ treatment. The primary outcome of Francis et al was to examine the role of neutralising antibodies on the efficacy of IFN-β-1a Rebif™. The primary outcome of Freedman et al was to demonstrate the efficacy of once weekly IFN-β-1a Rebif™ on the treatment of relapses. Li et al and Francis et al used the CPE assay to detect neutralising antibodies. However, Freedman et al did not mention the method used for detection of neutralising antibodies. Freedman et al and Li et al measured the neutralising antibodies every 3 and 6 months respectively. Francis et al did not mention the timing of neutralising antibody measurements. The neutralising antibody cut off positive titre was defined at ≥ 20 neutralising units (NU)/mL. The frequency of neutralising antibodies in these studies was 128 out of 584 (21.01 %) for Rebif™ 22 µg SC and 97 out of 577 (16.81 %) for Rebif™ 44 µg SC. The pooled estimate favoured Rebif™ 44 µg SC although the difference did not reach statistical significance (random effect, OR 1.27, 95 % CI 0.75, 2.14), the results show heterogeneity, I^2 = 63 % (refer Figure 2.6).

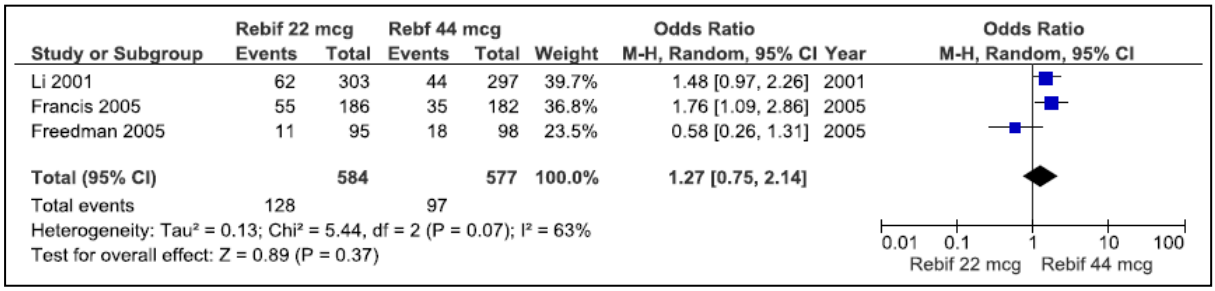


Figure 2.6: Forest plot. Individual and pooled OR for the outcome of neutralising antibodies in the studies considering Rebif™ 22 µg and 44 µg.

2.5.3 IFN-β-1a and IFN-β-1b

These two studies involved 586 participants provided data for this comparison (Barbero et al. 2006; Sorensen et al. 2003). Sorensen et al followed patients for up to 60 months, while Barbero et al followed patients for 24 months. The primary outcome of Sorenson et al was the formation of neutralising antibodies against IFN-β-1a and IFN-β-1b. The primary outcome of Barbero et al was the number of patients with at least one active MRI lesion using IFN-β-1a and IFN-β-1b. In Sorenson's study, patients received IFN-β-1a 30 µg once a week

administered IM or 22 µg once or three times a week SC and IFN-β-1b 250 µg every other day administered SC. The neutralising antibody was measured using CPE assay using different assay sensitivities every 12 months. In Barbero's study, patients received IFN-β-1a 30 µg once a week administered IM and IFN-β-1b 250 µg every other day administered SC. No information was provided on the neutralising antibody detection method but this was measured every 6 months. The neutralising antibody cut-off positive titre was defined at ≥ 20 neutralising units (NU)/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL). The frequency of neutralising antibodies in these studies reported was 100 out of 330 (30.30 %) for IFN-β-1a and 110 out of 256 (42.96 %) for IFN-β-1b. The pooled estimate favoured IFN-β-1a although the difference did not reach statistical significance (random effect, OR 0.31, 95 % CI 0.06, 1.72) and the results showed heterogeneity, I^2 = 88% (refer Figure 2.7).

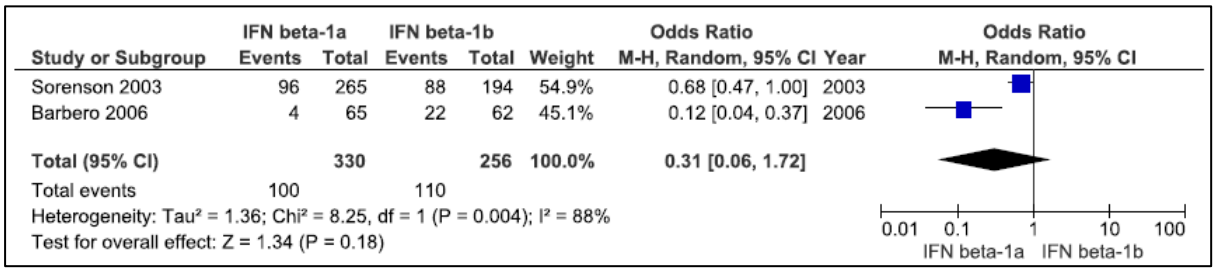


Figure 2.7: Forest plot. Individual and pooled OR for the outcome of neutralising antibodies in the studies considering IFN-β-1a and 1b.

2.6 Randomised controlled trails which were not included in meta-analysis

These six studies involved 3575 participants and were published between 1996 and 2013. ('North American Study Group' 2004; Goodin et al. 2012; Polman et al. 2003; Rudick et al. 1998; Shahkarami et al. 2013; UBC 1996) (UBC – University of British Columbia). The follow up of patients in all these studies varied from 24 months up to 60 months (refer Table 2.3).

The primary outcome of University of British Columbia (UBC) study was to validate the accuracy of neutralising antibodies against Betaseron/Betaferon™ during the trial and to discuss the clinical implications of neutralising antibodies. The primary outcome of Ruddick et al was to define the incidence and significance of neutralising antibodies against Avonex™ 30 µg QW IM and Betaseron/Betaferon™. The primary outcome of Polman et al was to explore the relationship between neutralising antibodies against Betaseron/Betaferon™ and clinical measures of the disease. The primary outcome of N American et al was to evaluate

the clinical efficacy and safety of Betaseron/Betaferon™. The primary outcome of Goodin et al was to assess neutralising antibodies against Betaseron/Betaferon™ 250 µg or 500 µg EOD SC in the trial. The primary outcome of Shahkarami et al was to evaluate the immunogenetic profile of Avonex™ 30 µg QW IM and Cinnovex™ (biosimilar) 30 µg QW IM. University of British Columbia study and Ruddick et al used the CPE assay to detect neutralising antibodies. Polman et al used both CPE and Myxovirus A protein (MxA) assay, N American et al used the MxA assay, Goodin et al used the MxA assay and Shahkarami et al used both CPE and the Luciferase assay to detect neutralising antibodies. The neutralising antibody cut-off positive titre was defined at ≥ 20 neutralising units (NU)/mL and was measured every 3 months (Goodin et al. 2012; Rudick et al. 1998; Shahkarami et al. 2013) or 6 months ('North American Study Group' 2004; Polman et al. 2003; Rudick et al. 1998; UBC 1996) (UBC – University of British Columbia) using Kawade titration method (TRU/ mL = ten times reduction units per mL).. The frequency of neutralising antibodies in Rudick's study was 57 out of 301 (18.93 %) for Avonex™ 30 µg QW IM. The frequency of neutralising antibodies in Goodin's study was 319 out of 861 (37.04 %) for Betaseron/Betaferon™ 250 µg and 340 out of 836 (40.66 %) for Betaseron/Betaferon™ 500 µg and for Shahkarami's study was 2 out of 42 (4.76 %) and 1 out of 42 (2.38 %) for Avonex™ and Cinnovex™ respectively. In all other studies where patients received Betaseron/Betaferon™ 250 µg EOD ('North American Study Group' 2004; Polman et al. 2003; Rudick et al. 1998; UBC 1996) (UBC – University of British Columbia), the frequency of neutralising antibodies was 195 out of 712 (27.38 %) for Betaseron/Betaferon™ and 5 out of 781 (0.64 %) for placebo.

2.7 Non-randomised controlled trials comparing formation of neutralising antibodies against:

2.7.1 IFN-β-1a Avonex™ and Rebif™

These five studies involved 763 participants and were published between 2002 and 2008 (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004; Minagar, Murray & Investigators 2008). They comprised a follow up study (Bertolotto et al. 2002), open label study (Malucchi et al. 2004), assess the frequency of neutralising antibodies using Luciferase assay (Farrell et al. 2008), correlation study (Gilli et al. 2007) and prospective and retrospective observational study (Minagar, Murray & Investigators 2008). The period of follow up in these studies ranged between 18 – 36 months and two studies reported that the CPE assay was used to detect neutralising antibodies (Bertolotto et al. 2002; Malucchi et al.

2004). However, Gilli et al did not report the follow up duration but used the MxA assay to detect neutralising antibodies. Farrell et al used the luciferase assay to detect neutralising antibodies (Farrell et al. 2008). Minager et al did not report the method used for detection of neutralising antibodies. The primary outcome of Bertolotto et al was to determine the incidence and prevalence of neutralising antibodies against three IFN-β formulations. The primary outcome of Malucchi et al was to investigate the impact of neutralising antibodies on clinical efficacy. The primary outcome of Farrell et al was the incidence of neutralising antibodies using the Luciferase assay. The primary outcome of Gilli et al was to correlate neutralising antibodies in both in vitro and in vivo bioactivity. The primary outcome of Minager et al was to compare the efficacy and tolerability of IFN-β-1a formulations. Four studies reported the neutralising antibody cut-off titre which was defined at ≥20 NU/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL) (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004). The frequency of neutralising antibodies in all of these studies was 42 out of 325 (12.92 %) for Avonex™ and 144 out of 438 (32.87 %) for Rebif™. The pooled estimate favoured Avonex™ although the difference did not quite reach statistical significance (random effect, OR 0.22, 95 % CI 0.05, 1.05) and the results showed heterogeneity, I^2 = 88 % (refer Figure 2.8).

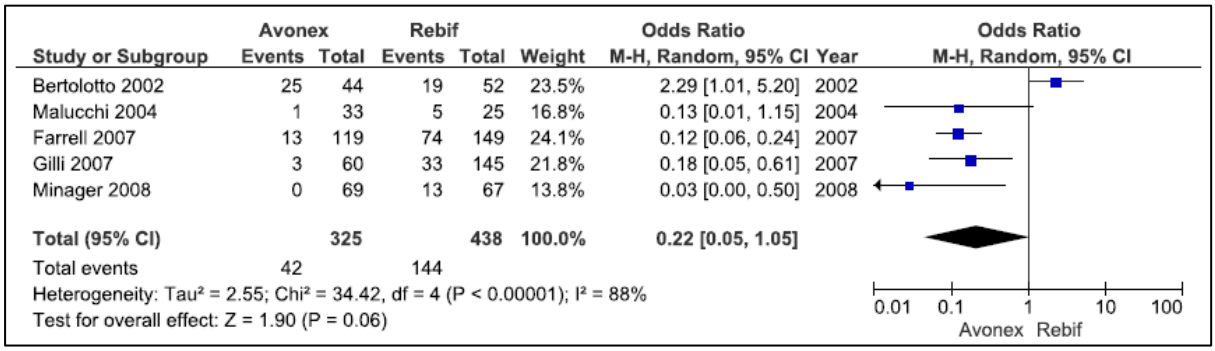


Figure 2.8: Forest plot. Individual and pooled OR for the outcome of formation of neutralising antibodies in the studies considering two formulations of IFN-β Avonex™ and Rebif™.

2.7.2 IFN-β-1a Rebif™ and IFN-β-1b Betaseron/Betaferon™

These five studies involved 617 participants and were published between 2002 and 2007 (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004; Scagnolari et al. 2002a). The details of four studies are mentioned in Section 2.3.2.9 (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004). The period of follow up

ranged between 12 – 36 months. The primary outcome of Scagnolaric et al was to quantitate the relative immunogenicity profile of various IFN-β formulations (Scagnolari et al. 2002a). The CPE assay was used to detect neutralising antibodies. However, no information on the cut-off used for positive titre was reported. The frequency of neutralising antibodies in all of these studies reported 143 out of 430 (33.25 %) for Rebif™ and 58 out of 187 (31.01 %) for Betaseron/Betaferon™. The pooled estimate was non-significant (random effect, OR 0.97, 95 % CI 0.41, 2.30) and the results showed heterogeneity, I^2 = 75 % (refer Figure 2.9).

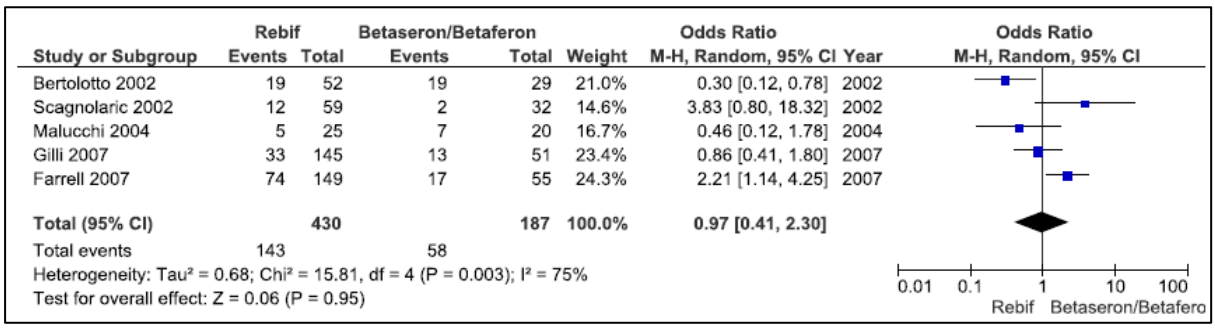


Figure 2.9: Forest plot. Individual and pooled OR for the outcome of formation of neutralising antibodies in the studies considering two formulations of IFN-β Rebif™ and Betaseron/Betaferon™.

2.7.3 IFN-β-1a Avonex™ and IFN-β-1b Betaseron/Betaferon™

These five studies involved 862 participants and were published between 2000 and 2007 (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004; Ross et al. 2000b). The details of these four studies are mentioned in Section 2.3.2.9. The period of follow up ranged between 18 – 36 months (Bertolotto et al. 2002; Farrell et al. 2008; Gilli et al. 2007; Malucchi et al. 2004). The primary outcome of Ross et al was to measure anti-drug antibodies and to assess the influence of IFN-β preparations, dosage, frequency and route of administration (Ross et al. 2000b). The CPE assay was used to detect neutralising antibodies. However, no information on cut off positive titres was reported. The frequency of neutralising antibodies in all of these studies was 77 out of 396 (19.44 %) for Avonex™ and 274 out of 466 (58.79 %) for Betaseron/Betaferon™. The pooled estimate was significant favouring Avonex™ (random effect, OR 0.22, 95 % CI 0.11, 0.44) but the results showed heterogeneity I^2 = 63 % (refer Figure 2.10).

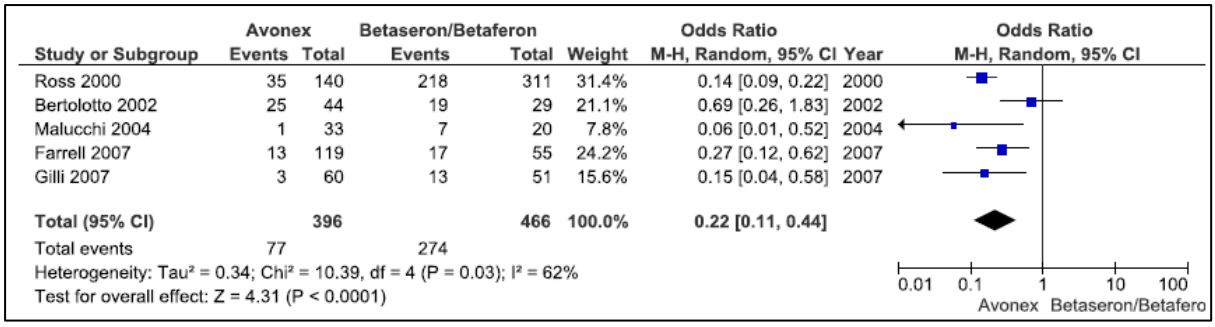


Figure 2.10: Forest plot. Individual and pooled OR for the outcome of formation of neutralising antibodies in the studies considering two formulations of IFN-β Avonex™ and Betaseron/Betaferon™.

2.8 Non-randomised controlled trails which were not included in the analysis

These 14 studies included 1951 participants and were published between 1993 and 2011 (Bellomi et al. 2003; Durelli et al. 2008; Durelli et al. 2009; Frank et al. 2004; Giovannoni et al. 2007; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Hegen et al. 2012a; Knobler et al. 1993; Pachner et al. 2009a; Petkau et al. 2004; Phillips et al. 2004; Sorensen et al. 2005d) and Clinical trial NCT00367484 2011. They comprised seven open label studies (Durelli et al. 2009; Frank et al. 2004; Giovannoni et al. 2007; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Phillips et al. 2004) and Clinical trial NCT00367484 2011, three longitudinal studies (Bellomi et al. 2003; Hegen et al. 2012a; Petkau et al. 2004), two observational studies (Durelli et al. 2009; Pachner et al. 2009a), one pilot study (Knobler et al. 1993) and one population based study (Sorensen et al. 2005d). The period of follow up in these studies ranged from 6 – 96 months, five used the CPE assay (Bellomi et al. 2003; Giovannoni et al. 2007; Knobler et al. 1993; Phillips et al. 2004; Sorensen et al. 2005d), six used the MxA assay (Durelli et al. 2008; Durelli et al. 2009; Frank et al. 2004; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Hegen et al. 2012a) and two used both the CPE and MxA assays (Pachner et al. 2009a; Petkau et al. 2004) to detect neutralising antibodies. Pachner et al did not report the follow up period (Pachner et al. 2009a) while the Clinical trial NCT00367484 2011 did not report the method used for detection of neutralising antibodies. Of 14 studies, 12 defined neutralising antibody cut-off titre as ≥20 NU/mL and was measured using Kawade titration method (TRU/ mL = ten times reduction units per mL) (Durelli et al. 2008; Durelli et al. 2009; Frank et al. 2004; Giovannoni et al. 2007; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Hegen et al. 2012a; Knobler et al. 1993; Pachner et al. 2009a; Petkau et al. 2004; Phillips et al. 2004) and Clinical trial NCT00367484 2011.

Bellomi et al and Sorenson et al have not reported the cut off neutralising antibody titres (Bellomi et al. 2003; Sorensen et al. 2005d). The primary outcomes of these studies are shown in the Table 2.7.

Primary outcomes of NRS not included in the analysis		
Studies		**Primary outcomes**
Knobler	1993	Evaluation of safety and side effect of IFN-β-1b
Bellomi	2003	Evaluate neutralising antibodies in long term IFN-β treatment
Frank	2004	Determine the effect of IFN-β-1b on clinical measures
Phillips	2004	Determine the safety and immunogenicity of IFN-β-1a Avonex™
Petkau	2004	Impact of neutralising antibodies on efficacy of IFN-β-1b
Sorenson	2005	Investigate the appearance and persistence of neutralising antibodies to various IFN-β preparations
Gottesman	2006	Dose escalation of IFN-β-1b Betaseron/Betaferon™ and adverse effects management
Giovannoni	2007	Immunogenicity and tolerability analyses of IFN-β-1a
Durelli	2008	Investigate magnetic resonance imaging (MRI) and neutralising antibodies occurrence to IFN-β-1b
Durelli	2009	Optimise IFN-β-1b dosage 250 µg and 375 µg
Pachner	2009	Assess neutralising antibodies on IFN-β-1b therapeutic efficacy
Hartung	2011	Evaluate frequency and implications of neutralising antibodies against IFN-β-1b
Hegan	2011	Investigate the significance of various IFN-β formulations specific neutralising antibodies cut-off titres
Clinical trial NCT00367484	2011	Evaluate the safety and immunogenicity of IFN-β-1a Rebif clone 484-39

Table 2.7: Primary outcomes of NRS not included in the analysis.

The Figure 2.11 represents the percentage of frequency of neutralising antibodies development against different IFN-β formulations in eleven NRS (Bellomi et al. 2003; Durelli et al. 2008; Durelli et al. 2009; Frank et al. 2004; Giovannoni et al. 2007; Gottesman & Friedman-Urevich 2006; Hartung et al. 2011; Hegen et al. 2012a; Knobler et al. 1993; Pachner et al. 2009a; Petkau et al. 2004; Phillips et al. 2004; Sorensen et al. 2005d) and Clinical trial NCT00367484 2011. The mean with 95 % CI was calculated. The remaining three studies (Bellomi et al. 2003; Hegen et al. 2012; Sorensen et al. 2005) were not included because of inconsistency in reporting the frequency of neutralising antibodies to each IFN-β formulations.

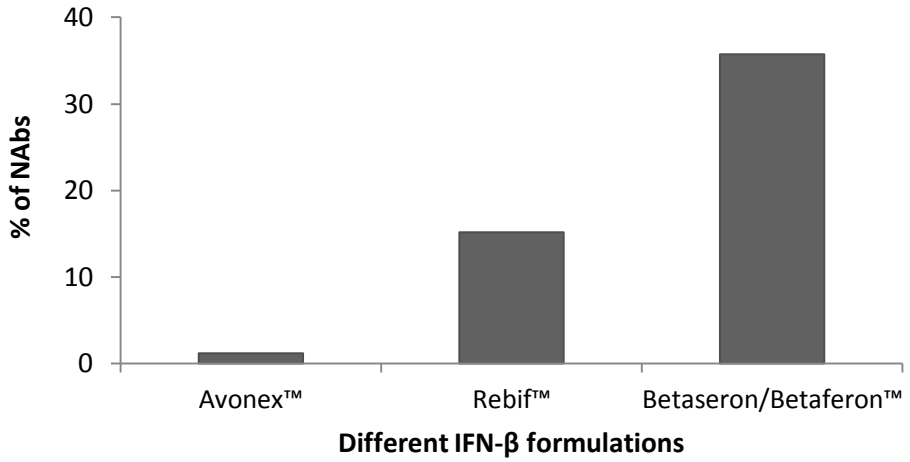


Figure 2.11: The frequency of neutralising antibodies of 11 NRS. The figure represents the % of frequency of development of neutralising antibodies in three different IFN-β formulations in eleven NRS.

2.9 Discussion

In this systematic review the development of neutralising antibodies against three different formulations of IFN-β Avonex™, Rebif™ and Betaseron/Betaferon™ were compared to each other in the treatment of relapsing forms of multiple sclerosis for the first time using meta-analysis. The results from the meta-analysis of RCTs demonstrated Avonex™ has 78 % less risk against Rebif™ (refer Figure 2.2), Rebif™ has 39 % less risk against Betaseron/Betaferon™ and Avonex™ has 89 % less risk against Betaseron/Betaferon™ (refer Figure 2.3) in the development of neutralising antibodies. The subgroup meta-analysis of Avonex™ 30 µg and 60 µg revealed 30 µg has 64 % less risk against 60 µg (refer Figure 2.4), meta-analysis of Rebif 22 µg and 44 µg revealed 22 µg has 27 % high risk against 44 µg (refer Figure 2.5) and meta-analysis of IFN-β-1a and 1b revealed IFN-β-1a has 69 % less risk against IFN-β-1a (refer Figure 2.6) in the development of neutralising antibodies. The comparison was also established in NRS and results demonstrate Avonex™ has 79 % less risk against Rebif™ (refer Figure 2.7), the comparison of Rebif™ against Betaseron/Betaferon™ was non-significant (refer Figure 2.8) and Avonex™ has 78 % less risk against Betaseron/Betaferon™ (refer Figure 2.9). Overall our results from all the studies included in this review suggest 1.22 % to 19.44 % of patients developed NAbs to Avonex™, 15.20 % to 33.25 % of patients developed NAbs to Rebif™ and 27.38 % to 58.79 % of patients developed NAbs to Betaseron/Betaferon™.

The results presented in this review summarises the incidence of NAbs against different IFN-β formulations. Several problems exist in comparison of relative immunogenicity among different IFN-β formulations and this can be due to differences in the studies, NAbs detection methods, laboratories, the duration of treatment and criteria for definition of NAbs positive titres. The differences between natural IFN-β and IFN-β-1b contribute for greater development of neutralising antibodies to Betaseron/Betaferon™ compared to Rebif™ and Avonex™. Betaseron/Betaferon™ is produced in *E.coli* and is not glycosylated. Furthermore, Betaseron/Betaferon™ has difference in the amino acid sequence compared to natural IFN-β, it consists of 165 amino acids due to lack of N terminal methionine and cysteine is substituted with serine at position 17. However, Rebif™ and Avonex™ are produced in CHO cells and have identical amino acid sequence to that of natural IFN-β and are glycosylated similar to natural IFN-β. The recent studies on recombinant therapeutic proteins which are produced in non-human mammalian cells can synthesise non-human sialic acid (Neu5Gc). The biologic cetuximab contains Neu5Gc, and forms immune complexes with anti-Neu5Gc antibodies that pre-exist in human plasma (Ghaderi et al. 2010). The studies also shown the galactose-alpha-1, 3-galactose (alpha-Gal) epitope on cetuzimab induce anaphylaxis in patients with pre-existing anti-Gal autoantibodies. The characteristics of different IFN-β formulations are provided in Table 2.1. The protein without carbohydrate (as in case with Betaseron/Betaferon™) has a tendency to form aggregates. The formulations for example large protein excipients like human serum albumin used in the IFN-β formulations can give rise to aggregates. The studies also suggest Avonex™ form less aggregates when compared to Rebif™ and Betaseron/Betaferon™ (Barnard, Babcock & Carpenter 2013). The posttranslational modifications, manufacturing, purification and storage also contribute for immunogenicity. The development of neutralising antibodies is also dependent on dosing regimen. Our meta-analysis data on comparison of Avonex™ 30 µg versus 60 µg and Rebif™ 22 µg versus 44 µg revealed Avonex 30 µg and Rebif 44 µg developed less neutralising antibodies compared to Avonex 60 µg and Rebif 22 µg. The paradox still exists on low dose and high dose administration of therapeutic proteins. It has also been shown higher doses of remicade in treatment of rheumatoid arthritis might be less immunogenic when given in higher doses possibly because of high zone tolerance. Therefore, optimising dosage can be an important factor to consider in terms of immunogenicity. The IV route of administration was found less immunogenic than SC and IM because route of administration can influence the risk of sensitisation because immune-competent cells are mainly found in areas such as skin and mucosa. In addition, the frequency of administration also contributes

for immunogenicity. The IM IFN-β-1a Avonex™ 30 µg is given once a week basis. However, SC IFN-β-1a Rebif™ and SC IFN-β-1b Betaseron/Betaferon™ is given three times a week and every other day basis. In this review, because of limited number of studies and variability in reporting we could not find a pattern.

The mechanism for development of anti-drug antibodies can be T-cell dependent and T-cell independent. In T-cell dependent the T-cells recognise the immunogenic peptides and are presented to antigen presenting cells via MHC class II molecules. However, in T-cell independent mechanism the antigen binds to BcR and cause B cell activation without help of T-cells. The generation of anti-drug antibodies will be of IgM isotype converting to IgG isotype. In the following experimental chapters we have characterised anti-IFN-β antibodies in a cohort of IFN-β treated patients and have explored the neutralising capabilities of anti-IFN-β subclass specific anti-drug antibodies and we also addressed the role of T helper cells in anti-drug antibodies to IFN-β (refer chapters 4 and 5).

Our systematic review has number of limitations. First of all, we included only English language studies introducing potential for language bias and limited our search to studies identified through MEDLINE on Ovid, EMBASE on Ovid, CENTRAL on The Cochrane Library, Clinicaltrials.gov and bibliographic searches. Second, some relevant trials were excluded as we did not include unpublished articles, thesis or conference proceedings. Third, the variations on the quality of studies such as inconsistent sample size, disproportionate group size across different treatments and high drop-out rates after randomisation. Clear heterogeneity was observed in the NRS included for analysis. Possibly including all different languages articles limits the potential for language bias. Searching all databases, tertiary sources and restricted interface search engines for examples 1. Agency for Health and Research Quality http://www.ahrq.gov/ 2. BioSciences Information Service of Biological Abstracts Citation Index via webofknowledge.com 3. British Library Direct http://www.bldss.bl.uk/BLDSS/ 4. Current Controlled trials http://www.controlled-trials.com/ 4. Database of Abstracts of Reviews of Effectiveness http://www.crd.york.ac.uk/CRDWeb/ 5. Iowa Drug Information Service http://www.uiowa.edu/idis/ 6. Medscape http://www.medscape.com/ 7. Science Citation Index via http://www.ebscohost.com/ 8. Scirus http://www.scirus.com/ 9. Toxicology Information Online – US National Library of Medicine via http://www.proquest.com 10. TRIP database http://www.tripdatabase.com/ 11. UK Clinical Research Network Portfolio Database http://public.ukcrn.org.uk/search/ 12. US Food and Drug Administration http://www.fda.gov/ can reveal all the possible relevant

studies. The pooling of the data systematicaly from all the identified studies and making contacts with the authors can avoid missing data.

This systematic review shows the relative differences in immunogenicity of different IFN-β formulations Avonex™, Rebif ™ and Betaseron/Betaferon™. In conclusion Avonex™ has less risk, Rebif™ has moderate risk and Betaseron/Betaferon™ has high risk for development of neutralising antibodies. The observed differences in development of neutralising antibodies among the three IFN-β formulations are likely due to differences in the molecular structure or post translational modifications; but the dosing regimen may also contribute.

2.10 Conclusions

- The risk of development of neutralising antibodies was less in IFN-β-1a Avonex™, moderate in IFN-β-1a Rebif™ and high in IFN-β-1b Betaseron/Betaferon™.
- Based on our findings IFN-β-1a Avonex™ 30 µg IM should be the drug of choice in patients with relapsing forms of multiple sclerosis.
- Clinical testing for anti-IFN-β antibodies both binding and neutralising antibodies have to be incorporated in the clinical practice and nationwide procedures for detection of anti-IFN-β antibodies have to be implemented.
- Treatment with IFN-β should be discontinued with high titres of neutralising antibodies and therapeutic options should be re-evaluated. The sequential step wise treatment strategies (the present drug of choice is glatiramer acetate) have to be followed in case of treatment failures.

Inconsistencies in methodology and in reporting data in the studies included in this review has limited the extent of our analyses. Longitudinal studies can help achieve time to first event analysis and can also characterise the kinetics of development of neutralising antibodies. Future clinical trials that include harmonised methodology and incorporate baseline testing for anti-IFN-β antibodies are required for a more robust meta-analysis to be conducted.

Chapter 3: Characterisation of anti-drug antibodies against therapeutic Interferon beta in multiple sclerosis patients

3.1 Introduction

The therapeutic protein interferon beta (IFN-β), available under the trade names Avonex™, Rebif™, Betaferon/Betaseron™ and Extavia™, is used for the treatment of multiple sclerosis. IFN-β exhibits anti-viral properties and numerous immunomodulatory functions. In terms of disease progression, treatment with IFN-β has been shown to dampen plaque formation, decrease relapses and eventually improve disease prognosis (Killestein & Polman 2011).

IFN-β is well-tolerated in the treatment of multiple sclerosis. However, a relatively common adverse effect of protein therapeutics is the development of anti-drug antibodies (De Groot & Scott 2007; Porter 2001; Strayer & Carter 2012). Development of anti-drug antibodies can be non-neutralising (N-NAb) or neutralising antibodies (NAb) depending on the site at which they bind. Nearly 50-80 % of patients develop N-NAb to different formulations of IFN-β. The presence of N-NAb in the absence of NAb has no significant clinical problem in terms of clinical efficacy (Francis et al. 2005; Pachner et al. 2009a; Pachner et al. 2009b; Rudick et al. 1998; Zang et al. 2000). Formation of NAb blocks the bioactivity of IFN-β by binding to IFNAR receptor which is crucial for drug-target interactions (Giovannoni & Goodman 2005). Up to 45 % (mean approx. 25 %) of patients develop NAb to different formulations of IFN-β. The presence of anti-IFN-β antibodies has been associated with total loss or reduced efficacy of the drug, altered pharmacokinetics, infusion reactions and cross reactions with endogenous protein counterparts (Bendtzen 2010). Immunogenicity is the major complication associated with protein therapeutics and has become the key focus from drug development to post-marketing studies. Detection and quantification of anti-drug antibodies has been implemented into routine clinical practice (Bertolotto 2009; van der Voort et al. 2009). The European National Neurological Societies (EFNS) task force recommends measurements of N-NAb before performing a NAb assay, with N-NAb positive samples further analysed for NAb. To date, there is no recommended assay available for the detection of N-NAb. However, the task force insists different N-NAb assays should be considered, evaluated and compared using large sample sets to identify the best sensitivity and specificity for further NAb detection (Sorensen et al. 2005a). The task force also recommends the use of a human lung adenocarcinoma epithelial cell line stimulated with a fixed amount of the same formulation of IFN-β used in the patients (Sorensen et al. 2005a) for the detection of NAb.

The anti-IFN-β antibodies are reported to be predominantly of the immunoglobulin G (IgG) class. Studies on distribution of IFN-β specific NAb IgG subclasses (IgG1-4) in multiple sclerosis patients following IFN-β treatment are limited (Deisenhammer, Reindl & Berger 2001; Gibbs & Oger 2007). Type I interferons are crucial in the regulation of host immune responses and play an important role in first line of defence against viral infections. However, the potential for neutralisation of endogenous IFN-β by anti-IFN-β antibodies is unclear. The interaction of anti-drug antibodies with the therapeutic can result in formation of immune complex. Immune complexes can activate the complement system, which comprises 30 soluble proteins circulating in the blood in inactive forms and plays an important role in innate and adaptive immunity (Carroll 2004). The evidence also suggests a role for activated complement in antigen processing and presentation (Strainic et al. 2008; Weaver et al. 2010). Hence, increased complement activation can enhance antigen processing and presentation which may contribute to progression of immunogenic responses against biologics. The identification of the relevant epitopes for anti-IFN-β antibodies is crucial for understanding drug-target interactions (Gneiss et al. 2004). Hence, an attempt has also been made to identify specific binding patterns of anti-IFN-β antibodies on the IFN-β molecule using continuous B-cell epitope mapping using multipin peptide technology and *in vitro* competitive binding assay approach.

The aim of this study was to characterise the anti-IFN-β antibody response in a cohort of IFN-β (Rebif™; Merck-Serono) treated patients and to determine the relative contribution of anti-IFN-β antibodies in neutralising IFN-β bioactivity. Furthermore, the study sought to establish whether NAb cross react with Type I interferons, to investigate the ability of immune complexes to activate the complement cascade and to identify the antibody binding regions on IFN-β-1a Rebif™ molecule.

3.2 Methods

The flow diagram of the methods employed in this chapter is shown in Figures 3.1 and 3.2.

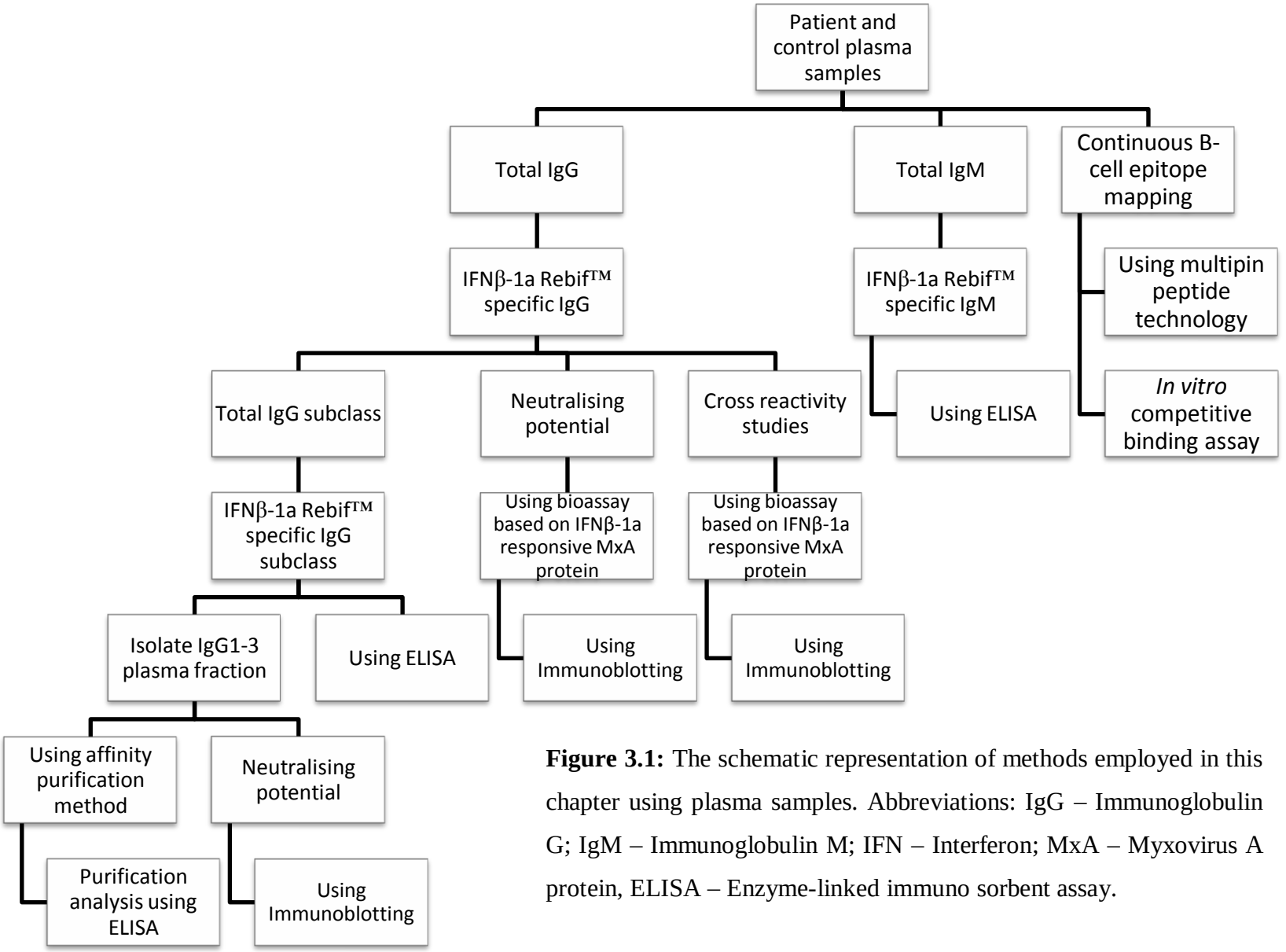


Figure 3.1: The schematic representation of methods employed in this chapter using plasma samples. Abbreviations: IgG – Immunoglobulin G; IgM – Immunoglobulin M; IFN – Interferon; MxA – Myxovirus A protein, ELISA – Enzyme-linked immuno sorbent assay.

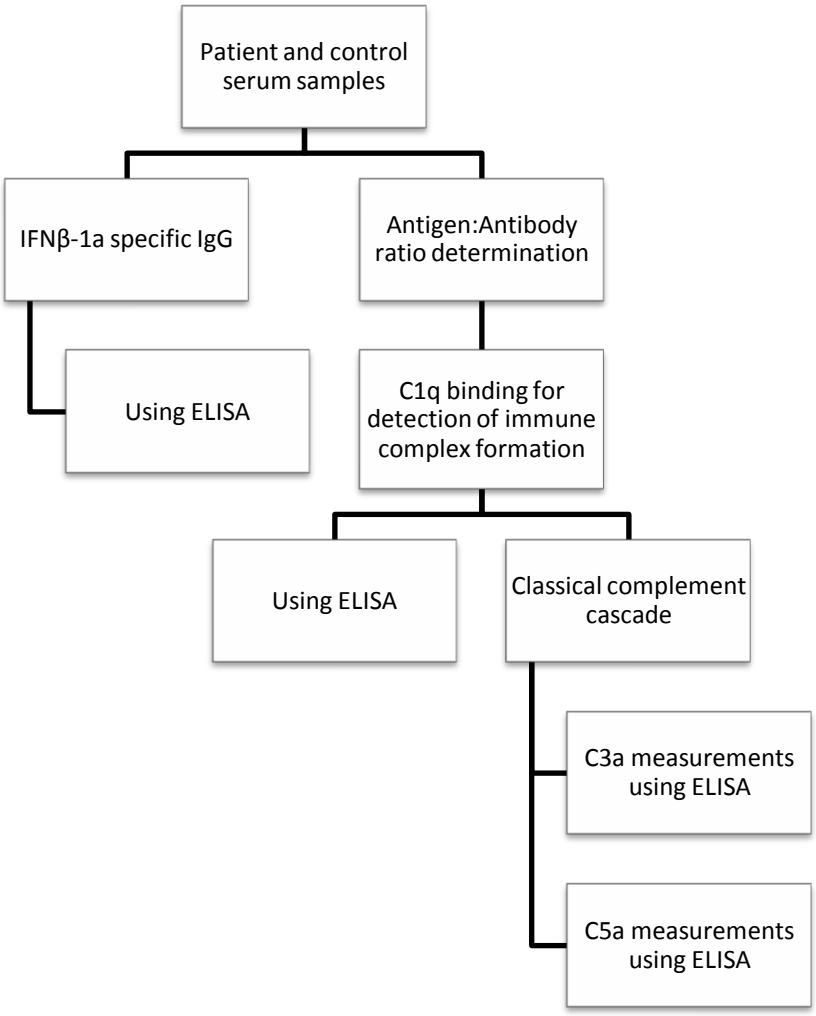


Figure 3.2: The schematic representation of methods employed in this chapter using serum samples. Abbreviations: IFN – Interferon; C1q – Complement 1, Q Subcomponent; ELISA – Enzyme-linked immuno sorbent assay, C3a – Complement 3a, C5a – Complement 5a.

3.2.1 Chemicals and reagents

Nunclon cell culture flasks, dishes and multi-well plates were obtained from Nalgene-Nunc International (VWR International, Lutterworth, UK). Protein assay dye reagents, Precision Plus protein Kaleidoscope standards, non-fat dry milk and the GS-710 calibrated imaging densitometer were from Bio-Rad (Hemel Hemstead, UK). NuPAGE Novox 4-12 % Bis Trisgels, NuPAGE lithium dodecyl sulfate (LDS) sample buffer, sample reducing agent and antioxidant, the XCell Surelock mini-cell were from Invitrogen (Paisley, UK). The THP-1 cell line was a kind gift from Dr Dominic Williams, Department of Molecular and Clinical Pharmacology, University of Liverpool, UK. Western Lightening chemiluminescence reagents were from PerkinElmer, Beaconfield, UK. Hyperfilm enhanced chemiluminescence (ECL) was from Amersham (Little Chalfont, UK). Tetanus toxoid was purchased from

Staten's Serum Institute, Denmark. IFN-β-1a Rebif™ was from Merck Serono; London, UK. Fetal bovine serum (FBS) was from Gibco, UK. Gammacell 1000 containing Caesium-137 (^{137}Cs) emitting gamma radiation was from Field Emission; Berkshire, UK. Pooled Human AB Serum was from Innovative research Inc; MI, USA. Roswell Park Memorial Institute medium-1640 (RPMI-1640), hydroxyethyl piperazineethanesulfonic acid (HEPES), 2-Mercaptoethanol (2-ME), Lipopolysaccharide (LPS), Polyinosinic:polycytidylic acid (Poly I:C), phytohaemagglutinin (PHA), Cyclosporin-A (CSA), Penicillin-streptomycin solution, Trypan blue solution, Dimethyl sulfoxide (DMSO), Hank's Balanced Salt Solution (HBSS), (Bovine serum albumin) BSA, 3-(N-morpholino)propanesulfonic acid (MOPS), Radio-Immunoprecipitation assay (RIPA) buffer, 3,3',5,5'-Tetramethylbenzidine (TMB), Kodak BioMax MS intensifying screen, Kodak developer and fixer solutions, Ponceau S solution, Tween 20, Phosphate Buffered Saline (PBS) tablets, paraformaldehyde were from Sigma-Aldrich (Poole, UK). All other reagents unless otherwise mentioned were of analytical or molecular grade, and were from Sigma-Aldrich.

3.2.2 Whole blood samples

Whole venous blood samples to isolate plasma, serum and peripheral blood mononuclear cells (PBMCs) for the study were obtained from The Walton Neurological Centre, NHS Foundation Trust, Liverpool, UK. A total of 20 multiple sclerosis patients and 10 healthy volunteers were recruited (refer Table 3.1). Ten patients were on IFN-β-1a Rebif™ therapy previously. They had been withdrawn from IFN-β-1a Rebif™ therapy due to development of neutralising antibodies (NAb) and are on alternative therapy. The determination of NAb status was established at Walton Neurological Centre as part of periodic or routine screening. The other 10 patients are still on FN-β-1a Rebif™ therapy and have not been reported to have developed NAb at the time of recruitment. Interferon beta therapy involves IFN-β-1a Rebif™ 22 or 44 µg subcutaneous (SC) injections three times per week. The blood samples were collected on 3 different occasions. The total volume of 10 mL, 100 mL and 100 mL were obtained in the 3 different visits respectively. The first 10 mL blood sample was taken to confirm the IFN-β-1a Rebif™ antibody status. The next 2 blood samples were taken 3 weeks apart and not more than 3 months apart. In addition to patients, 10 healthy donors were also recruited from the population based within the staff and student body of Department of Molecular and Clinical Pharmacology, University of Liverpool, UK. The total volume of 100 mL of blood was collected in a single visit for healthy donors. The study was approved by Liverpool local research ethics committee (reference No. 09 – H1005 – 84).

Donor Identity	Age	Sex	Group	IFN-β-1a therapy status	Rebif (s.c) Dose (μg)	Duration (months**)	
						Therapy	Since WD
P1	57	F	RRMS	WD	22	46	72
N2	55	F	RRMS	WD	22	43	5
P3	46	M	RRMS	WD	22	24	57
N4	47	M	RRMS	ON	22	48	N.A
N5	57	M	RRMS	ON	44	32	N.A
N6	30	F	RRMS	ON	44	>120	N.A
P7	54	M	RRMS	WD	22	36	44
N8	43	M	RRMS	ON	22	>120	N.A
H9	24	F	Healthy	N.A	N.A	N.A	N.A
P10	30	M	RRMS	WD	22	84	48
H11	42	M	Healthy	N.A	N.A	N.A	N.A
N12	32	F	RRMS	ON	44	141	N.A
P13	49	M	RRMS	WD	22	15	34
P14	43	F	RRMS	WD	22	12	13
P15	43	M	RRMS	WD	22	72	53
N16	56	F	RRMS	ON	22	>120	N.A
N17	37	F	RRMS	ON	44	96	N.A
H18	47	F	Healthy	N.A	N.A	N.A	N.A
H19	30	F	Healthy	N.A	N.A	N.A	N.A
H20	43	F	Healthy	N.A	N.A	N.A	N.A
H21	33	F	Healthy	N.A	N.A	N.A	N.A
H22	43	M	Healthy	N.A	N.A	N.A	N.A
H23	30	F	Healthy	N.A	N.A	N.A	N.A
H24	27	M	Healthy	N.A	N.A	N.A	N.A
H25	32	F	Healthy	N.A	N.A	N.A	N.A
N26	50	F	RRMS	ON	22	60	N.A
N27	45	F	RRMS	ON	22	120	N.A
P28	46	F	RRMS	WD	22	48	31
P29	53	F	RRMS	WD	22	36	57
P30	41	F	RRMS	WD	22	18	54

Table 3.1: Demographic and clinical features of 20 MS Patients and 10 Healthy controls involved in this study. The total of 20 RRMS patients were recruited in which 10 patients were IFN-β-1a Rebif™ NAb positive designated P1-P10 and 10 patients were IFN-β-1a Rebif™ NAb negative N1-N10. Patient P1 was discontinued from the study due to ill health. In addition to patients, 10 healthy donors designated as H1-H10 were also recruited as controls. Abbreviations: RRMS; Relapsing-Remitting Multiple Sclerosis; Rebif, IFN-β-1a; s.c, subcutaneous; WD, withdrawn/withdrawal; ON, ongoing; N.A, not applicable; F, female; M, male; ** treatment duration in months.

3.2.3 Separation of Peripheral blood mononuclear cells and Plasma

Peripheral blood mononuclear cells and plasma were isolated from venous blood using density gradient centrifugation. Heparinised whole venous blood was gently layered on lymphoprep (specific gravity = 1.077, Axis-Shield; Cheshire, UK) and was centrifuged for 25 minutes at 720 g without brake. Granulocytes and erythrocytes have a higher density than mononuclear cells and therefore sediment through the lymphoprep layer during centrifugation. Using a sterile Pasteur pipette the topmost layer containing plasma was collected into a new sterile tube and stored at – 80 °C until further use. The layer of cells (PBMCs) at the interface between the layered blood and lymphoprep was collected in another new sterile tube. The isolated cells were washed with HBSS and centrifuged for 10 minutes at 580g. The obtained cells were counted using trypan blue and frozen (cryopreservation in Fetal bovine serum + 10% Dimethyl sulfoxide) at – 150 °C until further use.

3.2.4 Separation of Serum

Whole venous blood samples were collected in clotting activated silicon-coated blood collecting tubes (Vacuette; Gloucestershire, UK). The clotting of blood was allowed to take place at room temperature (around 20 °C) for 30 – 60 minutes. The initial centrifugation was carried out in a centrifuge at 2000 g for 10 minutes. The serum was removed and centrifuged again at 8000 g for 5 minutes to remove all cell debris. Isolated serum was aliquoted and frozen at – 80 °C until future use.

3.2.5 Detection and quantification of total and Interferon beta 1a Rebif™ specific immunoglobulins

Total IgM, IgG and IgE and IFN-β-1a Rebif™ specific IgM, IgG and IgE in the plasma were quantified using human IgM, IgG and IgE sandwich type ELISA quantitation kits (Bethyl Laboratories, Inc. USA). Total IgG subclass levels in the plasma were quantified by ELISA using human IgG subclass profile kit (Invitrogen – Life Technologies, UK). IFN-β-1a Rebif™ specificIgG subclasses were measured by ELISA using human IgG subclass screening kit (Cygnus Technologies, Inc. USA) according to the manufacturer's protocol (please refer Table 3.3 for ELISA steps and Figure 3.3 for schematic representation.

For quantification of total IgM, IgG and IgE immunoglobulins in plasma samples the affinity purified human IgM, IgG and IgE coating antibodies were diluted in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6). The plates were coated with 100 µl of 1:100 diluted affinity purified coating antibodies and incubated for 16 hours at 4 °C. The plates were washed using

wash buffer (50 mM Tris, 0.14 M Nacl, 0.05% Tween 20, pH 8.0) 200 µL per well five times and blocked using blocking solution (50 mM Tris, 0.14 M Nacl, 1 % BSA, pH 8.0) for 1 hour at room temperature. The plates were washed again and 100 µl of plasma samples in 1:10000 for IgM, 1:100000 for IgG and 1:200 for IgE dilutions were added and respective standards was incubated for 1 hour at room temperature. The plates were washed again and 100 µl of HRP-conjugated anti-human IgM, IgG and IgE detection antibodies were added at 1:50000, 1:100000 and 1:50000 dilutions respectively and incubated for 1 hour at room temperature. The plates were washed and 100 µl of freshly prepared TMB substrate was added and incubated in the dark for 15 minutes. The reaction was stopped by addition of 100 µl of 0.18 M H_2SO_4 and absorbance was measured at 450 nm. For measuring IFN-β-1a Rebif™ specific immunoglobulins IgM, IgG, and IgE a similar method was used. However, the plates were coated with 100 µl per well of IFN-β-1a (1.5 µg/mL Rebif™) instead of coating antibodies for 16 hours at 4 °C and the plasma dilutions used were 1:2 for IgM, 1:20 for IgG and neat samples was used for IgE detection. The absolute concentrations for total immunoglobulins types and IFN-β-1a Rebif™ specific immunoglobulins types were calculated based on the standards provided in the kit.

For quantification of total IgG subclass, the plasma samples were diluted at 1:2500. Fifty microliters of diluted plasma samples, standards and human serum control was added into IgG antibody-coated microtiter plates. The plate was incubated at room temperature for 30 minutes. The plate was washed using 200 µl of wash buffer three times. 100 µl of 1:50 diluted peroxidase anti-human IgG secondary antibody was added into the plates and incubated at room temperature for 30 minutes. The plate was washed again using 200 µl of wash buffer three times. 100 µl of TMB solution was added into each well and the plate was incubated at room temperature for 30 minutes. 100 µl of stop solution was added into each well and the absorbance was measured on a plate reader at 450 nm. For measuring IFN-β-1a Rebif™ specific subclasses the plates were coated with 100 µl per well of IFN-β-1a Rebif™ (1.5 µg/mL) and incubated for 16 hours at 4 °C. The plates were washed using wash buffer 200 µL per well five times and blocked using blocking solution for 1 hour at room temperature. The plates were washed again and 100 µl of 1:20 diluted plasma samples were added and incubated for 1 hour at room temperature. The plates were washed again and 100 µl of HRP-conjugated IgG subclass (IgG1-IgG4) specific detection antibodies were added at 1:100 dilutions respectively and incubated for 1 hour at room temperature. The plates were washed and 100 µl of freshly prepared TMB substrate was added and incubated in the dark

for 15 minutes. The reaction was stopped by addition of 100 µl of 0.18 M H_2SO_4 and absorbance was measured at 450 nm. The absolute concentrations for total IgG subclass were calculated based on the standards provided in the kit. However, IFN-β-1a Rebif™ specific IgG subclass was based on optical density values as no standards for quantification was available.

ELISA	Total immunoglobulins	IFN-β-1a Rebif™ specific immunoglobulins
Step 1	The plates coated with coating antibodies and incubated for 16 hours at 4 °C	The plates coated IFN-β-1a Rebif™ for 16 hours at 4 °C
Step 2	Diluted plasma samples were added and incubated for 1 hour at room temperature	The plasma samples were added and incubated for 1 hour at room temperature
Step 3	HRP-conjugated human detection antibodies were added and incubated for 1 hour at room temperature	HRP-conjugated human detection antibodies were added and incubated for 1 hour at room temperature
Step 4	TMB substrate was added and incubated in dark for 15 minutes	TMB substrate was added and incubated in dark for 15 minutes
Step 5	Stop solution was added and absorbance was measured using 450 nm	Stop solution was added and absorbance was measured using 450 nm

Table 3.2: Enzyme linked immuno-sorbent assay (ELISA) steps involved in detection and quantification of total and IFN-β-1a Rebif™ specific immunoglobulins.

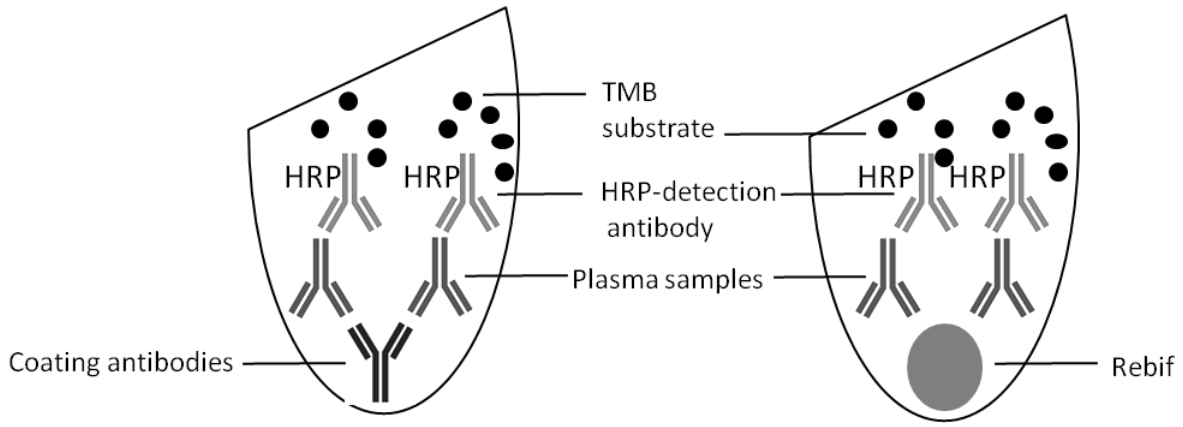


Figure 3.3: The schematic representation of total immunoglobulins and IFN-β-1a Rebif™ specific immunoglobulins ELISA. The details of the experiments are provided in Section 3.2.5.

3.2.6 Neutralisation assay: Interferon beta 1a induced MxA bioassay

Myxovirus resistance protein A (MxA) expression is an established biomarker for IFN-β bioactivity (Pachner et al. 2005). The neutralising potential of anti-IFN-β antibodies was

determined utilising IFN-β induced MxA protein expression in THP-1 cells, using western blotting.

3.2.6.1 THP-1 cell culture

The monocytic cell line THP-1 (Tsuchiya et al. 1980), was maintained in culture medium RPMI-1640 supplemented with 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and L-glutamine 2 mM. Cells were maintained in a humidified incubator, at 37 °C, in 5 % carbon dioxide (CO_2) atmosphere. Cells were grown in 75 cm^2 Nunclon culture flasks and routinely passaged every alternate day, at around 80% confluency. For the analysis of Myxovirus A (MxA) protein, cells were seeded onto 24-well culture dishes, at 3 x 10^6 cells per well, in a total volume of 800 µL culture medium.

3.2.6.2 Incubation of plasma, IFN-β-1a Rebif™ and THP-1 cells

Under sterile conditions, in a U-bottom 96-well plate, 100 U of IFN-β-1a Rebif™ was incubated with varying dilutions of plasma samples (1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000). The plasma samples were obtained from IFN-β-1a Rebif™ treated NAb positive and NAb negative RRMS patients and a total volume of 200 µL in RPMI-1640 culture medium. Healthy donor plasma samples were used as controls. The mixture was incubated at 37 °C, in a 5 % CO_2 atmosphere for an hour. Two hundred microliters ofIFN-β-1a Rebif™ incubated plasma samples were then added to THP-1 cells and incubated for 18 hours at 37 °C, in a 5 % CO_2.

3.2.6.3 Preparation of cell lysates

The incubated THP-1 cells were harvested and the cells were pelleted by centrifuging at 300 g for 5 minutes at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 200 µL of ice cold RIPA lysis buffer. Cells were allowed to lyse on ice for 30 minutes, vortexing every 10 minutes. Cellular debris was removed by centrifugation at 8000 g for 10 minutes at 4 °C. The supernatants (cell lysates) obtained were stored at – 80 °C prior to analysis by Western blot.

3.2.6.4 Determination of protein content

The total protein content of cell lysates was determined using Bradford Protein assay (Bradford 1976). This method involves the binding of Coomassie Brilliant Blue G-250 dye to basic and aromatic amino acids, an event that results in a change in colour of the dye (red to blue), and a consequent change in absorbance maximum from 465 to 595 nm. Hence, the

increase in absorbance at 570 nm, is proportional to the amount of bound dye, and thus to the amount of protein present.

In brief, a standard curve 0, 0.5, 1, 2.5, 3.5, 5, 7.5, 10 µg of bovine serum albumin (BSA) was prepared using stock concentration of 0.1 mg/mL. The cell lysates were diluted at 1:200. One hundred microliters of Bradford detection reagent (Coomassie Brilliant Blue G, ethanol, phosphoric acid and deionised water) was added and the samples were vortexed and incubated for 5 minutes at room temperature. Two hundred microliters of samples and standards were transferred to 96 – well flat bottom plate for spectrophotometric analysis and measured using a MRX microplate reader (Dynatech Labs; Billingshort, UK).

3.2.6.5 Western blot analysis

10 µg of protein was denatured via the addition of loading buffer (70 % (v/v) NuPAGE sample loading buffer, 30 % (v/v) NuPAGE reducing agent) and incubated at 99 °C for 5 minutes. The pre-cast 4 – 12 % NuPAGE Novex bis-tris polyacrylamide gels or 10 % SDS-polyacrylamide electrophoresis (SDS-PAGE) gels (resolving gel made of deionised water, 30 % acrylamide, 1.5 M Tris pH 8.8, 10 % SDS, 10 % APS, TEMED and stacking gel was made of deionised water, 30 % acrylamide, 1 M Tris pH 6.8, 10 % SDS, 10 % APS, TEMED) was used for loading the samples alongside PrecisionPlus protein Kaleidoscope standards. Samples were resolved by electrophoresis in a XCell Surelock mini-cell, using a 3-(N-morpholino) propanesulphonic acid (MOPS) running buffer (50 mM MOPS, 50 mM Tris base, 3.5 mM SDS, 1 mM EDTA, 0.25 % (v/v) NuPAGE antioxidant or 25 mM Tris base, 250 mM glycine pH 8.3, 0.1 % SDS at 90 V for 10 minutes, followed by 120 minutes at 130 V for pre-cast gels and 10 % SDS-PAGE gels respectively). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane using transfer buffer (12 mM Tris, 96 mM Glycine, 20 % Methanol) for 60 minutes keeping current constant (at 0.30 A). The PVDF membranes were pretreated with 100 % methanol and washed with deionised water prior to transferring proteins. To confirm whether the transfer process was successful, membranes were stained for 10 seconds with Ponceau S staining solution. Ponceau S is used for the detection of total proteins on PVDF membranes. Membranes were blocked for 60 minutes, on an orbital shaker, in 5 % tris-buffered saline (TBS; 0.15 M NaCl, 25 mM Tris base, 3 mM KCL, pH 7.0) containing 0.1 % (v/v) Tween 20 and 5 % (weight/volume; w/v) non-fat dry milk. Blocked membranes were probed for 16 hours at 4 °C with rabbit anti-MxA primary antibody from Santa Cruz biotechnology, Middlesex, UK (1:500 in TBS-Tween containing 5 % (w/v) non-fat dry milk). Following 3 washes in TBS-Tween, membranes were

then probed for 1 hour with goat anti-rabbit (1:5000 in TBS-Tween containing 5 % (w/v) non-fat dry milk) horseradish peroxidase (HRP) – conjugated secondary antibody (New England Biolabs, Hertfordshire, UK). Immunoblots were visualized with Western Lightening chemiluminescence reagents and exposed to Hyperfilm ECL under darkroom conditions, using a Kodak BioMax MS intensifying screen. Blots were developed using Kodak developer and fixer solutions. In order to determine equal loading of samples, membranes were probed with mouse anti-β-actin primary antibody from abcam, Cambridge, UK (1:20000 in TBS-Tween containing 5 % (w/v) non-fat dry milk) and rabbit anti-mouse (1:20000 in TBS-Tween containing 5 % (w/v) non-fat dry milk) HRP – conjugated secondary antibody (New England Biolabs, Hertfordshire, UK). Films were scanned using a GS-710 calibrated imaging densitometer and analysis was performed using TotalLab software.

3.2.7 IgG4 depletion

The human IgG4 affinity matrix (Capture select, BAC BV, The Netherlands) is a unique resin containing 12 kDa Llama antibody fragments which recognises human IgG4. The affinity matrix is directed towards a particular domain on the Fc portion of the human IgG4 that enables binding of human IgG4 without cross reacting with other human IgG subclasses. The amount of resin required for the IgG4 depletion in the total plasma was calculated as described below. The anti-IFN-β antibodies positive plasma samples contained 0.1 – 0.8 mg/mL of total IgG4. The recommended ratio indicated for use is 1 mL of resin to 6 mg/mL of total IgG4. Hence, 200 µL of resin was used for every mL of plasma samples. The total IgG4 levels in anti-IFN-β-1a Rebif™ antibodies plasma samples were quantified using human IgG subclass profile kit (Invitrogen – Life Technologies, UK).

The Mobicol "F" spin columns (MoBiTec GmbH, Germany) was used for depleting IgG4 in plasma samples. The columns are designed for wide range of applications which includes fractionation and purification of antibodies when used with affinity matrix. The matrix and the plasma samples of interest were incubated in these columns.

The columns were equilibrated using equilibration buffer (100 mM Sodium Citrate, 150 mM NaCl pH 6.5-7.0) using gravity flow-through mode. Two milliliters of human IgG4 affinity matrix resin was centrifuged for 2 minutes at 500 g and supernatants were discarded. 1 mL of equilibration buffer was added and was centrifuged and supernatants were discarded. This equilibration step was repeated 3 times. 1 mL of neat plasma was incubated with the resin for 3 minutes at room temperature in a Mobicol "F" spin column. The IgG4 depleted plasma

samples were collected using quick spin on a mini centrifuge. The flow-through plasma samples obtained was depleted of total IgG4 (80-99 %). The depletion percentage of total IgG4 was quantified by ELISA using human IgG subclass profile kit (Invitrogen – Life Technologies, UK) according to manufacturer's instructions.

3.2.8 Cross reactivity of anti-Interferon beta 1a Rebif™ antibodies to endogenous interferon beta

Lipopolysaccharide (LPS) and Polyinosinic: polycytidylic acid (Poly I:C) are Toll-like receptor (TLR) TLR-4 and TLR-3 ligands respectively and are able to induce endogenous production of IFN-β in many cell types including THP-1 cells. In this experiment, LPS and Poly I:C were used to stimulate the THP-1 cells to produce endogenous IFN-β and the production of IFN-β was determined using MxA protein expression (refer Figure 3.4). The addition of anti-IFN-β-1a Rebif™ antibodies reacts with endogenous IFN-β and hence reduces the MxA protein expression.

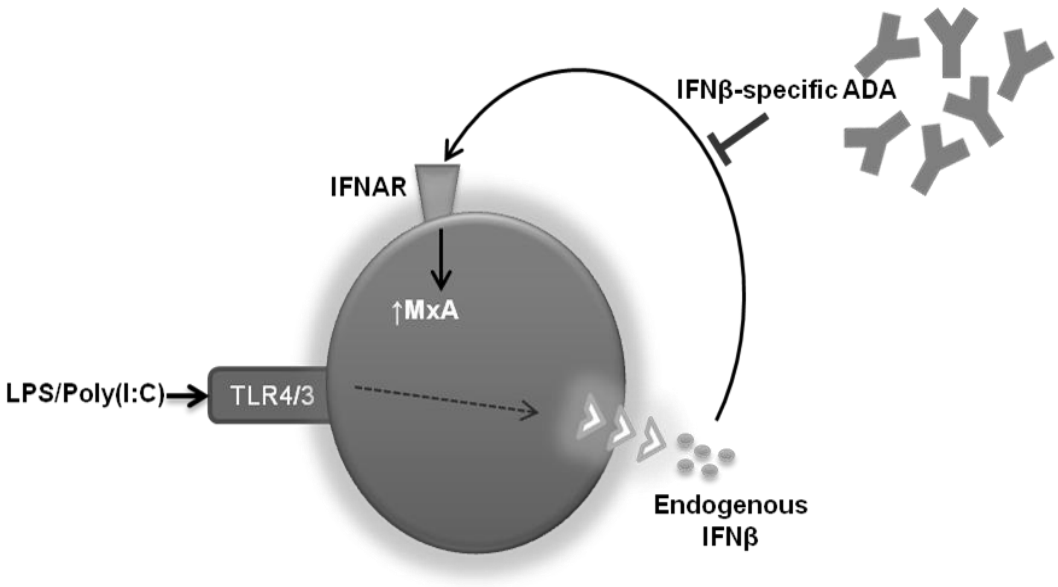


Figure 3.4: Schematic representation of the rationale and strategy for the cross reactivity assessment of anti-IFN-β-1a Rebif™ antibodies to endogenous IFN-β. In order to assess the potential of anti-IFN-β-1a Rebif™ antibody cross reactivity – LPS 1 µg/mL and Poly I:C 10 µg/mL was used to trigger endogenous IFN-β secretion in THP-1 cells and subsequent MxA protein expression due to autocrine effect of secreted IFN-β on THP-1 cells. Cross reactivity of anti-IFN-β-1a Rebif™ antibodies was assessed by co-incubating anti-IFN-β-1a Rebif™ antibodies positive patient plasma at 1:1000 dilutions with LPS and Poly I:C in

THP-1 cells followed by culture at 37 °C for 24 hours. After 24 hours, the MxA protein levels were quantified using western blotting as described in Section 3.2.7.5.

3.2.9 Mapping of continuous B-cell epitopes

Analysis of continuous B-cell epitope mapping on IFN-β-1a Rebif™ protein was carried out using the Multipin Peptide Technology (PepSets) of Mimotopes (Victoria, Australia). The amino acid sequence of the IFN-β-1a Rebif™ protein was used to synthesise a complete set of overlapping (12 mer, 5 offset) peptides covalently attached to the surfaces of derivatised polyethylene pins in a format compatible with standard enzyme-linked immunosorbent assays (refer Figure 3.5). These overlapping peptides covered the entire sequence of the protein. The final PepSets consisted of a total of 31 peptides plus 2 control peptides. The reactivities of P3, P10, P13, P14 and P15 patient's plasma samples containing anti-IFN-β-1a Rebif™ antibodies with the pin-bound peptides were detected by modified enzyme immunoassay.

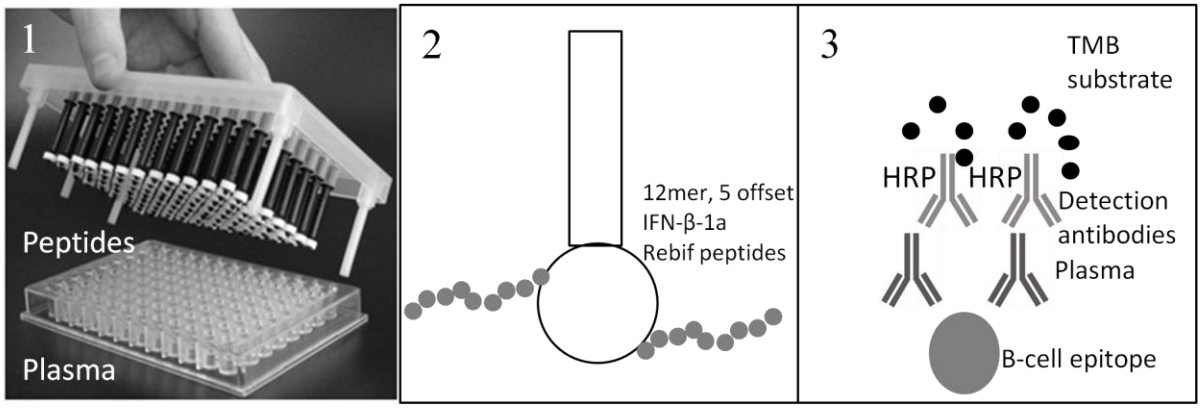


Figure 3.5: Schematic representation of continuous B-cell epitope mapping using multipin peptide technology (Step 1 photograph was taken from Mimotopes (Victoria, Australia)**.** The pins were pre-coated for 1 hour at room temperature with 200 µL of pre-coating buffer (0.01 M PBS pH 7.2 and 0.1 % v/v Tween 20) per well in the wells of a microtiter flat bottom plate. They were then incubated overnight at 4 °C with 200 µL of a 1:1000 dilution of anti-IFN-β-1a Rebif™ antibodies positive plasma sample in pre-coating buffer. The plate containing the plasma was discarded, and the pin block was washed four times for 10 minutes each with PBS. Then, the peroxidase-conjugated secondary antibody goat anti-human IgG (H+L) F(ab')2 (Kirkegaard and Perry; Maryland, USA) at 1:2000 dilution in conjugate buffer (0.01M PBS pH 7.2, 0.1 % v/v Tween-20 and 0.1 % w/v Sodium caseinate) was added to the wells of a microtiter plate and the pin block was inserted and incubated for 1 hour at room temperature. After being washed as before, the block was

inserted in a new microtiter plate containing 200 µL of 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Kirkegaard and Perry; Maryland, USA) per well and developed for 45 minutes with gentle agitation. The plate was read at 405 nm absorbance using a MRX microplate reader. To reuse the pin block, it was placed in an ultrasonic bath with disruption buffer (0.1 M PBS, 1 % w/v SDS and 0.1 % v/v 2-mercaptoethanol pH 7.4) heated to 60 °C. The pin block was sonicated for 10 minutes, rinsed and washed in deionised water with an initial temperature of 60 °C (twice for 30 seconds each time and once for 30 minutes). The confirmation of antibody removal was checked by conjugate test. The test evaluates the reactivity of the conjugate with the peptides before actual epitope mapping test using patient's plasma. When the pin block was not in use, the pins were air dried after immersion in 100 % methanol and stored at 4 °C.

3.2.10 Linear binding epitope detection

Liner binding epitopes in anti-drug antibodies against IFN-β-1a Rebif™ were detected using an *in vitro* competitive binding assay approach. The *in vitro* approach followed was combination of competition binding assay and ELISA. Peptides (12 amino acids long with 5 amino acids overlap) for IFN-β-1a Rebif™ were custom synthesised (Mimotopes, Australia) for this assay (refer Table 3.3). A total of 32 lyophilised peptides (2.6 mg each) were dissolved in 1.6 ml of 100 % DMSO to yield an average of 1.7 µmoles per peptide. Concentrations of anti-drug antibodies in moles were determined based on anti-drug antibodies levels in serum as described in chapter 3 Figure 3.14a. Eight peptide pools were prepared by mixing 4 consecutive peptides for assessments (Pool 1: Peptide 1-4, Pool 2: Peptide 5-8, Pool 3: Peptide 9-12, Pool 4: Peptide 13-16, Pool 5: Peptide 17-20, Pool 6: Peptide 21-24, Pool 7: Peptide 25-28, Pool 8: Peptide 28-32). Peptides (8 pools) and anti-drug antibodies containing serum n=8 were incubated at 37 °C for 1 hour. To facilitate maximum binding of the test peptides, saturating levels – 40 times: 2X (for IgG valency) + 20X (excess), of peptides were added to the serum (1:20 dilution). The 100 µl of peptide: anti-drug antibodies mixture was added onto wells in an ELISA plate coated with IFN-β-1a Rebif™ (1.5 µg/ml; overnight incubation at 4 °C) and human IgG bound to IFN-β-1a Rebif™ was measured colorimetrically at 450 nm. Serum containing anti-drug antibodies (n=8) without peptides was used as controls for each anti-drug antibodies positive sample. A decrease in the optical density values in the peptide: anti-drug antibodies mixture compared to its corresponding anti-drug antibodies only category indicates reduced binding of the anti-drug antibodies to the coated IFN-β-1a Rebif™. This indicates binding that the incubated

peptides to the anti-drug antibodies which prevents the binding of the anti-drug antibodies to the coated IFN-β-1a Rebif™. No change or slight increases in optical density values indicate lack of binding of the peptides to the anti-drug antibodies. Dr Swaminathan Sethu kindly conducted this experiment and analysed the data.

1	MSYNLLGFLQRS	9	PEEIKQLQQFQK	17	ETIVENLLANVY	25	HLKRYYGRILHY
2	LGFLQRSSNFQC	10	QLQQFQKEDAAL	18	NLLANVYHQINH	26	YGRILHYLKAKE
3	RSSNFQCQKLLW	11	QKEDAALTIYEM	19	VYHQINHLKTVL	27	HYLKAKEYSHCA
4	QCQKLLWQLNGR	12	ALTIYEMLQNIF	20	NHLKTVLEEKLE	28	KEYSHCAWTIVR
5	LWQLNGRLEYCL	13	EMLQNIFAIFRQ	21	VLEEKLEKEDFT	29	CAWTIVRVEILR
6	GRLEYCLKDRMN	14	IFAIFRQDSSST	22	LEKEDFTRGKLM	30	VRVEILRNFYFI
7	CLKDRMNFDIPE	15	RQDSSSTGWNET	23	FTRGKLMSSLHL	31	LRNFYFINRLTG
8	MNFDIPEEIKQL	16	STGWNETIVENL	24	LMSSLHLKRYYG	32	YFINRLTGYLRN

Table 3.3: The amino sequences of thirty two 12mer 5offset overlapping IFN-β-1a Rebif™ peptides. The overlapping peptide libraries are important biological research tools used for screening of large number of peptides in order to discover critical bioactive peptides.

3.2.11 Measurement of immune complex

The formation of immune complex *in vitro* was induced by incubating IFN-β-1a Rebif™ with patient serum for 1 hour at 37 °C followed by quantification of IgG complexes bound to C1q using IMTEC-CIC IgG ELISA kit (Imtec, Human Gesellschaft für Biochemica und Diagnostica mbH, Germany).

Hundred microliters of serum samples diluted at 1:100, standards and controls were added to C1q coated microtitre plate and incubated for 1 hour at room temperature. The plate was washed 3 times with wash buffer and 100 µL of conjugate solution was added and incubated for 30 minutes at room temperature. The plate was washed again and 100 µL of TMB solution was added and incubated for 10 minutes at room temperature. Following color development the reaction was stopped using addition of 100 µL of ready for use, 0.18 M H_2SO_4. The absorbance was read at 450 nm using a MRX microplate reader.

3.2.12 Measurement of immune complex induced C3a and C5a

The immune complex induced samples were analysed for activated complement factors, C3a and C5a using C3a and C5a ELISA kits (Hycult Biotech, Netherlands). Hundred microliters of immune complex induced samples (diluted at 1:4000 and 1:100 using dilution buffer for C3a and C5a respectively), standards and controls were added to microtitre plate coated with C3a or C5a specific antibody and incubated for 1 hour at room temperature. The plate was washed 4 times with wash buffer and 100 µL of biotinylated tracer antibody solution was

added to the microtitre plates and incubated for 1 hour at room temperature. The plate was washed again and 100 µL of streptavidin-peroxidase was added and incubated for 1 hour at room temperature. The wash procedure was repeated and then 100 µL of TMB solution was added and incubated for 20-30 minutes at room temperature. The reaction was stopped by addition of 100 µL of stop solution and the plate was read at 450 nm using a plate reader.

3.2.13 Statistical analysis

The data set for each group was tested for normality using the Shapiro-Wilk normality test. Descriptive statistical analysis was performed on each data group. For normal data sets, the mean and standard deviation (SD) was determined for each group. For non-normal data sets, the median and interquartile range was determined for each group. To determine statistical significance, comparisons were made using the Student's t-test (for two parametric groups), the Mann Whitney U test (for two non-parametric groups), the ANOVA and ANOVA with Dunnett's post hoc test (for more than two parametric groups) and the Kruskall-Wallis test for more than two non-parametric groups (Statsdirect; Cheshire, UK). The graphs were made using GraphPad Prism 5 software (GraphPad Saftware; CA, USA). Statistical significance was set at $P < 0.05$.

3.3 Results

3.3.1 Interferon beta 1a Rebif™ anti-drug antibodies are predominantly of the IgG1 and IgG4 subclass

The aim was to detect anti-drug antibodies against IFN-β-1a Rebif™ and to investigate the relative contribution of IgG subclasses in the formation of anti-drug antibodies. The distribution of IgG subclasses partially depends on the properties of antigen, with protein therapeutics normally eliciting IgG1 and IgG3 subclass specific response, whereas IgG2 dominates with carbohydrate antigens (Ferrante, Beard & Feldman 1990; Siber et al. 1980). Therefore, we investigated the relative contribution of IgG subclasses to immunogenicity of IFN-β-1a Rebif™. In this study we found eight out of nineteen RRMS patients had detectable levels of IFN-β-1a Rebif™ specific IgG antibodies (refer Figure 3.6b). The donor P1 was discontinued due to poor health. The samples were classified as IFN-β-1a Rebif™ specific anti-drug antibody positive when the IFN-β-1a Rebif™ specific IgG concentrations were above the threshold value of the mean plus two times the standard deviation of the value in healthy control plasma. The IFN-β-1a Rebif™ specific IgG concentration ranged between 0.3 µg/ml and 2.0 µg/ml in anti-drug antibodies positive samples. The data sets are non-

parametric and hence the median and interquartile range was determined. No significant difference was noticed in the levels of total IgG 6.850 (4.575, 7.850) and 7.200 (6.200, 9.400), total IgM 0.2650 (0.2025, 0.04925) and 0.3300 (0.2000, 0.5100) and IFN-β-1a Rebif™ specific IgM 43.56 (42.41, 53.91) and 45.01 (43.39, 51.27) between healthy donors and RRMS patients respectively (refer Figure 3.6a, 3.6c and 3.6d). IFN-β-1a Rebif™ specific IgG subclass revealed IFN-β-1a Rebif™ specific IgG1 and IgG4 in the plasma of eight anti-drug antibodies positive patients and were significantly high when compared to IgG2 and IgG3 (refer Figure 3.7b). Distribution of IFN-β-1a Rebif™ specific IgG subclasses for each of the sample indicated IgG1 0.1550 (0.0950, 0.3325) and IgG4 0.2850 (0.0225, 0.4225) at varying levels predominantly contributed to the anti-drug antibodies followed by IgG3 0.0200 (0.00575, 0.0200) and IgG2 0.0100 (0.0010, 0.0400) (refer Figure 3.7c). The donor P29 IFN-β-1a Rebif™ specific subclass IgG subclass profile was found to be primarily made up of IFN-β-1a Rebif™ specific subclass IgG1 and IgG3 with IFN-β-1a Rebif™ specific IgG subclass IgG4 being undetectable (refer Figure 3.7c). There were no significant differences in the total IgG subclass levels between healthy donors and RRMS patients (refer Figure 3.7a). Our data suggests that predominant Ig isotype of IFN-β-1a Rebif™ anti-drug antibodies is IgG and is dominated by IgG1/IgG4 subclass specific response in multiple sclerosis patients.

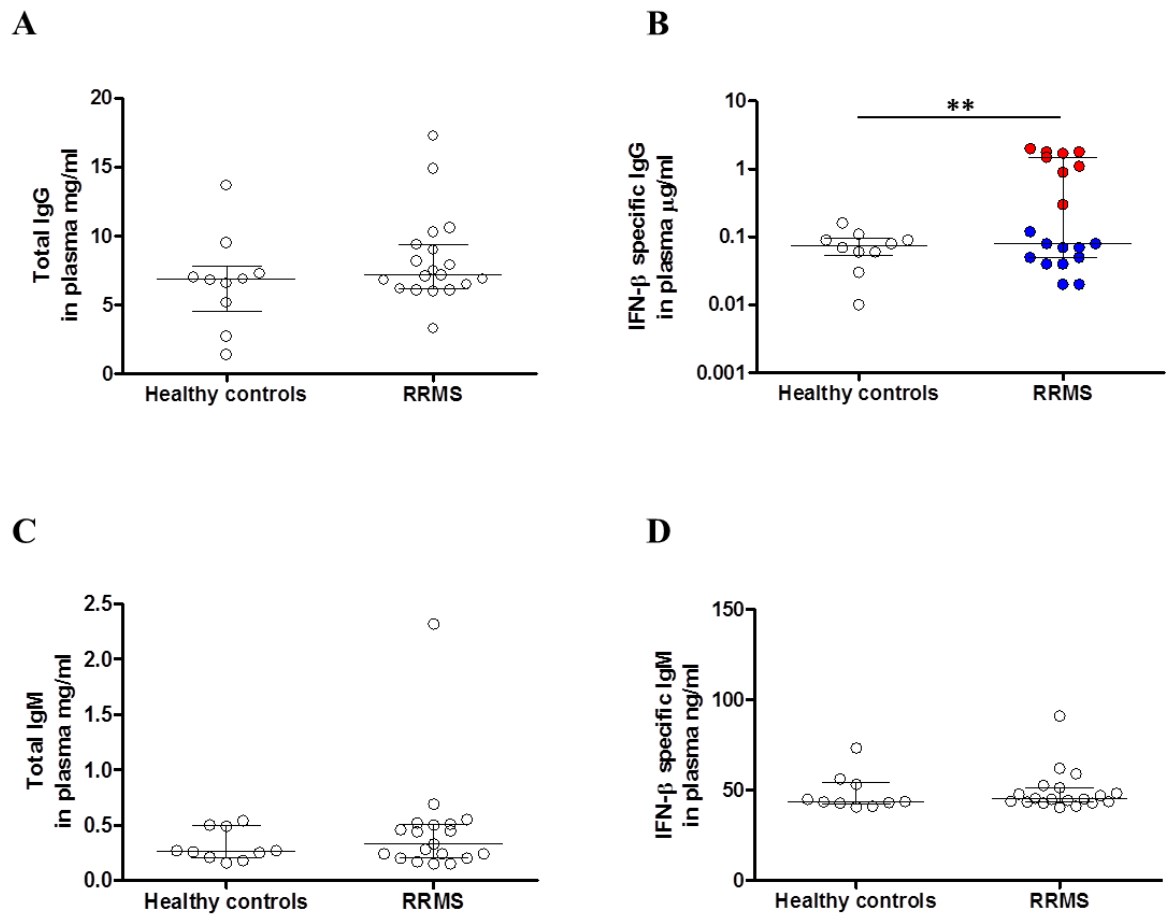


Figure 3.6: Immunoglobulin G and IgM of IFN-β-1a Rebif™ anti-drug antibodies. Total and IFN-β-1a Rebif™ specific IgG and IgM was quantified in plasma of healthy donors (n=10) and in RRMS patients (n=19) treated with IFN-β-1a Rebif™. Each open circle represents an individual total and IFN-β-1a Rebif™ specific IgG and IgM levels. Bars represent the IQR (25^{th} and 75^{th} percentiles) from the median (horizontal line). Figure A represents total IgG, the medians (25^{th}, 75^{th} percentiles) include 6.850 (4.575, 7.850) and 7.200 (6.200, 9.400) for the 2 groups respectively. Figure B represents IFN-β-1a Rebif™ specific IgG, the medians (25^{th}, 75^{th} percentiles) include 0.0750 (0.0525, 0.0950) and 0.0800 (0.0500, 1.500) for the 2 groups respectively. The anti-drug antibody positive samples circles are coloured in red, while negative samples circles are coloured in blue. Figure C represents total IgM, the median (25^{th}, 75^{th} percentiles) include 0.2650 (0.2025, 0.04925) and 0.3300 (0.2000, 0.5100) for the 2 groups respectively. Figure D represents IFN-β-1a Rebif™ specific IgM, the medians (25^{th}, 75^{th} percentiles) include 43.56 (42.41, 53.91) and 45.01 (43.39, 51.27) for the 2 groups respectively. The data point in each graph is a mean of triplicate measurements and is representative of three independent experiments. Statistically significant differences are shown in panels and comparisons were determined using Mann Whitney U test for two non-parametric groups.

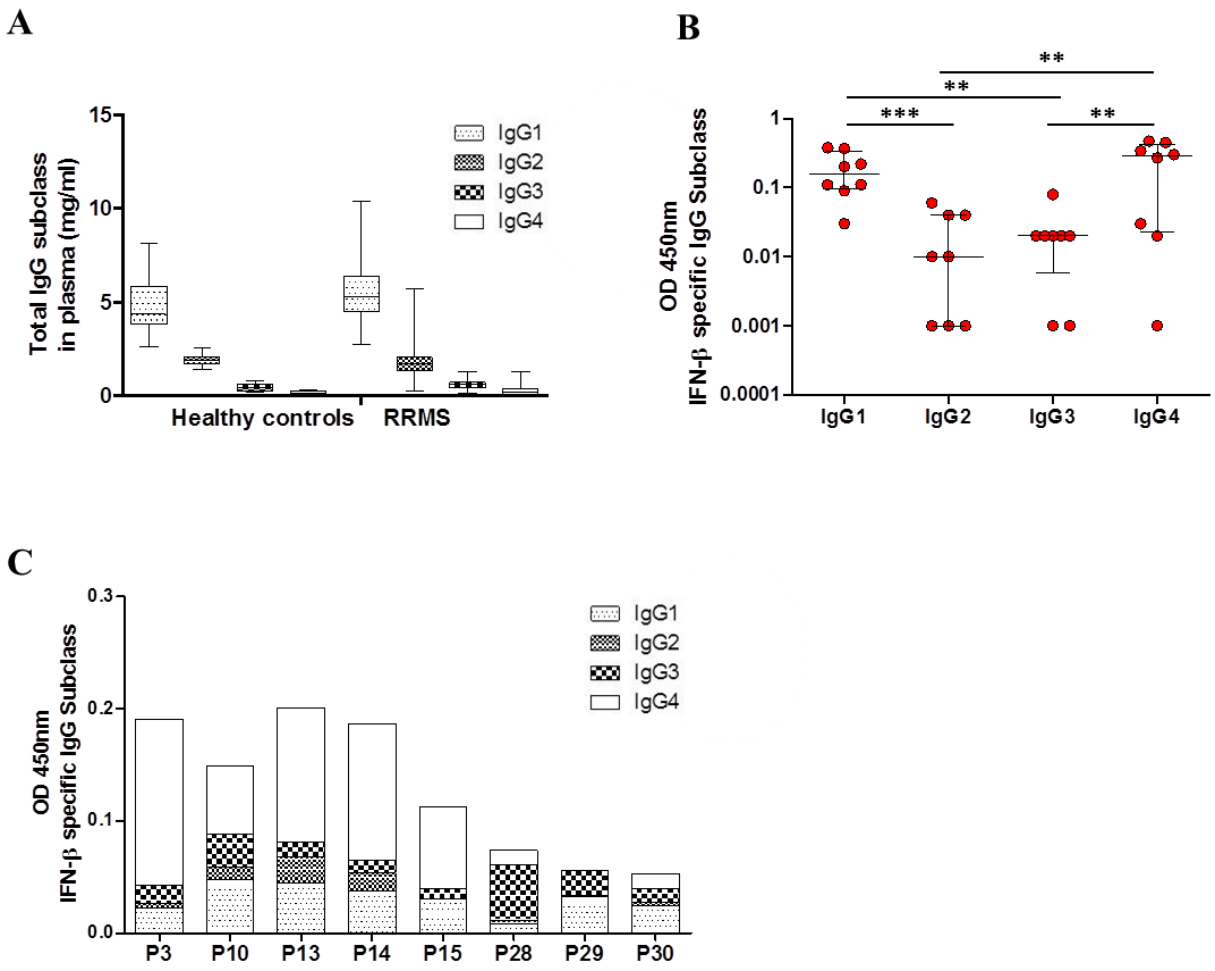


Figure 3.7: Immunoglobulin G subclass (IgG1-IgG4) of IFN-β-1a Rebif™ anti-drug antibodies. Total IgG subclass was quantified in plasma of healthy donors and in RRMS patients. IFN-β-1a Rebif™ specific IgG subclass was determined in IFN-β-1a Rebif™ anti-drug antibodies positive samples (n=8). Bars represent the IQR (25^{th} and 75^{th} percentiles) from the median (horizontal line). Figure A represents total IgG subclass (IgG1-IgG4), the medians (25^{th}, 75^{th} percentiles) include 4.354 (3.814, 5.853) and 5.279 (4.484, 6.403) for IgG1, 1.878 (1.705, 2.104) and 1.748 (1.370, 2.067) for IgG2, 0.4680 (0.2563, 0.6425) and 0.6400 (0.4520, 0.7460) for IgG3 and 0.1530 (0.08625, 0.2448) and 0.2040 (0.0910, 0.4100) for IgG4 in the 2 groups respectively. Figure B represents IFN-β-1a Rebif™ specific IgG subclass (IgG1 – IgG4) in anti-drug antibody positive samples. Each red circle represents an individual IFN-β-1a Rebif™ specific IgG subclass (IgG1 – IgG4). The medians (25^{th} and 75^{th} percentiles) include 0.1550 (0.0950, 0.3325), 0.0100 (0.0010, 0.0400), 0.0200 (0.00575, 0.0200) and 0.2850 (0.0225, 0.4225) for IFN-β-1a Rebif™ specific IgG1, IgG2, IgG3 and IgG4 respectively. Figure C represents the proportion of IFN-β-1a Rebif™ specific IgG subclass (IgG1-IgG4) in each anti-drug antibody positive plasma samples P3, P10, P13, P14,

P15, P28, P29 and P30 respectively. The data point in each graph is a mean of triplicate measurements and is representative of three independent experiments. Statistically significant differences are shown in panels and comparisons were determined using Kruskall-Wallis test for more than two nonparametric groups.

3.3.2 Neutralising potential of Interferon beta 1a Rebif™ anti-drug antibodies

In order to assess the neutralising potential of IFN-β-1a Rebif™ anti-drug antibodies, we used IFN-β-1a Rebif™ induced intracellular MxA expression in human acute monocytic leukemic cells line, THP-1. Semi-quantitative assessment of neutralising potential of IFN-β-1a Rebif™ anti-drug antibodies was performed using a bioassay based on IFN-β responsive MxA protein. MxA protein levels were measured by immunoblotting (refer Figure 3.8). Different concentrations of research grade IFN-β-1a and commercially available IFN-β-1a Rebif™ at 1U, 10U, 100U, 500U, 1000U and 5000U at different incubation times either overnight and 18 hours was also evaluated to induce interferon induced MxA protein. The decision was established to use 100U of IFN-β-1a Rebif™ and 18 hours of incubation time before preparation of protein lysates (refer Figure 3.9a).

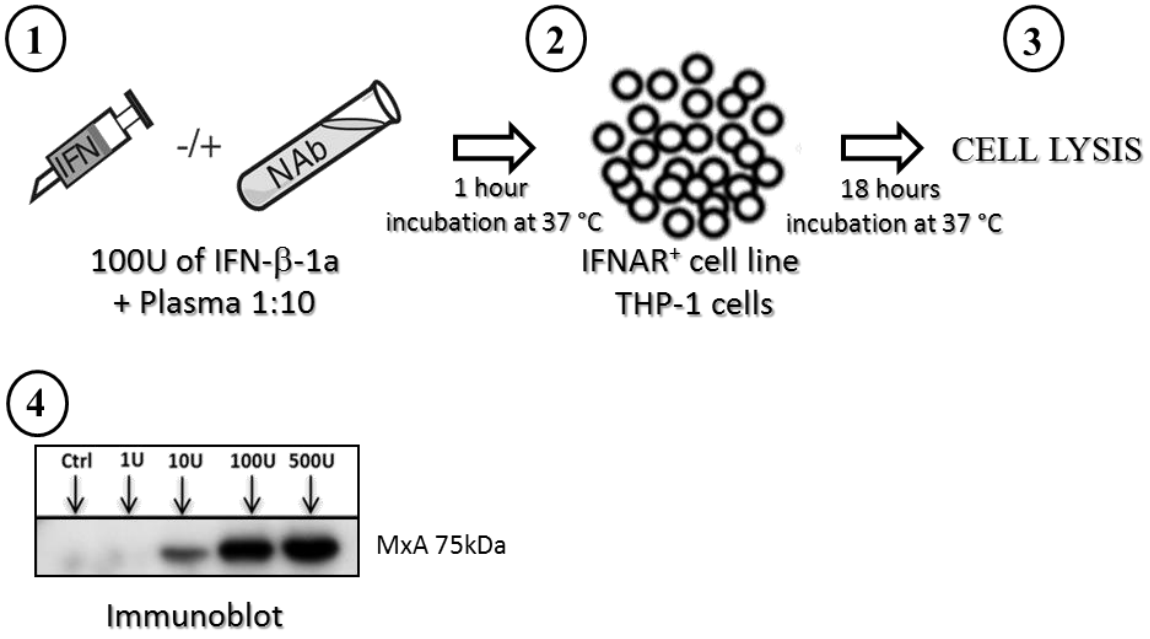


Figure 3.8: Schematic representation of in vitro bioassay using Type 1 IFN-receptor positive THP-1 cells.

Our data indicate seven out of eight IFN-β-1a Rebif™ anti-drug antibodies positive samples had the potential to neutralise the IFN-β-1a Rebif™ induced MxA (refer Figure 3.8b).

However, donor P29 did not neutralise IFN-β-1a Rebif™ indicating the anti-drug antibodies were of non-neutralising type. None of the anti-drug antibodies negative samples and healthy donors neutralised IFN-β-1a Rebif™ induced MxA (refer Figure 3.9b). Our data was consistent with the data obtained from other neutralisation assays carried out independently at National Institute of Biological Standards and Control (NIBSC), UK (refer Appendix 7).

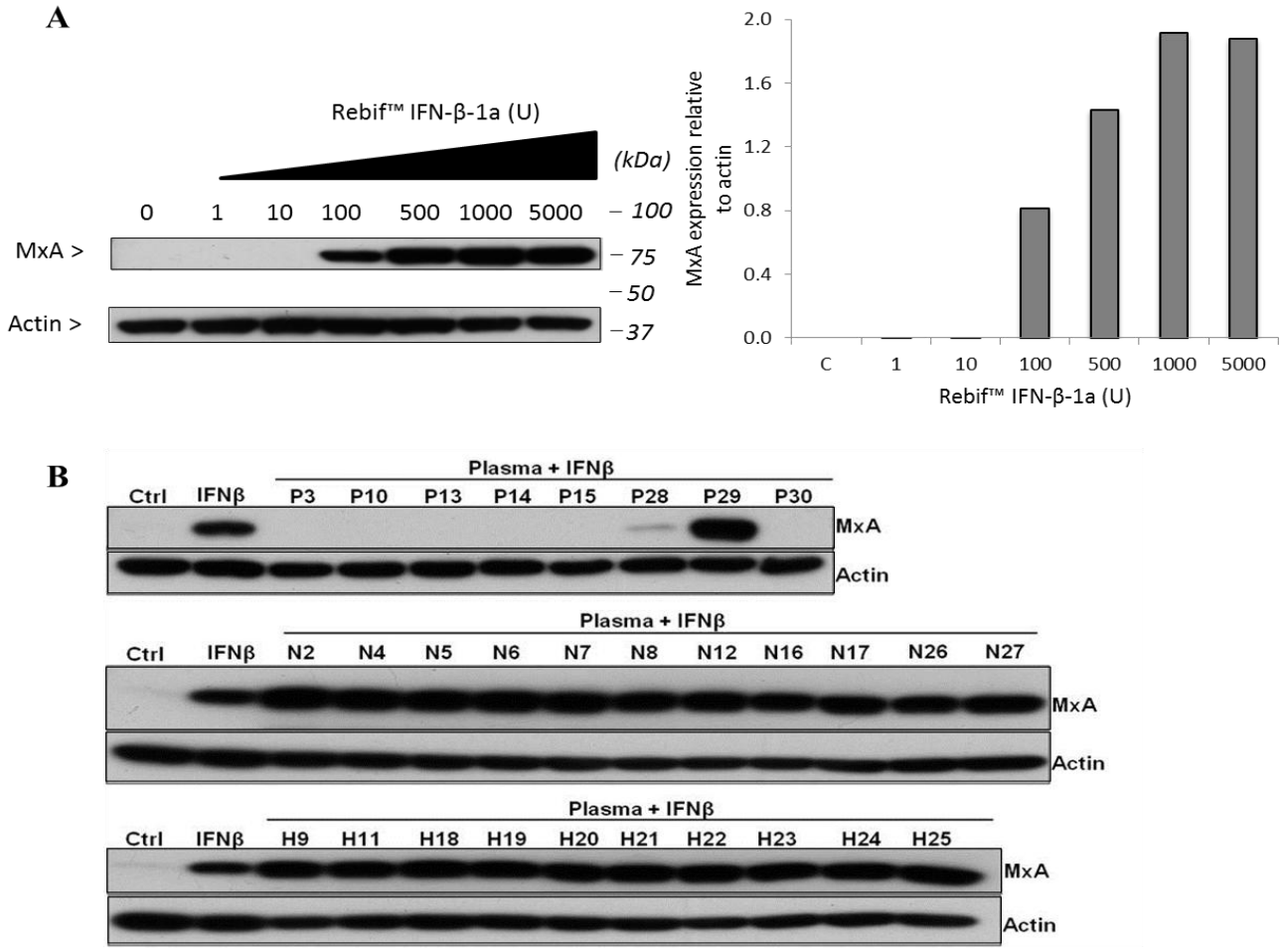
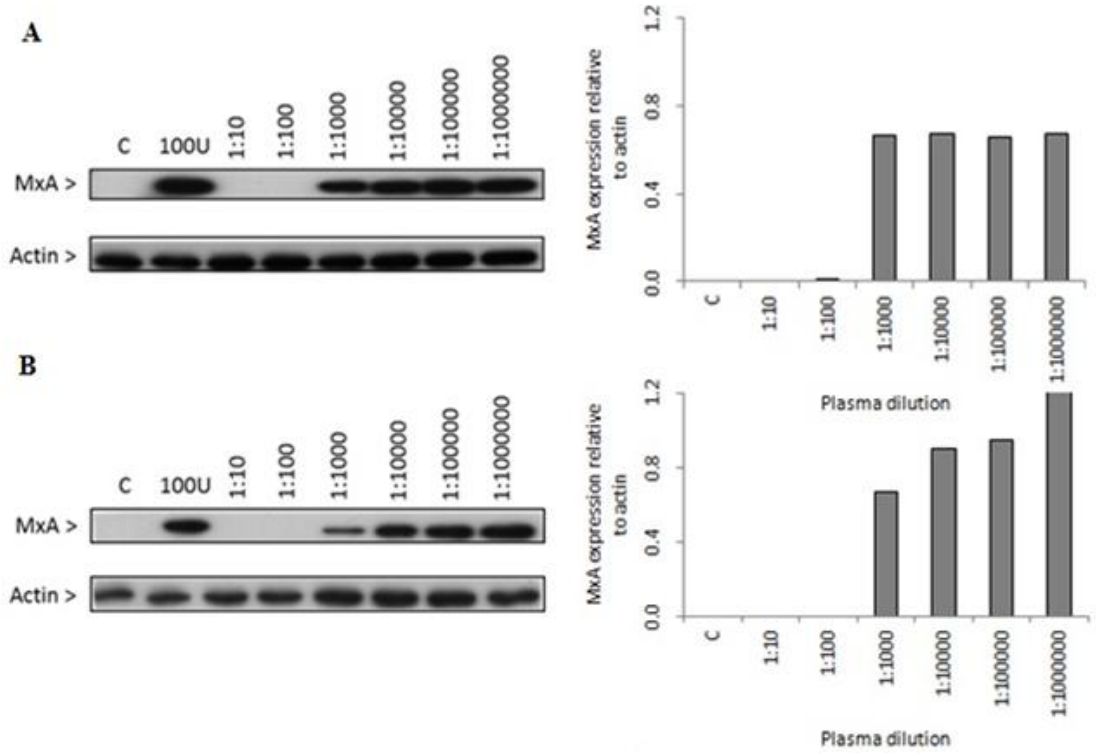


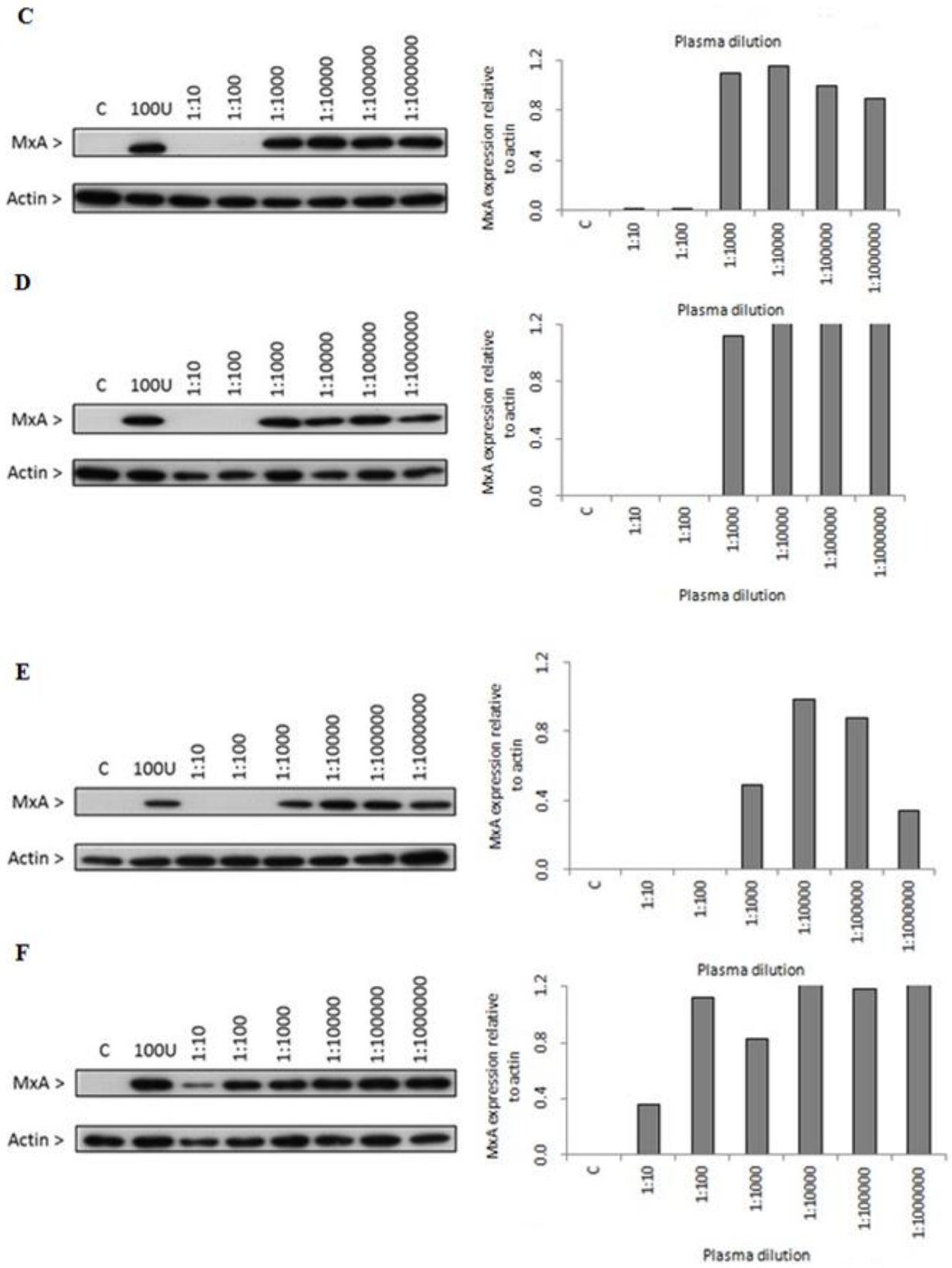
Figure 3.9: Neutralisation potential of IFN-β-1a Rebif™ anti-drug antibodies. Figure A represents the western blot for MxA protein expression. THP-1 cells were treated with increasing doses of IFN-β-1a Rebif™ (1-5000 U) for 18 hours and whole cell lysates were prepared. The protein lysates (10 µg) were subjected to SDS-PAGE and the levels of MxA and the loading control protein *β*-actin were determined by western blot analysis. Figure B represents the western blot for IFN-β-1a Rebif™ induced MxA protein levels in THP-1 cells. 100 U of IFN-β-1a Rebif™ was pre-incubated with 1:10 dilution of plasma samples of healthy controls and IFN-β-1a Rebif™ anti-drug antibody positive and negative samples for 1 hour at 37 °C and was then added to THP-1 cells for 16 hours. Proteins from whole cell

lysates were separated by SDS-PAGE and western blotting was performed. The membranes were probed for human MxA and β-actin. Ctrl indicates the cell lysates prepared from cells without any treatment and IFNβ indicates the lysates from cells treated with 100U IFN-β-1a Rebif™ only. The images are representative of three independent experiments.

3.3.3 Magnitude of neutralising potential of Interferon beta 1a Rebif™ anti-drug antibodies

In order to determine the magnitude of neutralising potential in each of the IFN-β-1a Rebif™ anti-drug antibodies positive samples dose response neutralisation bioassay was performed (refer Section 3.3.2). The plasma samples were incubated for 1 hour at 37 °C with 100 U of IFN-β-1a Rebif™ at 1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000 dilutions. Later, the mixture was added to THP-1 cells and incubated for 18 hours at 37 °C and MxA induction was measured. The Figure 3.10 represents the dose response neutralisation bioassay immunoblots for P3, P10, P13, P14, P15, P28, P29 and P20 samples. The neutralisation capabilities for five samples P3, P10, P13, P14 and P15 was until 1:1000 dilutions (refer Figure 3.10Ai – v). However, P28 and P30 neutralisation capacity was until 1:100 dilutions (refer Figure 3.10Avi and viii). The donor P29 failed to neutralise IFN-β-1a Rebif™ induced MxA protein and therefore was classified as IFN-β-1a Rebif™ neutralising antibody negative sample (refer Figure 3.10vii). The remaining was classified as IFN-β-1a Rebif™ neutralising antibodies positive samples in this study.




C
C 100U 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
MxA >
Actin >
Plasma dilution
MxA expression relative to actin
0.0 0.4 0.8 1.2
C 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
D
C 100U 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
MxA >
Actin >
Plasma dilution
MxA expression relative to actin
0.0 0.4 0.8 1.2
C 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
Plasma dilution
E
C 100U 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
MxA >
Actin >
MxA expression relative to actin
0.0 0.4 0.8 1.2
C 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
Plasma dilution
F
C 100U 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
MxA >
Actin >
MxA expression relative to actin
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C 1:10 1:100 1:1000 1:10000 1:100000 1:1000000
Plasma dilution

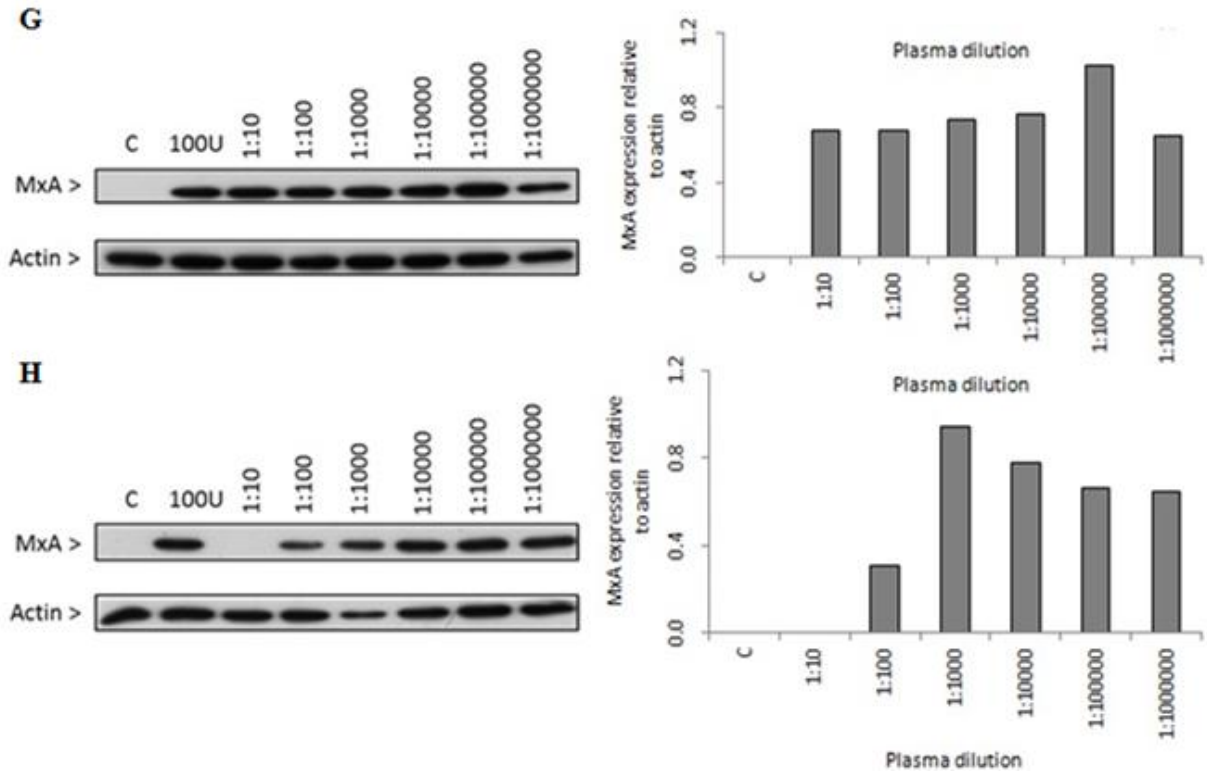


Figure 3.10: Inter-individual variations in neutralisation potential of IFN-β-1a Rebif™ anti-drug antibodies. Western blots showing IFN-β-1a Rebif™ induced MxA protein levels in THP-1 cells. 100 U of IFN-β-1a Rebif™ was pre-incubated with increasing dilutions 1:10, 1:100, 1:1000, 1:10000, 1:100000, 1:1000000 of plasma samples of IFN-β-1a Rebif™ anti-drug antibody positive samples **A.** P3, **B.** P10, **C.** P13, **D.** P14, **E.** P15, **F.** P28, **G.** P29 and **H.** P30 for 1 hour at 37 °C and was then added to THP-1 cells for 18 hours. Proteins from whole cell lysates were separated by SDS-PAGE and western blotting was performed. The membranes were probed for human MxA and *β*-actin. **C** on the blots indicates the cell lysates prepared from cells without any treatment and **100 U** on the blots indicates the lysates from cells treated with 100 U IFN-β-1a Rebif™ only. MxA protein bands were quantified by densitometry, expressed relative to *β*-actin and subsequently converted into ratio of MxA expression. The images are representative of three independent experiments.

3.3.4 IgG4 depletion for Interferon beta 1a Rebif™ anti-drug antibodies positive samples

Our data suggests IgG1 and IgG4 contribute to IFN-β-1a Rebif™ anti-drug antibodies profile. The earlier studies have implicated IgG4 anti-drug antibodies have greater neutralisation potential compared to other IgG subclasses. Hence, IgG4 anti-drug antibodies was depleted in order to establish the correlation between IgG4 anti-drug antibodies and neutralising potential in all the IFN-β-1a Rebif™ anti-drug antibodies positive samples P3, P10, P13, P14, P15, P28 and P30. However, quantification of IgG subclasses revealed loss of IgG1, IgG2 and IgG3 in addition to the expected depletion of IgG4 (refer Table 3.4). Hence, adjustments of the samples were performed based on total IgG1 levels (refer Figure 3.11).

A

Samples	Total plasma µg/ml				Unadjusted IgG4 depleted fraction µg/ml			
	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
P3	13077.62	1016.59	1097.85	1084.32	5284.94	531.67	547.57	0.00
P10	5849.32	1730.04	2089.52	543.62	3513.97	1046.30	1239.00	0.00
P13	7652.09	2395.45	685.38	574.82	3718.17	1479.85	317.09	0.00
P14	7422.87	2305.98	653.03	614.48	4517.56	1316.04	334.99	0.00
P15	6246.60	1055.45	408.50	214.30	2926.98	572.36	278.20	0.00
P28	8607.40	2040.60	2383.32	598.20	4460.91	1442.52	1348.33	0.00
P30	7893.31	2052.79	621.70	46.78	4183.43	1206.12	343.56	0.00

B

Samples	adjusted IgG4 depleted fraction µg/ml			
	IgG1	IgG2	IgG3	IgG4
P3	11858.15	1168.02	1642.61	0.00
P10	6917.24	1622.62	1889.80	0.00
P13	7090.32	2823.06	722.88	0.00
P14	7469.18	1980.21	467.45	0.00
P15	9919.18	1101.80	578.74	0.00
P28	7370.64	2092.88	2200.84	0.00
P30	7692.11	1745.03	628.16	0.00

Table 3.4: Quantification of total IgG subclasses in total plasma, adjusted and unadjusted IgG1-3 plasma samples.

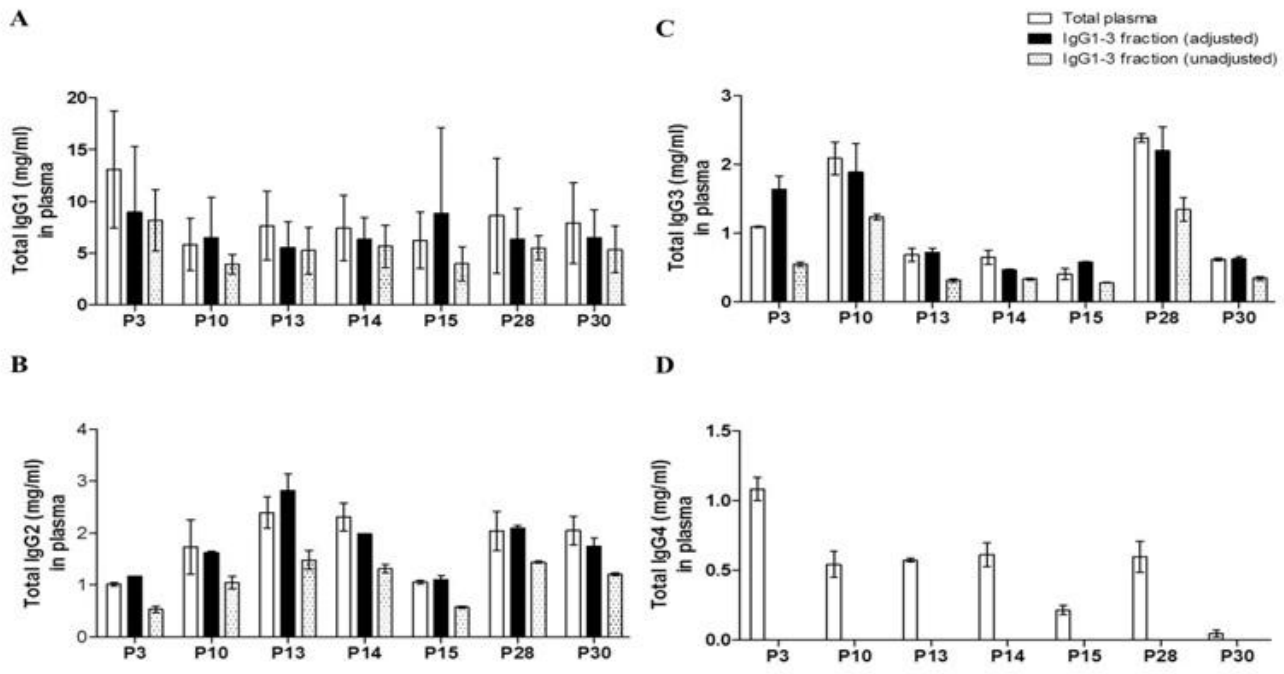


Figure 3.11: Immunoglobulin G subclass (IgG1-IgG4) in total plasma, adjusted and unadjusted IgG1-IgG3 plasma samples. The total plasma, adjusted and unadjusted IgG1-3 plasma samples from IFN-β-1a Rebif™ anti-drug antibody positive patients P3, P10, P13, P14, P15, P28 and P30 were analysed for total IgG subclass (IgG1-4) to assess the purity in obtained IgG1-3 fractions. Figure A represents total IgG1 levels in total, adjusted and unadjusted IgG1-3 plasma samples. Figure B represents total IgG2 levels in total, adjusted and unadjusted IgG1-3 plasma samples. Figure C represents total IgG3 levels in total, adjusted and unadjusted IgG1-3 plasma samples. Figure D represents total IgG4 levels in total, adjusted and unadjusted IgG1-3 plasma samples.

3.3.5 Neutralising potential of IgG4 depleted Interferon beta 1a Rebif™ anti-drug antibodies

In order to assess the neutralisation potential in total plasma and IgG4 depleted plasma (adjusted and unadjusted) the comparison was established using IFN-β induced MxA protein expression in THP-1 cells, using western blotting (refer Figure 3.12). Based on the results, depletion of IgG4 resulted in P3 donor 60-79 %, P10 donor 1-3 %, P14 donor 3-13 %, P14 donor 3-18 %, P15 donor 13-14 %, P28 50 % and P30 56-64 % reduction in the neutralisation potential in both IgG4 adjusted and unadjusted plasma samples. Our observation from this study indicates that IgG4 may likely contribute to the neutralisation activity of IFN-β-1a Rebif™ specific anti-drug antibodies and this effect may vary between patients.

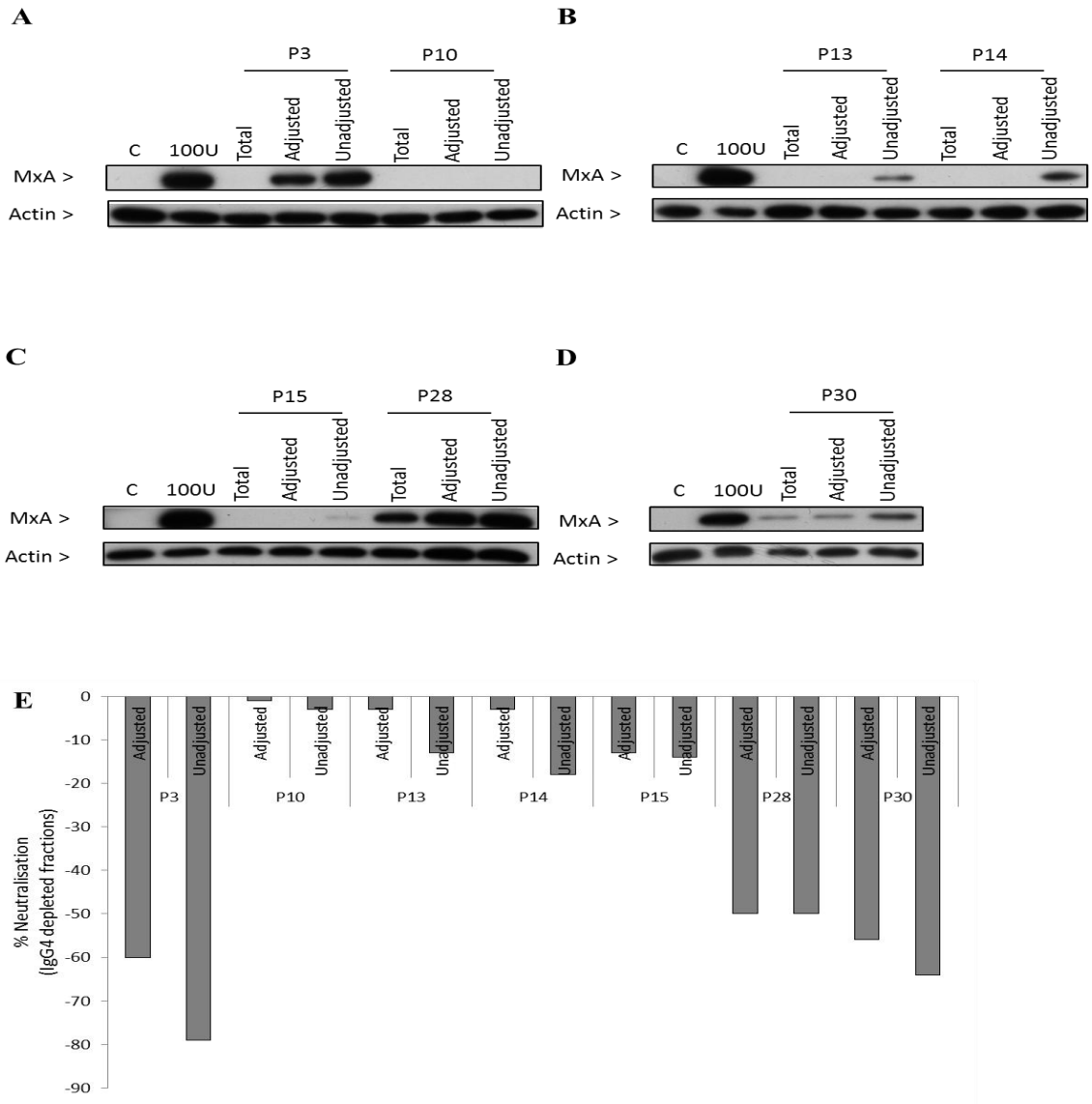


Figure 3.12: Neutralisation potential of IgG4 depleted IFN-β-1a Rebif™ anti-drug antibodies. Western blots showing variable differences in the potency of IFN-β-1a Rebif™ anti-drug antibody positive plasma samples with (total) and without (adjusted and unadjusted) IFN-β-1a Rebif™ specific IgG4 to neutralise IFN-β-1a Rebif™ induced MxA protein in THP-1 cells. IFN-β-1a Rebif™ 100 U pre-incubated with diluted plasma from 8 IFN-β-1a Rebif™ anti-drug antibody positive samples (1:1000 for P3, P10, P13, P14 and P15 and 1:10 for P28 an P30 with (total) and without (adjusted and unadjusted) IgG4 plasma samples for 1 hour at 37 °C and was then added to THP-1 cells for 16 hours. Proteins from whole cell lysates were separated by SDS-PAGE and western blotting was performed. The membranes were probed for human MxA and *β*-actin. **C** on the blots indicates the cell lysates prepared from cells without any treatment and **100 U** on the blots indicates the lysates from cells treated with 100 U IFN-β-1a Rebif™ only. MxA protein bands were quantified using densitometry. Neutralisation % is the percentage based on densitometric analysis of western blots assessing IFN-β-1a Rebif™ induced MxA neutralisation by IFN-β-1a Rebif™ anti-drug antibody positive plasma samples with (total) and without (adjusted and unadjusted) IgG4 relative to 100 U only category. The neutralisation % also indicates the difference in the neutralisation potential of IgG4 depleted plasma compared to its respective non-depleted fraction. The images are representative of three independent experiments.

3.3.6 Interferon beta 1a Rebif™ anti-drug antibodies cross reacts and neutralises the endogenous IFN-β induced bioactivity

The aim of this experiment was to elucidate the potential for IFN-β-1a Rebif™ anti-drug antibodies on neutralisation of endogenous IFN-β. LPS and Poly (I: C) was used to induce IFN-β in THP-1 cells (refer Figure 3.13A). The production of IFN-β was determined using MxA protein expression. Eight IFN-β-1a Rebif™ anti-drug antibodies positive samples were tested for their cross-reactivity to endogenous IFN-β. Five donors P3, P10, P13, P14 and P15 out of eight neutralised LPS and Poly I:C induced endogenous IFN-β at varying degrees (refer Figure 3.13B) P3 47.6 %, P10 77.5 %, P13 63 %, P14 60.5 % and P15 75 % reduction in the MxA protein expression to its positive control (Lane 2). The intensities of immunoreactive bands were quantified using densitometry. Our results indicate anti-drug antibodies generated in response to IFN-β-1a Rebif™ can neutralise endogenous IFN-β.

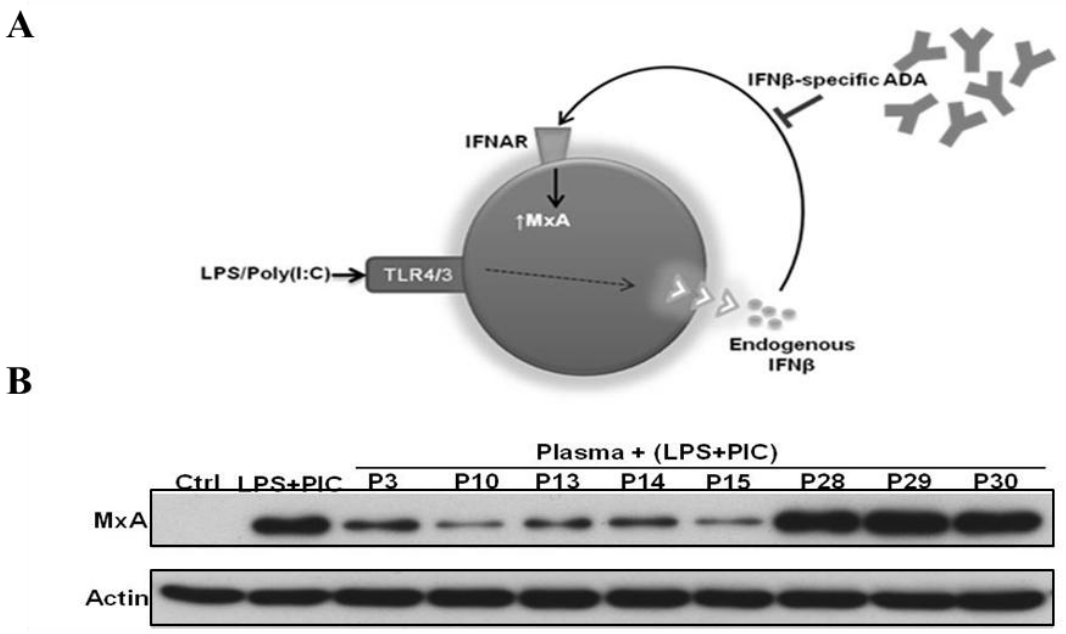


Figure 3.13: IFN-β-1a Rebif™ anti-drug antibodies cross reacts and neutralises the endogenous IFN-β induced bioactivity. Figure A represents the schematic representation of the rationale and strategy for the cross reactivity studies. In brief the bioactivity of endogenous IFN-β was assessed by triggering IFN-β induced MxA protein by TLR4/3 activation – LPS 1 µg/mL and Poly I:C 10 µg/mL. Cross reactivity of Rebif™ IFN-β-1a anti-drug antibodies to endogenous IFN-β was studied by investigating the ability of the anti-drug antibodies to block or inhibit the induction of MxA protein. Figure B represents a western blot for MxA protein expression induced using LPS and Poly I:C stimulation for 24 hours in

THP-1 cells. Induction of MxA protein is a result of endogenous Type 1 Interferons, which includes IFN-β triggered by LPS and Poly I:C. The western blot also indicates the changes in the level of induced MxA protein when cells are co-incubated with 1:10 dilution of plasma samples from Rebif™ IFN-β-1a anti-drug antibody positive samples (P3, P10, P13, P14, P15, P28 and P30) along with LPS and Poly I:C. Proteins from whole cell lysates were separated by SDS-PAGE and western blotting was performed. The membranes were probed for human MxA and *β*-actin. Ctrl indicates the cell lysates prepared from cells without any treatment and LPS+PIC indicates the lysates from cells treated with LPS+PIC only. The images are representative of three independent experiments.

3.3.7 Complement activation by Interferon beta 1a Rebif™ anti-drug antibodies immune complex

The formation of immune complexes between a biologic and anti-drug antibody have the potential to activate complement system by first binding to complement component C1q, which then leads to induction of activated complement. Serum from IFN□-1a Rebif™ anti-drug antibodies positive samples were used to investigate complements. Hence, we also quantified Rebif™ specific IgG in all anti-drug antibodies positive serum samples (refer Figure 3.14a). The quantification was found comparable to plasma samples. Optimal C1q binding of the IFN-β-1a Rebif™ anti-drug antibodies and IFN-β-1a Rebif™ immune complex was observed at an antigen-antibody (IFN-β-1a Rebif™: anti-drug antibody) ratio 5:1 (refer Figure 3.14b). Significant increase in immune complex formation was observed following IFN-β-1a Rebif™ incubation with IFN-β-1a Rebif™ anti-drug antibodies positive serum samples (refer Figure 3.14c). The magnitude of immune complex formation in individual samples was variable with values ranging from 0.2 to 2 fold increase from baseline (refer Figure 3.14d). Elevated C1q binding was accompanied by significant increase in the C3a levels in the serum samples incubated with IFN-β-1a Rebif™ (refer Figure 3.15a and b). However, no significant increase in C5a levels was observed (refer Figure 3.15c and d). Our data demonstrate IFN-β-1a Rebif™ anti-drug antibodies can form immune complexes with IFN-β-1a Rebif™ and therefore activate complement.

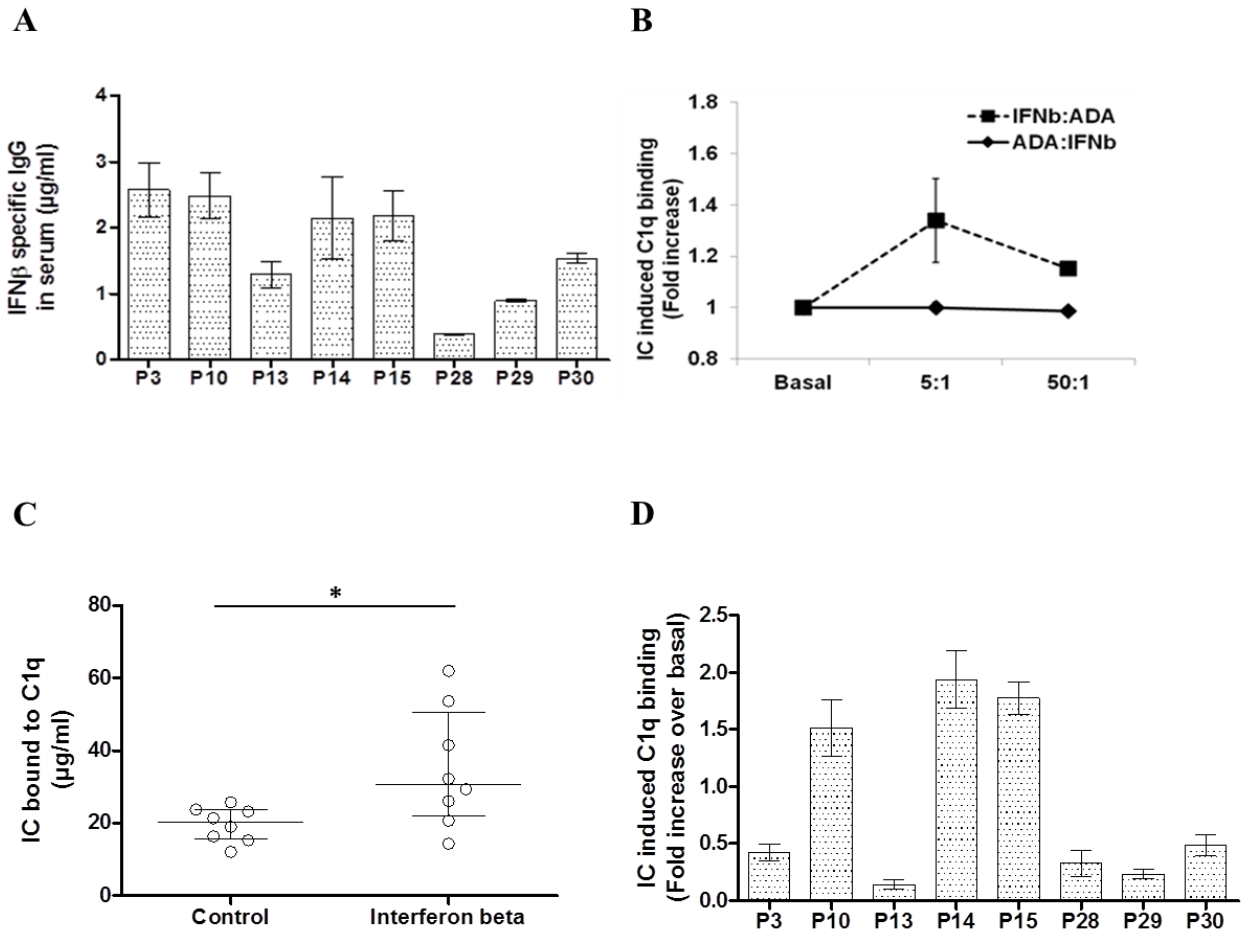


Figure 3.14: IFN-β-1a Rebif™ forms anti-drug antibody immune complexes that bind complement C1q. Figure A represents the IFN-β-1a Rebif™ specific IgG anti-drug antibodies in serum of P3, P10, P13, P14, P15, P28, P29 and P30 samples. Figure B represents Ag [IFN-β-1a Rebif™]:Ab [IFN-β-1a Rebif™ specific anti-drug antibody] ratio at which immune complexes were formed by incubating IFN-β-1a Rebif™ with serum containing IFN-β-1a Rebif™ specific IgG anti-drug antibodies for 1 hour at 37 °C. Figure C and D represents the levels of immune complex bound to C1q in 8 IFN-β-1a Rebif™ specific IgG anti-drug antibody positive P3, P10, P13, P14, P15, P28, P29 and P30 samples. Each open circle represents an individual immune complex bound to C1q. Bars represents the IQR (25^{th} and 75^{th} percentiles) from the median (horizontal line). The medians (25^{th}, 75^{th} percentile) include 20.20 (15.58, 23.65) and 30.80 (22.05, 50.65) for the 2 groups respectively. The data point in each graph is a mean of triplicate measurements and is representative of three independent experiments. Statistically significant differences are shown in panels and comparisons were determined using Mann Whitney U test for two nonparametric groups.

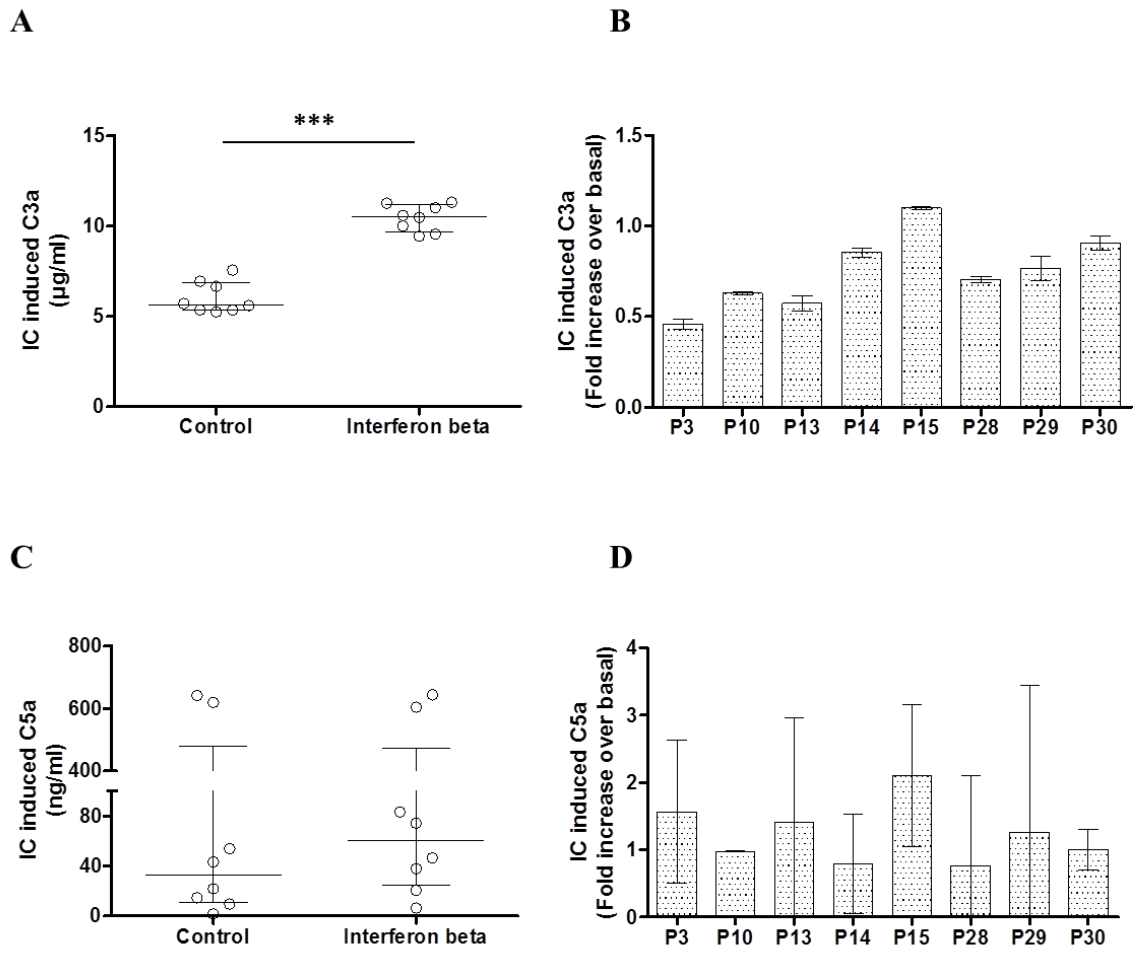


Figure 3.15: IFN-β-1a Rebif™ anti-drug antibody immune complex activates anaphylatoxins C3a and C5a. Figure A and B represent the subsequent changes in the C3a in 8 IFN-β-1a Rebif™ anti-drug antibody positive P3, P10, P13, P14, P15, P28, P29 and P30 serum samples. Each open circle represents an individual immune complex induced C3a. Bars represent the IQR (25^{th} and 75^{th} percentiles) from the median (horizontal line). The medians (25^{th}, 75^{th} percentile) include 5.662 (5.364, 6.887) and 10.54 (9.681, 11.22) for the 2 groups respectively. Figure C and D represent the subsequent changes in the C5a in 8 IFN-β-1a Rebif™ anti-drug antibody positive P3, P10, P13, P14, P15, P28, P29 and P30 serum samples. Each open circle represents an individual immune complex induced C5a. The medians (25^{th}, 75^{th} percentile) include 32.63 (10.93, 478.1) and 60.40 (25.03, 473.9) for the 2 groups respectively. The data point in each graph is a mean of triplicate measurements and is representative of three independent experiments. Statistically significant differences are shown in panels and comparisons were determined using Mann Whitney U test for two nonparametric groups.

3.3.8 Mapping of continuous antibody linear epitopes

To identify the potential antibody linear epitopes, we analysed the binding of IFN-β-1a Rebif™ anti-drug antibodies in five positive P3, P10, P13, P14 and P15 plasma samples (refer Figure 3.17a), one negative N5 plasma sample (refer Figure 3.17b) and one healthy control H25 plasma sample (refer Figure 3.17c) to 32 12mer (5offset) overlapping peptides spanning 166 amino acid residues representing the amino acid sequence of IFN-β-1a Rebif™ molecule (refer 3.16a and b). The overlapping peptide library spanning IFN-β-1a Rebif™ protein was generated. The choice of appropriate peptide length and offset was determined based on previous studies. In general for antibody (or B-cell) epitope mapping, 5 – 20 amino acid length and 1 offset will be ideal. However, we used 12mer and 5 offset in our studies using multipin peptide technology. The binding was considered significant when the OD value was 25 % of the average of overall least OD values plus 2 times standard deviation. According to this type of analysis, significant binding was seen in P10, P13, P14 and P15 at amino acid residues 1-32, 40-62, 70-86, 106-112 and 136-166 respectively. No significant binding was observed either in negative and healthy control plasma samples.

We also used an alternative approach to detect IFN-β-1a Rebif™ antibody linear epitopes using an *in vitro* competitive binding assay. This involves the combination of competitive binding assay and ELISA. In brief the anti-IFN-β-1a Rebif™ antibodies are incubated with IFN-β-1a Rebif™ peptides, the complexes are added to 96 well plate which are pre-coated with IFN-β-1a Rebif™, the unbound anti-IFN-β-1a Rebif™ antibodies are removed (peptide competition with anti-IFN-β-1a Rebif™ antibodies) , the secondary antibody specific to primary antibody which are conjugated with an enzyme is added and developed using chromogenic substrate. For this assay the higher the binding, the weaker is the eventual signal (Morris GE 2007). Therefore, there is IFN-β-1a Rebif™ peptide binding with anti-IFN-β-1a Rebif™ antibodies. The Figure 3.18 indicates no linear binding epitopes present against IFN-β-1a Rebif™ or the method developed for detection of linear epitopes was not specific or sensitive to detect the linear binding IFN-β-1a Rebif™ epitopes.

A

MSYNLLGFLQ	RSSNFQCQKL	LWQLNGRLEY	CLKDRMNFDI	PEEIKQLQQF	QKEDAALTIY
EMLQNIFAIF	RQDSSSTGWN	ETIVENLLAN	VYHQINHLKT	VLEEKLEKED	FTRGKLMSSL
HLKRYYGRIL	HYLKAKEYSH	CAWTIVRVEI	LRNFYFINRL	TGYLRN	

B

1	MSYNLLGFLQRS	9	PEEIKQLQQFQK	17	ETIVENLLANVY	25	HLKRYYGRILHY
2	LGFLQRSSNFQC	10	QLQQFQKEDAAL	18	NLLANVYHQINH	26	YGRILHYLKAKE
3	RSSNFQCQKLLW	11	QKEDAALTIYEM	19	VYHQINHLKTVL	27	HYLKAKEYSHCA
4	QCQKLLWQLNGR	12	ALTIYEMLQNIF	20	NHLKTVLEEKLE	28	KEYSHCAWTIVR
5	LWQLNGRLEYCL	13	EMLQNIFAIFRQ	21	VLEEKLEKEDFT	29	CAWTIVRVEILR
6	GRLEYCLKDRMN	14	IFAIFRQDSSST	22	LEKEDFTRGKLM	30	VRVEILRNFYFI
7	CLKDRMNFDIPE	15	RQDSSSTGWNET	23	FTRGKLMSSLHL	31	LRNFYFINRLTG
8	MNFDIPEEIKQL	16	STGWNETIVENL	24	LMSSLHLKRYYG	32	YFINRLTGYLRN

Figure 3.16: The amino acid sequences. The Figure A represents the amino acid sequence of IFN-β-1a Rebif™ molecule. The Figure B represents 32 12mer 5offset overlapping IFN-β-1a Rebif™ peptides.

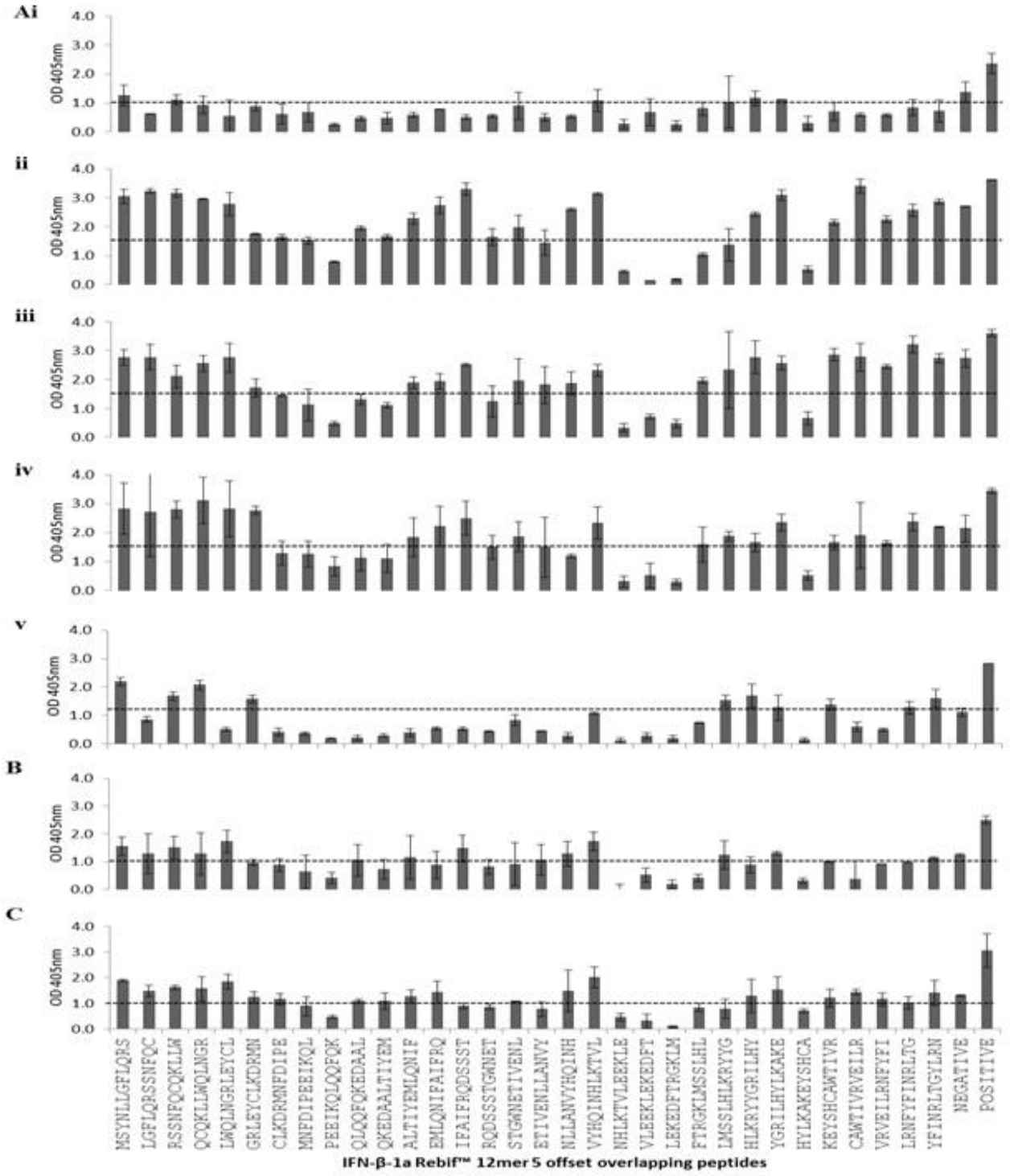


Figure 3.17: Mapping of continuous B-cell linear epitopes using 32 12mer 5 offset overlapping peptides representing the amino acid sequence of the IFN-β Rebif™ molecule. Figure Ai, ii, iii, iv and v represents IFN-β Rebif™ anti-drug antibody positive plasma samples from P3, P10, P13, P14 and P15 for continuous B-cell epitope mapping using multipin peptide technology. Figures B and C represent IFN-β Rebif™ anti-drug antibody negative plasma sample from N5 and healthy control plasma sample from H24 as controls for B-cell epitope mapping. No significant binding was seen in N5 or H25 plasma samples. The plasma samples were diluted at 1:1000. The control peptides included negative peptide GLAQGGGG and positive peptide PLAQGGGG. However, the negative control peptide cannot truly be considered as negative control as the peptide is binding to the antibody. The OD was measured at 405nm.

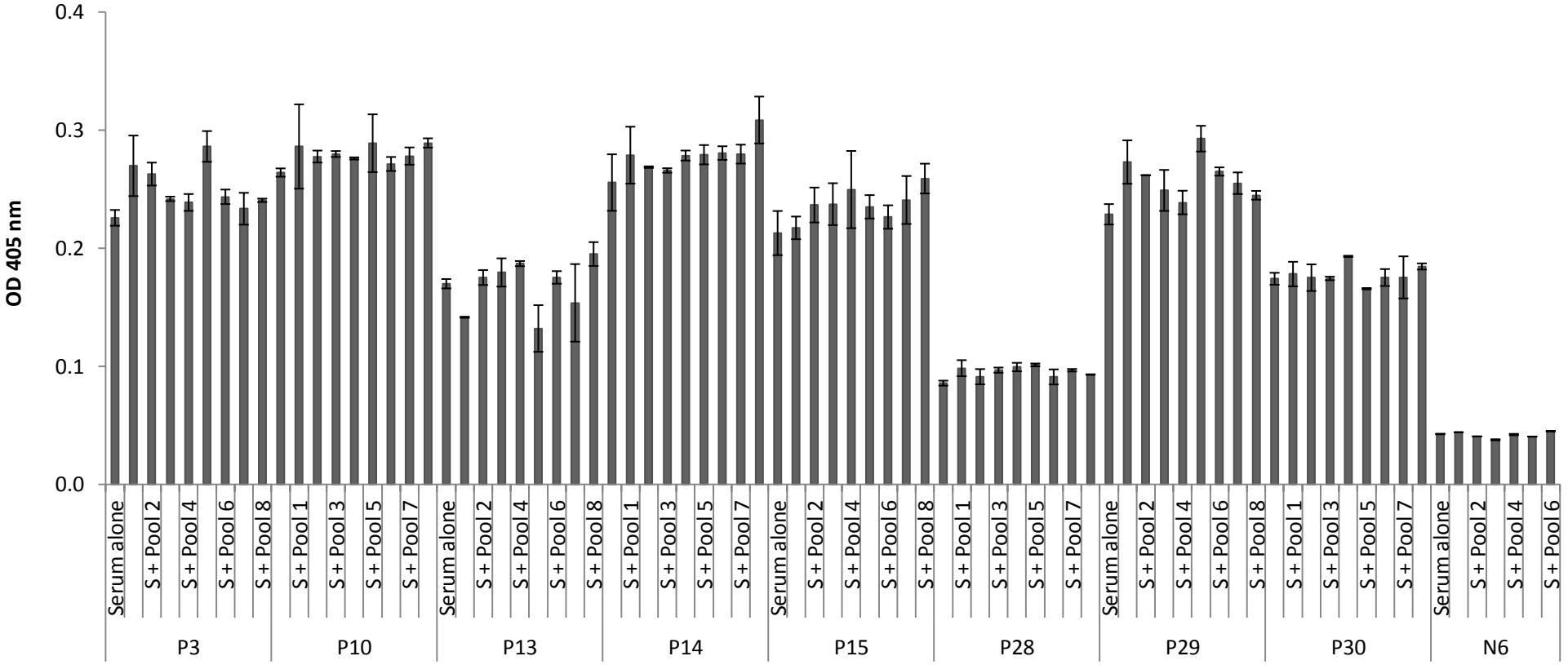


Figure 3.18: Linear binding epitopes detection in anti-IFN-β-1a Rebif™ antibodies. *In vitro* competitive binding approach was designed and developed for detection of linear binding IFN-β-1a Rebif™ epitopes in anti-drug antibodies positive serum samples P3, P10, P13, P14, P15, P28, P29 and P30. Serum from respective anti-drug antibodies positive samples containing anti-drug antibodies without IFN-β-1a Rebif™ peptides was used as controls. One anti-IFN-β-1a Rebif™ negative donor N6 was also used as a control. The OD was measured at 450 nm.

3.4 Discussion

The results presented in Figure 3.6 indicate the predominant immunoglobulin isotype of IFN-β Rebif™ anti-drug antibodies is IgG isotype, with no detectable levels of IgM. However, the detection of IgM isotype after 3 months of IFN-β treatment has been reported, but this was gradually replaced by IgG after 6 months of treatment (Di Marco et al. 2006). In our studies the plasma samples were obtained from individuals who had been on IFN-β Rebif™ treatment for several years (refer to Table 3.1 for demographic and clinical features of patients enrolled for our studies). It is possible that IgM might have been part of early phase of an IFN-β-1a Rebif™ immunogenic response but have switched to IgG in our samples (Heyman 2000). The data presented in Figure 3.7 demonstrate the distribution of IFN-β Rebif™ IgG subclass specific anti-drug antibodies in anti-drug antibody positive plasma samples. The data also show the presence of both IgG1 and IgG4 anti-drug antibodies contributing to IFN-β-1a Rebif™ anti-drug antibody profiles. The studies have shown predominance of IgG4 anti-drug antibodies against IFN-β-1a formulations (Gibbs & Oger 2007) and IgG2 and IgG4 anti-drug antibodies to IFN-β-1b formulation (Porter 2001). The observations are also similar to other treatments such as glatiramer acetate for multiple sclerosis where the predominant IgG subclass isotype was IgG4 anti-drug antibodies (Basile et al. 2006; Farina et al. 2002). It is also suggested that IgG4 anti-drug antibodies can be associated with increased neutralisation potential. Our studies in this chapter also attempt to demonstrate that IFN-β-1a Rebif™ IgG subclass specific anti-drug antibodies contribute to neutralisation potential.

In order to assess the neutralisation potential in our plasma samples an IFN-β-1a Rebif™ induced MxA neutralisation bioassay was developed. Myxovirus resistance protein A (MxA) expression is the established biomarker for IFN-β bioactivity (Pachner et al. 2005). Figure 3.9 demonstrates the immunoblots for neutralisation potential of IFN-β-1a Rebif™ anti-drug antibodies in all the plasma samples. Figure 3.10 demonstrates the inter-individual variation in neutralisation potential of IFN-β-1a Rebif™ anti-drug antibodies. The IgG4 was depleted from the IFN-β-1a Rebif™ anti-drug antibody positive plasma samples. The variations in the obtained plasma fractions were adjusted according to the IgG1 subclass. The neutralisation potential in total plasma, adjusted IgG4 depleted plasma and unadjusted IgG4 depleted plasma samples was determined using the IFN-β-1a Rebif™ induced MxA neutralisation bioassay. IgG4 antibodies have unique properties such as half-antibody exchange reactions because of two different Fabs arms rendering them bi-specific and hence functionally

monovalent for given antigen in circulation (Aalberse & Schuurman 2002). The evidence suggests that monovalent binding of IgG4 caused less neutralisation (Hofmeister Y et al 2011). Hence, in our study these IgG4 antibodies might possess less neutralisation capabilities. The IgG4 structure and function implicates to exhibit diverse functional mechanisms in vitro and in vivo. The cytokines such as IL-4, IL-10 and IL-12 promote IgG4 production and is dependent on the stimulating antigen (Nirula et al. 2011). Hence, evaluating cytokines can provide insights into immunogenicity reactions. The evidence also suggests prolonged exposure to allergens causes increase allergen-specific IgG4 response and shown T helper 2 cytokines such as IL-4, IL-5 and IL-13 in PBMCs cultures (Aalberse RC et al 1983, Akdis CA et al 1998). The densitometric analyses of the immunoblots show that the IgG4 anti-drug antibodies are likely to be the major contributor to the neutralisation potential (see Figure 3.12). However, this has to be investigated further using increased number of samples and with purified IFN-β-1a Rebif™ specific IgG4. Furthermore, longitudinal studies are required to better understand the evolution of IgG subclass specific anti-drug antibodies and their neutralisation potential.

The interaction of neutralising anti-drug antibodies against therapeutic proteins thrombopoietin and epoetin with endogenous protein has been studied (Bennett et al. 2004; Li et al. 2001). The data suggest that the antibodies have the ability to cross react with endogenous proteins and therefore compromise their normal physiological functions. The studies on therapeutic IFN-β have also shown cross reactivity with endogenous protein (Bertolotto et al. 2000; Khan & Dhib-Jalbut 1998). The recent reports also show IFN-β neutralising antibodies are able to neutralise fibroblast derived endogenous IFN-β (Shapiro et al. 2006; Sominanda et al. 2010). In our study we have shown in an in vitro model that IFN-β-1a Rebif™ specific neutralising antibodies were able to neutralise the endogenous IFN-β bioactivity in THP-1 cells. The endogenous IFN-β activity was not completely neutralised when compared to therapeutic IFN-β, suggesting the amounts of endogenous IFN-β produced may be too high to be completely neutralised by their anti-drug antibodies. To test this possibility, the amounts of IFN-β produced by LPS/PIC activated THP-1 cells need to be quantified. The other possibility to explore is that the epitopes of both endogenous IFN-β and therapeutic IFN-β might differ in terms of recognition by neutralising anti-drug antibodies and this requires further investigation. The potential consequences of neutralisation of endogenous IFN-β include susceptibility to viral infections and an impact on the normal physiological functions of endogenous IFN-β in various organs including the CNS. This is of

particular importance considering the fact that the high titres of neutralising antibodies to IFN-β were found to last for many years after termination of IFN-β treatment (Hegen et al. 2012; Sorensen et al. 2005b).

The antigen-antibody based immune complex can initiate the classical complement activation cascade with the release of complement anaphylatoxins C3a and C5a. The data shown in Figure 3.14 and 3.15 demonstrates that the interaction of anti-drug antibodies with IFN-β-1a Rebif™ resulted in the formation of immune complex and activated complement cascade following binding of C1q and subsequently C3a and C5a. C3a and C5a are implicated in enhancing antigen processing and presentation and also in the maintenance of tolerance (Hegen et al. 2012; Strainic et al. 2008; Weaver et al. 2010). Complement activation enhances antigen uptake, processing and presentation and might be the major contributing factor in the initiation of the immunogenic response to biologics. Complement activation could also potentially favour the formation of anti-drug antibodies with increasing neutralising potential. The release of C3a and C5a enhance the function of DCs and generate Th1 cells. The effects are mediated via increased expression of co-stimulatory molecules CD80 and CD40 (Sacks & Zhou 2012). Furthermore, CD21 (complement receptor 2) expression on B cells enhances the processing of macromolecular antigens in immune individuals (Boackle, Holers & Karp 1997). One other important observation is that the incidence of anti-drug antibodies in subcutaneous Rebif™ and intramuscular Avonex™ is significantly different. The cause for this difference may be due to the ability of the immune complex between the injected Rebif™ and anti-drug antibodies to activate complements in the subcutaneous interstitium and gain access to antigen presenting cells such as Langerhans cells and dermal dendritic cells. The interactions between the immune complexes, activated complement and skin antigen presenting cells can enhance antigen processing and presentation of the therapeutic protein. This could also be the mechanism by which low affinity and non-neutralising antibodies lead to the development of neutralising antibodies through immune complex formation and antigen processing or presentation. In this study we also observe that patient P29, who had limited or no neutralising antibodies, was able to form immune complex and activated complement in presence of IFN-β-1a Rebif™. Based on this observation we also speculate that if a patient with non-neutralising antibodies continues to receive IFN-β treatment, immune complexes can be formed which through efficient antigen processing and presentation together with epitope spreading, may cause formation of neutralising antibodies. The complements are also altered in relapsing remitting multiple

sclerosis disease (Boos et al. 2004; Ingram et al. 2012). In our study we observed that complement activation by IFN-β-1a Rebif™ in anti-drug antibody positive samples increases the possibility that repeated administration of IFN-β-1a Rebif™ to anti-drug antibody positive patients can cause disturbed complement status. The impact of such complement activation on disease progression is still unclear and therefore requires further investigations.

In order to further understand the binding patterns for IFN-β-1a Rebif™ anti-drug antibodies and receptor binding sites on IFN-β-1a Rebif™ molecule we also attempted to identify the binding regions on IFN-β-1a Rebif™ molecule and to examine if the neutralising antibody positive samples bind to the epitopes that are identical to the domains responsible for receptor binding. The B-cell epitope mapping was established in P3, P10, P13, P14 and P15 with differential amounts of IFN-β-1a Rebif™ anti-drug antibodies. Our preliminary data suggest the epitopes for antibodies against IFN-β-1a Rebif™ may be located on residues 1-32, 40-62, 70-86, 106-112 and 136-166 respectively. However, the signals obtained in experimental results are not robust enough to make a definite conclusion. Immunoglobulin purification from serum or plasma before subjecting samples to the peptide pin assay may increase the signal. Human plasma is a mixture of many antibodies and hence we can expect to see binding to many peptides and therefore it is important to establish antibody specificity. In order to establish this specificity affinity purified antibodies are required. One other possible strategy is to use the peptide pins to affinity purify antibodies and then elute the antibodies from the pins that test each antibody for binding to the IFN-β-1a Rebif™ molecule. The major limitation of this set-up is that data are limited because of linear epitopes. It is difficult to establish evidence that antibodies binding to linear epitopes in the IFN-β Rebif™ molecule will recognise the same in endogenous IFN-β. The negative control peptide used in this set-up cannot truly be considered as negative control as the peptide is binding to the antibody. An alternative strategy for identifying the binding patterns for IFN-β-1a Rebif™ anti-drug antibodies the *in vitro* competitive binding assay was also utilised. However, the data obtained indicate there are no linear binding epitopes in anti-IFN-β-1a Rebif™ antibodies or the method developed for the same was not specific or sensitive to achieve the objective. Further optimisation of peptide: anti-drug antibodies ratio mix (eg. using checkerboard titration) needs to be performed to rule this out as a potential cause for failure to reveal linear epitopes. Several studies have used peptide competitive binding assays to detect B-cell epitopes for examples *Mycobacterium tuberculosis* protein and fumonisin-a mycotoxin found in food (Yang H et al 2013, Liu X et al 2013). Some other epitope mapping techniques are

available to identify linear or conformational epitopes. X-ray crystallography (Amit et al. 1986), nuclear magnetic resonance (Anglister et al. 1995) and electron microscopy (Belnap et al. 2003; Dore et al. 1988) can identify the contact amino acid residues on antibody-antigen complexes.

In this study we characterised neutralising capabilities, the IgG subclass profile, cross-reactivity to endogenous IFN-β and have also shown complement activation in multiple sclerosis patients. An attempt has also been made to identify the binding regions on the IFN-β Rebif™ molecule. Clear understanding of the evolution of anti-drug antibody formation and relevant immune responses can inform the development of better clinical approaches and regimens for biologics treatment.

3.5 Conclusions

- IgG4 and IgG1 contribute to IFN-β neutralising anti-drug antibodies profile.
- Neutralising IFN-β anti-drug antibodies cross reacts and blocks endogenous IFN-β activity.
- IFN-β anti-drug antibodies results in immune complex formation and complement activation.

Chapter 4: Role of T helper cells in anti-drug antibody development to Interferon beta in multiple sclerosis patients

4.1 Introduction

The previous chapter focused on humoral immune responses to therapeutic IFN-β. In this chapter we address the role of T helper cells in anti-drug antibodies development to IFN-β. Several studies have reported the clinical link between the existence of T helper cell epitopes, the titre and persistence of anti-drug antibodies for several therapeutic proteins (Barbosa et al. 2006; Koren et al. 2007; Tatarewicz et al. 2007). For example as in the case of recombinant fusion protein FPX, which consists of two similar biologically active peptides bound to human Fc fragment and recombinant-methionyl human glial cell line-derived neurotrophic factor (r-metHuGDNF) (Koren et al. 2007; Tatarewicz et al. 2007). There has also been some indication that T helper cells are involved in anti-drug antibodies development to therapeutic IFN-β (Barbosa et al. 2006). The anti-drug antibodies that are of high-affinity, high titre and IgG class is usually indicative of T helper cell involvement (Baert et al. 2003; Casadevall et al. 2002; Li et al. 2001; Swanson et al. 2004). Protein therapeutics that contain within them MHC class II restricted helper T-cell epitopes are more likely to elicit anti-drug antibodies. In brief the protein therapeutic could be taken up by antigen presenting cell wherein they can be broken down into peptide fragments. The peptide fragments are then presented on the surface of antigen presenting cells (including B cells) as a complex with MHC class II molecules. T helper cells then interact with B cells presenting these peptides on appropriate MHC class II molecules, cause B cell activation and drive B cell maturation which then can secrete antibodies. One of the important determinants of T-cell epitope immunogenicity is the fit or binding strength between T-cell epitope and MHC molecules (Lazarski et al. 2005). Evidence suggests only 2 % of peptide fragments derived from proteins have the exact amino acid side chains that fit into MHC class II binding groove (de Groot et al. 2009).

Several computer algorithms for example Immune epitope database analysis resource (IEDB–AR) (Zhang et al. 2008) are available to map the potential MHC class II restricted T-cell epitopes within proteins. MHC binding peptide prediction methods are classified into three major groups - motif based, statistical or mathematical expression and structure based methods. Structure based methods is considered as the gold standard method for MHC binding peptide prediction and this method calculates the binding energy of peptide – MHC complex and energetically favourable peptides are predicted as binders. The IEDB employs a consensus approach based on Sturniolo et al, automated generation and evaluation of specific

MHC binding predictive method (ARB) and stabilisation matrix align (SMM_align) (Bui et al. 2005; Nielsen, Lundegaard & Lund 2007; Sturniolo et al. 1999). ProPred is another server that employs quantitative matrices derived from the method described by Sturniolo et al. The server also can assist in locating promiscuous HLA-II binding regions on peptides. Promiscuous binding involves identification of antigen or region of antigen that can bind to several HLA alleles. Predicting promiscuous region is important for vaccine development (Singh & Raghava 2001). In silico predictions of T helper cell epitopes have been successfully applied to design vaccines (Ahlers et al. 2001; Reek et al. 2002). The IEDB analysis resource tool utilises consensus approach using NN–align (Nielsen & Lund 2009), SMM–align (Nielsen, Lundegaard & Lund 2007) and combinatorial library methods (Sidney et al. 2008 to predict MHC class II epitopes. Neural network based method (NN–align) is used to recognise the MHC class II binding core and the binding affinity. The stabilisation matrix align (SMM–align) is used to predict peptide: MHC binding affinities. The combinatorial library methods use a pool of peptides libraries to measure the contribution of each amino acid at each of the nine positions at the binding peptide. The IEDB exploits the features of the above three methods to predict T-cell epitopes. Propred use quantitative matrices which include all the fifty one human MHC class II alleles and predicts the promiscuous binding regions preferably for vaccine candidates {Sturniolo, 1999 #1237).

The peptide libraries provide powerful tool in screening of large number of peptides that have a systemic arrangement of amino acids. The peptide libraries are ideal for T-cell assays and can be used for T-cell epitope mapping. Overlapping peptides are ideal for T-cell epitope mapping as T-cell epitopes are short linear peptides from the native protein sequence. The identified T-cell epitopes using in silico technologies should be successfully validated using functional T-cell assays.

T-cell epitopes can be identified using several functional T-cell epitope mapping assays such as Carboxy Fluorescein Succinimidyl Ester (CFSE) T-cell proliferation assay, IFN-γ secretion using ELISpot, cytokine gene expression assay and tritiated thymidine incorporation (3HT) T-cell proliferation assay. Several studies suggest using naive primary T-cell proliferation assay for peptide epitope mapping which identifies the peptide epitope sequences that can elicit T helper cell proliferation (and therefore indicate the potential T helper cell involvement in anti-drug antibodies development). Human leukocyte antigen (HLA) proteins can present peptides for immune recognition (Leckband et al. 2013). Utilising HLA-genotyped PBMCs for T-cell assays can provide critical information of peptide binding

to different HLA haplotypes and identification of HLA allele responsible to elicit T helper cell proliferation. Numerous studies have identified HLA association in the development of anti-drug antibodies against biologics. HLA-DRB1*0401, HLA-DRB1*1601, HLA-DRB1*0408 (Buck et al. 2011; Hoffmann et al. 2008), HLA-DRB1*0701 (Barbosa et al. 2006), HLA-DR2, HLA-DQ6, DQB1*0602, HLA-DR15 (Stickler et al. 2004) for IFN-β, HLA-DQA1*0102, HLA-DR4.1, DQ4, DQA1*0301 (Ohta et al. 1999; Simonney et al. 1985), HLA-DRA-DRB1*1104 (Ettinger et al. 2010) for factor VIII and HLA-DRB1*09-DQB1*0309 for erythropoietin (Praditpornsilpa et al. 2009). Furthermore, Dendritic cell T-cell assay can also be used to screen immunogenicity against biologics, especially when antigen modifies the function to T-cells.

T regulatory cells (Tregs), subset of T-cells, play important role in immune regulation. Deficiencies in the number and function of Tregs are associated with several autoimmune diseases including multiple sclerosis. Tregs regulate autoantibody responses through receptor editing, anergy, follicular exclusion and lack of T-cell help. In addition, the autoreactive B cells can be controlled by Tregs. Several studies have identified the association of Tregs with anti-drug antibodies development to biologics (Cousens et al. 2013). The evidence also suggests IFN-β treatment can restore Tregs function in multiple sclerosis (Korporal et al. 2008).

The aim of this study was to identify IFN-β-1a Rebif™ T helper cell epitopes using T-cell epitope prediction tools and functional assays. We utilised T-cells from multiple sclerosis patients, healthy volunteers and HLA genotyped cell archive from healthy volunteers. Furthermore, the study also investigated any protential correlation between the anti-drug antibody response to IFN-β-1a Rebif™ and the frequency and/or functional activity of Tregs cells.

4.2 Methods

The flow diagram of the methods employed in this chapter is shown in Figures 4.1 and 4.2.

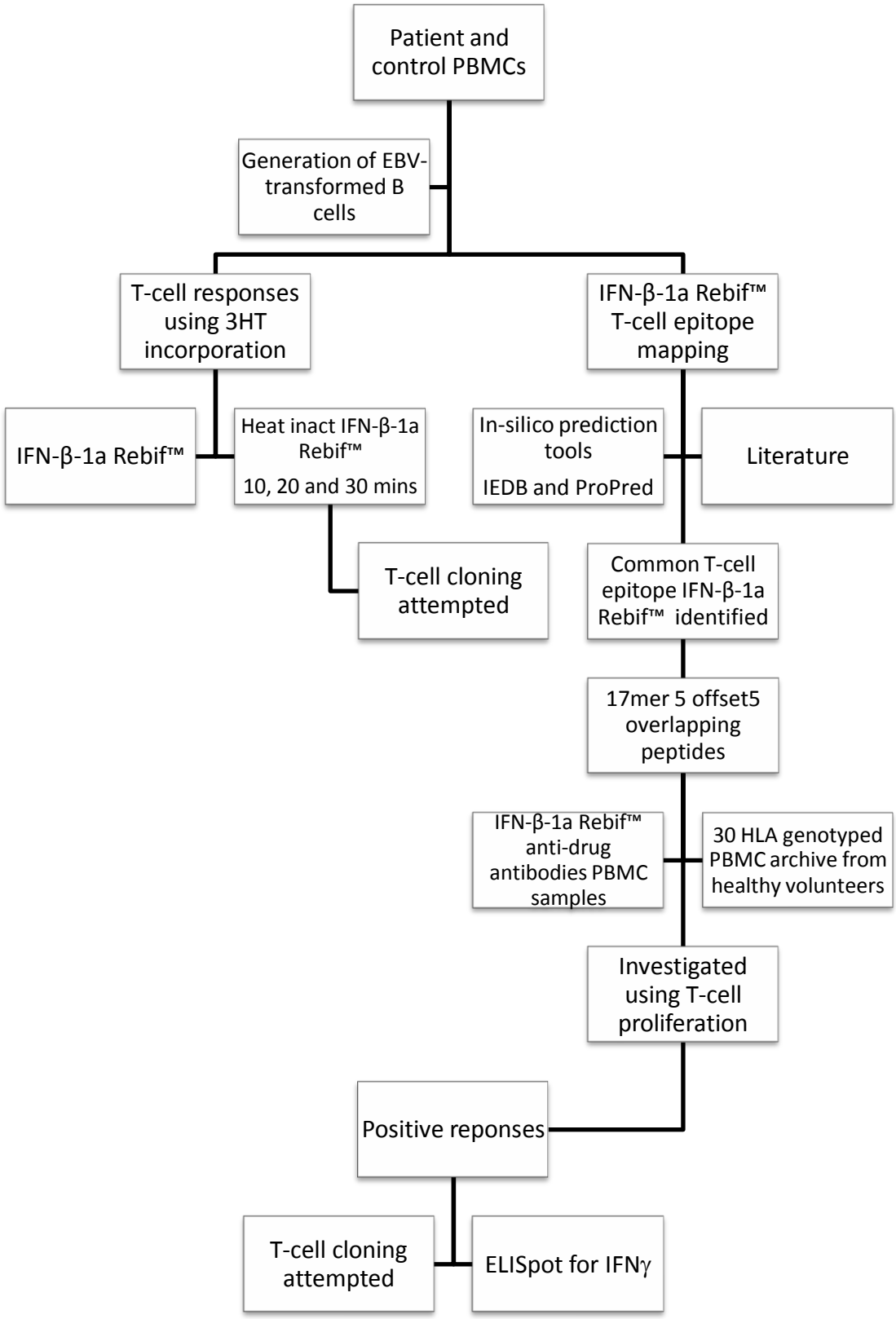


Figure 4.1: The schematic representation of methods employed in T-cell study. Abbreviations: PBMCs – Peripheral blood mononuclear cells; EBV – Epstein-Barr virus; 3HT – 3H-thymidine, IFN – Interferon; IEDB – Immune epitope database; HLA – Human leukocyte antigen; ELISpot – Enzyme-linked immunospot

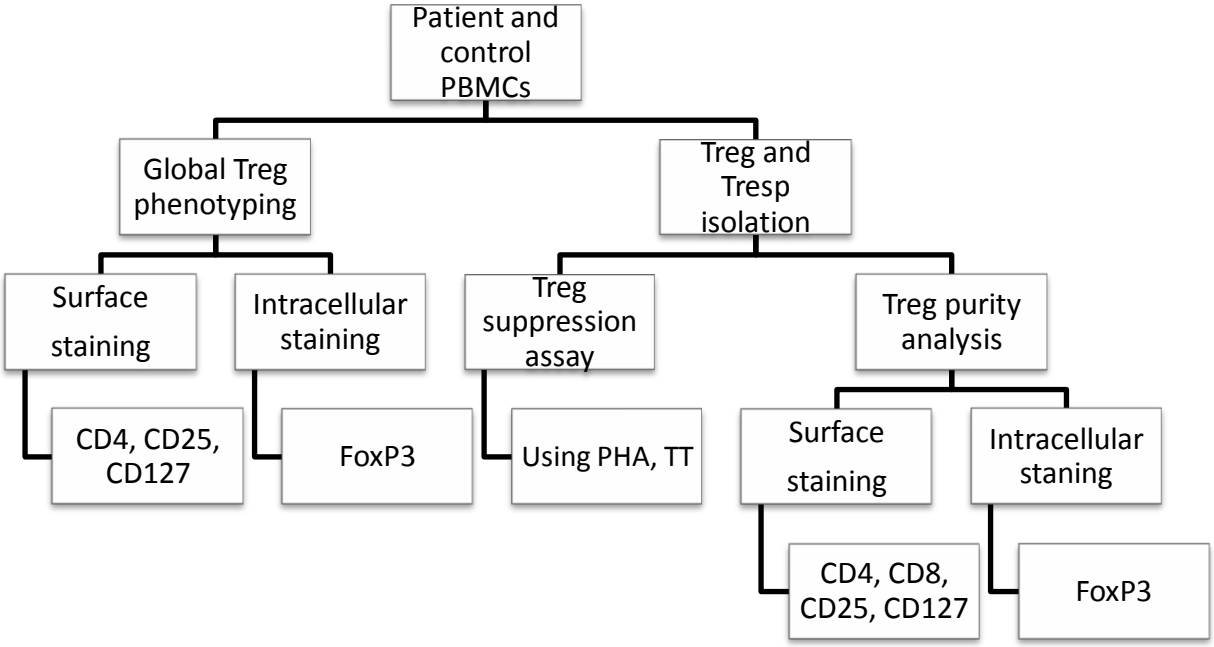


Figure 4.2: The schematic representation of methods employed in Treg study. Abbreviations: PBMCs – Peripheral blood mononuclear cells; Treg – Regulatory T-cells; Tresp – Responder T-cells, CD – Cluster of differentiation; PHA – Phytohemagglutinin; TT – Tetanus toxoid

4.2.1 Chemicals and reagents

Please refer to Section 3.2.1 for details.

4.2.2 Whole blood samples

Please refer to Section 3.2.2 for details.

4.2.3 Human leukocyte antigen genotyped donor details

Department of Molecular and Clinical Pharmacology, University of Liverpool, UK has an established HLA genotyped cell archive from 400 healthy volunteers (Alfirevic et al. 2012). This cell bank was generated from blood collected from the volunteers from which PBMCs and deoxyribonucleic acid (DNA) were isolated. The high-resolution sequence based HLA typing was performed from Histogenetic labs, NY, USA at the following loci HLA – A, – B, – C, – DRB1 and – DQB1. Peripheral blood mononuclear cells from ten randomly chosen were used in IFN-β-1a Rebif™ study (refer Table 4.1).

Donor Identity	Age	Sex	HLA–A	HLA–B	HLA–C	HLA–DRB1	HLA–DQB1
1	45	F	A*2402 A*2601	B*3503 B*5501	C*0303 C*1203	DRB1*0101 DRB1*1401	DQB1*0501 DQB1*0503
2	28	F	A*0201 A*2402	B*0702 B*2705	C*0202 C*0702	DRB1*1501 DRB1*0401	DQB1*0301 DQB1*0602
3	29	F	A*0201 A*0301	B*4001 B*4001	C*0304 C*0304	DRB1*0401 DRB1*0401	DQB1*0302 DQB1*0302
4	21	F	A*0101 A*1101	B*0801 B*4002	C*0202 C*0701	DRB1*0301 DRB1*1301	DQB1*0201 DQB1*0603
5	25	M	A*0302 A*0301	B*4001 B*4402	C*0304 C*0501	DRB1*0401 DRB1*1001	DQB1*0302 DQB1*0501
6	25	F	A*0101 A*3101	B*0801 B*3501	C*0401 C*0701	DRB1*0103 DRB1*1302	DQB1*0501 DQB1*0604
7	43	M	A*0301 A*3101	B*0702 B*0702	C*0702 C*0702	DRB1*1501 DRB1*0801	DQB1*0402 DQB1*0602
8	25	F	A*1101 A*2601	B*0702 B*4001	C*0304 C*0702	DRB1*1601 DRB1*0901	DQB1*0303 DQB1*0502
9	27	F	A*0301 A*3301	B*4403 B*5101	C*0202 C*1502	DRB1*0102 DRB1*0402	DQB1*0302 DQB1*0501
12	22	F	A*0101 A*0201	B*1501 B*4402	C*0102 C*0501	DRB1*0401 DRB1*0404	DQB1*0302 DQB1*0301
11	57	F	A*0201 A*0101	B*4403 B*4402	C*0704 C*1601	DRB1*0101 DRB1*0701	DQB1*0201 DQB1*0501
12	52	F	A*0101 A*0201	B*0801 B*4402	C*0501 C*0701	DRB1*0301 DRB1*0401	DQB1*0201 DQB1*0301
13	21	F	A*0201 A*2501	B*4402 B*4402	C*0501 C*0501	DRB1*0401 DRB1*0401	DQB1*0301 DQB1*0301
14	56	F	A*1010 A*0201	B*0801 B*4002	C*0202 C*0701	DRB1*0401 DRB1*0701	DQB1*0302 DQB1*0303
15	19	F	A*0201 A*2402	B*1501 B*4402	C*0102 C*0501	DRB1*0101 DRB1*0301	DQB1*0201 DQB1*0501
16	21	F	A*0101 A*0101	B*0801 B*1402	C*0701 C*0802	DRB1*0301 DRB1*0301	DQB1*0201 DQB1*0201

17	46	F	A*0101 A*0201	B*1801 B*3701	C*0602 C*0701	DRB1*1501 DRB1*1101	DQB1*0301 DQB1*0602
18	18	M	A*1101 A*2402	B*1502 B*1502	C*0801 C*0801	DRB1*1501 DRB1*1201	DQB1*0301 DQB1*0601
19	25	M	A*0101 A*2402	B*3701 B*4006	C*0602 C*1502	DRB1*1501 DRB1*1404	DQB1*0503 DQB1*0601
20	24	F	A*2402 A*2901	B*0705 B*2707	C*0702 C*1502	DRB1*1501 DRB1*1101	DQB1*0301 DQB1*0301
21	21	M	A*0101 A*0222	B*1401 B*5101	C*1203 C*1505	DRB1*1104 DRB1*0401	DQB1*0301 DQB1*0301
22	19	F	A*0201 A*0201	B*1501 B*1801	C*0303 C*1203	DRB1*1602 DRB1*0401	DQB1*0302 DQB1*0502
23	19	M	A*0201 A*0201	B*1501 B*1801	C*0303 C*1203	DRB1*1602 DRB1*0401	DQB1*0302 DQB1*0502
24	24	F	A*0101 A*2601	B*4001 B*5701	C*0304 C*0602	DRB1*0101 DRB1*1301	DQB1*0501 DQB1*0603
25	23	M	A*0101 A*0201	B*0801 B*2701	C*0102 C*0701	DRB1*0103 DBR1*0301	DQB1*0201 DQB1*0301
26	27	N	A*0301 A*3101	B*1501 B*4403	C*0304 C*1601	DRB1*0701 DRB1*0901	DQB1*0201 DQB1*0303
27	20	M	A*0101 A*0205	B*5001 B*5201	C*0602 C*1202	DRB1*1502 DRB1*1101	DQB1*0301 DQB1*0601
28	58	M	A*0101 A*0301	B*0801 B*1402	C*0701 C*0802	DRB1*0301 DRB1*1302	DQB1*0201 DQB1*0609
29	20	M	A*2402 A*6801	B*0705 B*1518	C*0704 C*0702	DRB1*1501 DRB1*0403	DQB1*0302 DQB1*0601
30	24	F	A*0101 A*2601	B*1518 B*4006	C*0704 C*1502	DRB1*1404 DRB1*1404	DQB1*0503 DQB1*0503
31	26	M	A*0101 A*0205	B*0801 B*5001	C*0602 C*0701	DRB1*0301 DRB1*1305	DQB1*0201 DQB1*0301
32	27	M	A*0201 A*2402	B*3501 B*4402	C*0401 C*0501	DRB1*0101 DRB1*0103	DQB1*0501 DQB1*0501
33	19	F	A*0101 A*0201	B*0801 B*3502	C*0401 C*0701	DRB1*0301 DRB1*1104	DQB1*0201 DQB1*0603

34	24	F	A*0201 A*0301	B*1801 B*4402	C*0501 C*0701	DRB1*0401 DRB1*0801	DQB1*0301 DQB1*0402
35	19	M	A*0201 A*2501	B*0702 B*1801	C*0702 C*1203	DRB1*0103 DRB1*1501	DQB1*0501 DQB1*0602
36	18	F	A*0101 A*0101	B*0801 B*5201	C*0701 C*1202	DRB1*1502 DRB1*0301	DQB1*0201 DQB1*0601
37	36	F	A*0101 A*0201	B*0702 B*0801	C*0701 C*0702	DRB1*1501 DRB1*0301	DQB1*0201 DQB1*0602
38	42	F	A*0201 A*2402	B*0702 B*3503	C*0702 C*1203	DRB1*1501 DRB1*0408	DQB1*0304 DQB1*0602
39	24	M	A*0224 A*2601	B*0702 B*0702	C*0702 C*0702	DRB1*1501 DRB1*0701	DQB1*0201 DQB1*0603
40	43	F	A*0101 A*0201	B*0801 B*3501	C*0401 C*0701	DRB1*0101 DRB1*0701	DQB1*0201 DQB1*0501

Table 4.1: Human leukocyte antigen (HLA) and demographic features of 40 Healthy controls involved in this study. The total of 40 randomly selected healthy donors was used for this study. Abbreviation: HLA, human leukocyte antigen

4.2.4 Separation of Peripheral blood mononuclear cells and Plasma

Please refer to Section 3.2.3 for details.

4.2.5 T-cell proliferation assay

Isolated PBMCs were plated in triplicate at a density of 1.5 x 10^5 per well in 96-well U-bottom plates (Nunc; Fisher Scientific, UK). The cultures were incubated with increasing concentration of heat inactivated IFN-β-1a Rebif™ at 37 °C under 95 % air 5 % CO_2 for 5 days. Tritiated-thymidine (0.5 µCi per well) was added for the last 16 hours of culture. IFN-β-1a Rebif™ was heat inactivated by placing it at 100 ^oC for 30 minutes (Stickler et al 2004). Heat inactivation was performed to inhibit the suppressive effect on T-cell proliferation whilst retaining the antigenic properties of IFN-β-1a Rebif™. Cells were harvested onto filter mats using TomTec Mach III, CT, USA and counted in the scintillation counter (Microbeta Trilux, Perkin Elmer, MA, USA). The incorporation of tritiated-thymidine was measured as cpm and the data was processed to calculate the Stimulation index (SI). SI ≥ 1 was accepted as a cut off for antigen-induced proliferation. SI is calculated by dividing the mean cpm of stimulated cultures by the mean cpm of non-stimulated cultures.

4.2.6 Generation of Epstein-Barr virus transformed Lymphoblastoid Cell lines

Lymphoblastoid cell lines were generated by Epstein-Barr Virus (EBV) transformation of the B-lymphocytes component within the peripheral blood lymphocyte population. The generated lymphoblastoid cell lines were used as antigen-presenting cells to evaluate antigen-specificity using T-cell proliferation assay as mentioned in Section 3.13.

B95-8 (marmoset cell line) cells were used as a source of Epstein-Barr virus DNA which exists as circular episomes inside the nucleus of these infected cells 5-800 copies/cell. 5 mL of cell-free and filtered B95-8 supernatant was used to resuspend PBMCs. Cyclosporin-A (CSA) at 1 µg/mL was added to the cells to prevent T-cell growth and the tubes were cultured overnight 37^{o}C, 5% CO_2. After 16 hours, the cells were harvested using centrifugation for 5 minutes at 300 g. Supernatant was discarded and cells were reconstituted in 2 mL of culture medium (RPMI-1640, 10 % v/v FBS, Penicillin 100 IU/mL, Streptomycin 100 µg/mL, HEPES buffer 25 mM, L-glutamine 2 mM) supplemented with 1 µg/mL CSA. Cells were then serially diluted in a 24-well plate; keeping the total volume per well at 1 mL. Cells were fed every 3 days with fresh medium containing CSA (1µg/mL). After 3 weeks the addition of CSA was stopped and cells were expanded using the same culture medium without CSA in 25 cm^2cell culture flasks according to their rate of growth.

4.2.7 Generation of heat-inactivated Interferon beta 1a Rebif™ T-cell clones

The isolated PBMCs from P3 and P10 patients at 1 x 10^6 cells per well were incubated in 48-well plate for 2 weeks in the presence of heat inactivated IFN-β-1a Rebif™.The cultures were incubated at 37^{o}C and 5% CO_2. The cells werefed on days 6 and 9 with complete medium supplemented with (Interleukin) IL-2 at 60 U/mL.

On day 14, heat inactivated IFN-β-1a Rebif™treated cultures were pooled; cells were washed in culture medium (RPMI-1640, 10% v/v pooled heat-inactivated human AB-serum, Penicillin 100 IU/mL, Streptomycin 100 µg/mL, HEPES buffer 25 mM, L-glutamine 2 M, Transferrin 25 µg/mL) and counted using trypan blue dye exclusion. The cells were serially diluted by seeding them in 96-well U-bottom plates in three concentrations 0.3, 1 and 3 cells per well, in culture medium supplemented with IL-2 at 200 U/mL and PHA at 5 µg/mL. Forty five gray irradiated allogeneic lymphocytes were used as feeder cells and added to each well at the concentration of 5x10^4 cells per well, 100 µL volume. Serial dilution cultures were incubated at 37^{o}C, 5% CO_2 for 2 weeks, fed every 48 hours with culture medium supplemented with IL-2 at 60 U/mL.

The cells in the serial dilution plates was restimulated using 45 Gy irradiated allogeneic lymphocytes as feeder cells at 5 x 10^4 cells per well, IL-2 500 U/mL and PHA 10 µg/mL to maintain the T-cell expansion. The restimulated cultures were incubated at 37°C, 5% CO_2 for 2 weeks. The culture medium was supplemented with IL-2 60 U/mL every 48 hours. During this culture period, the well-growing cells were transferred into 96-well U-bottom plates and expanded when required.

The generated T-cells were tested for antigen-specificity using T-cell proliferation assay. The T-cells were incubated in duplicates with and without heat-inactivated IFN-β-1a Rebif™ in presence of 60 Gy irradiated autologous EBV 10^4 cells per well, as antigen presenting cells. Cultures were incubated for 48 hours at 37°C, 5% CO_2 before the addition of 0.5 µCi/well tritiated-thymidine for the last 16 hours of the incubation. The cells were harvested on glass fibre filter mats and proliferation was detected as incorporated tritiated-thymidine using scintillation counter. The SI was calculated and SI $\geq$ 2 was accepted as an antigen-specific cell proliferation and the data was presented as cpm. T-cells shown to respond or proliferate in presence of antigen were further expanded to generate enough cells to further charaterisation. Expansion was carried out by expanding antigen specific cultures in 48-well plates by addition of 45 Gy irradiated allogeneic lymphocytes, IL-2 500 U/mL and PHA 10 µg/mL total volume 1 mL. The cultures were maintained at 37°C, 5% CO_2 and fed every 48 hours with culture medium supplemented with IL-2 60 U/mL.

4.2.8 T-cell epitope mapping

In order to identify immunogenic epitopes of IFN-β-1a Rebif™, a combination of T-cell epitope prediction tools ProPred and Immune epitope database (IEDB) was used. ProPred is a MHC class-II binding peptide prediction server which can predict MHC class-II binding regions in an antigen sequence (Singh & Raghava 2001; Sturniolo et al. 1999). The prediction server will assist in locating promiscuous binding site i.e. region of an antigen that can bind to several HLA alleles. Immune epitope database include T-cell epitope prediction tool and employs a consensus method to predict MHC class-II epitopes built on Sturnilo, ARB and SMM_align (Nielsen, Lundegaard & Lund 2007; Peters et al. 2005). The potential MHC class-II immunogenic sequence of 36 amino acids "HYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN" within IFN-β-1a Rebif™, was identified and overlapping (17mer, 5 offset) IFN-β-1a Rebif™ peptides were synthesised (Severn Biotech Ltd, UK). T-cell proliferation assay was performed using IFN-β-1a Rebif™ peptides as mentioned in Section 3.13. The IFN-β-1a Rebif™ peptides, PBMCs from P3,

P10, P13, P14 and P15 patients were co-cultured for 5 days with 0, 2.5, 5, 10 and 20 µM of IFN-β-1a Rebif™ peptides. T-cell proliferation was detected using tritiated-thymidine incorporation.

4.2.9 Peptides quality checked by Liquid Chromatography-Mass Spectrometry analysis

For LC-MS/MS analysis, aliquots of 1 µL (1 µM) IFN-β-1a Rebif™ peptides were analysed. Samples were delivered into a QSTAR™ Pulsar I-hybrid mass spectrometer (Applied Biosystems) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and 75 µm x 15 cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10 µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% ACN/0.05% TFA (v/v) to 48% ACN/0.05% TFA (v/v) in 60 minutes was applied at a flow rate of 300 nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA; Analyst, Applied Biosystems). Survey scans of 1second were acquired for *m/*z 400-2000, and the 3 most intense ions were selected for MS/MS, with accumulation times of 1second and with a dynamic exclusion of 40seconds. The data were assessed manually to confirm the sequence of peptides. Dr Xiaoli Meng kindly carried out the processing and analysis of samples on LC-MS/MS.

4.2.10 Measurement of Interferon gamma cytokine secretion

ELISpot assays employ sandwich type ELISA technique. The monoclonal or polyclonal antibody specific for the chosen analyte is pre-coated onto a PVDF microtitre plate. The cells and the stimulants are added into the wells and the plate is placed into a humidified 37 ^{o}C CO_2 incubator for a specific period of time. During incubation period the immobilised antibody in presence of secreting cells binds to secreted analyte. After washing the unbound cells and unbound substances, the biotinlyated antibody specific for the chosen analyte is added into the wells. Following a wash any unbound biotinylated antibody will be removed. Alkaline-phosphatase conjugated to steptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution 4-bromo-5-chloro-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) is added. The blue-black coloured precipitate forms and distinct spots appear at the sites of cytokine localisation, with each individual spot representing an individual analyte-secreting cell. The spots are counted using an automated ELISpot reader or using a stereomicroscope.

The ELISpot strips (Millipore; Watford, UK) were activated using 15 µL of 35 % (v/v) ethanol for 1 minute. The strips was washed five times using 250 µL of deionised water. IFN- specific coating antibody was diluted (15 µg/ml) using sterile HBSS. The antibody solution at 100 µL per well was added to ELISpot plate and incubated for 16 hours at 4 ^{o}C. Coating antibodies was removed and the plate was washed five times with sterile HBSS 200 µL per well. 250 µL per well of culture medium was added and incubated for 30 minutes at room temperature. The culture medium was removed and the PBMCs (5 x 10^6 cells/mL, 100 µL) from P3, P10 and P15 were added. IFN-β-1a Rebif™ responsive peptides, PHA 5 µg/ml as positive control and medium alone of 100 µL was added as negative control. The plate was incubated in a 37 ^{o}C humidified incubator with 5 % CO_2 for 24 hours. The cells were removed and the plate was washed for five times with 200 µL per well using sterile HBSS. The biotinylated detection antibody was diluted to 1 µg/mL in HBSS containing 0.5 % FBS. 100µL/well of the detection antibody solution was added to each well and incubated for 2 hours at room temperature. The plate was washed with HBSS 250 µL per well for five times. Streptavidin-ALP was diluted at 1:1000 in HBSS containing 0.5 % FBS 100 µL per well was added and incubated for 1 hour at room temperature. The plate was washed as described before. 100 µL per well of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution was added and developed until distinct spots emerge. The colour development was stopped rinsing the plate extensively in tap water. The plate was left to dry for 16 hours and the spots was counted using ELISpot reader (Autoimmune Diagnostika GmbH; Strassberg, Germany). The ELISpot kit was obtained from Mabtech; Nacka Strand, Sweden.

4.2.11 Global regulatory T-cells phenotyping

Isolated PBMCs (0.2 x 10^6 cells) was surface stained with $CD4^{FITC}$, $CD25^{APC}$ cocktail (eBioscience; Hatfield, UK) and $CD127^{PECy5}$ (Biolegend, Cambridge, UK) anti-human monoclonal antibodies (mAbs) at 4 °C for 20 minutes in the dark. At the end of the incubation, the cells were washed using FACS buffer (PBS + 10 % v/v FCS + 0.02 % v/v sodium azide) followed by fixation and permeabilisation of cells for 45 minutes at 4 °C in the dark. The cells were washed again and intracellular staining was performed using Foxp3-PE and with the PE-isotype control for 45 minutes at 4 °C in the dark. At the end of this incubation, the cells were washed and resuspended in 0.5 mL FACS buffer, for flow cytometric analysis (FACS Calibur, BD Biosciences, UK). On average 60,000 events was captured for analysis. Data were processed using Cyflogic software.

4.2.12 Isolation of regulatory T-cells, responder T-cells and feeder cells

Magnetic-activated cell sorting was used to isolate various cell populations depending upon their surface molecules. The technique allows cells to be separated by incubating with magnetic microbeads coated with antibodies against a particular surface molecule. The cells expressing the surface molecule attach to the magnetic microbeads. The cells solution is transferred on a column placed in a magnetic field. The cells with microbeads expressing the surface molecule stay on the column, while other cells not expressing the surface molecule flow-through. Using this method the cells can be separated positively or negatively. In positive selection the cells expressing surface molecules are attached in the magnetic column and are washed through in the different tube after removing the column from the magnet. Cells labeled with MACS microbeads can be positively selected using MS, LS and XS columns. In negative selection the cells expressing surface molecules which are attached in the magnetic column are not of interest and the flow-through is collected. Cells can be negatively selected efficiently using LD, CS and D columns.

Isolated PBMCs were counted using Trypan blue dye exclusion. The cell suspension was centrifuged at 300 g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 90 µL of Magnetic-activated cell sorting (MACS) buffer (HBSS + 5 % v/v BSA + EDTA 2 mM) per 10^7 total cells. The cells were incubated with 10 µL of $CD4^+$ T-cell biotin-antibody cocktail (Miltenyi Biotech; Surrey, UK) at 4 °C for 10 minutes. 20 µL of anti-biotin microbeads was added and incubated at 4 °C for 15 minutes. At the end of incubation, the cells were washed and resuspended in 0.5 mL of MACS buffer for magnetic separation. Using LD column (Miltenyi Biotech; Surrey, UK) non $CD4^+$ feeder cells and $CD4^+$ cells were separated. $CD4^+$ cells were centrifuged again at 300g for 10 minutes. The supernatant was discarded and the cells were incubated with 10 µL of CD25 microbeads. Using MS column (Miltenyi Biotech; Surrey, UK) Tregs ($CD4^+CD25^{high}$) and Tresp cells ($CD4^+CD25^{low}$) were separated. The separated cells were kept on ice until beforetheir addition to Tregs suppression assay (refer Section 3.21). The obtained cells fractions were surface stained for % of purity and were measured using facs.

4.2.13 Regulatory T-cells suppression assay

Isolated Tregs 5 x 10^4 were co-cultured with 5 x 10^4 Tresp cells at different ratios in the presence of stimulus. The stimulus includes phytohaemagglutinin (PHA) and Tetanus toxoid (TT). Tregs without stimulus shows hypoproliferative or anergic response. Tresp cells alone shows proliferative response to the stimuli. Co-culturing Tregs with Tresp cells results in

reduced proliferation of Tresp cells. The irradiated (45 Gy for 15 minutes) non-$CD4^+$ feeder cells were added at 5 x 10^4 cells per well followed by addition of stimulus. The addition of irradiated non-$CD4^+$ feeder cells effectively stimulated T-cells to proliferate. The cultures were incubated at 37 °C in humidified atmosphere of 95 % air 5 % CO_2 for 3-5 days. Cells were pulsed with 0.5 µCi tritiated-thymidine per well for the last 16 hours of the incubation. The cells were harvested on filter mats and tritiated-thymidine incorporation was measured using scintillation counter. The incorporation of tritiated-thymidine was measured as counts per minute (cpm). The assay was performed with a dilution series ranging from a ratio of 1:1 to 8:1 of Tresp cells: Tregs cells. As additional controls, the Tresp and Treg cells were cultured alone with and without addition of stimulus. The dilution series were carried out in triplicates.

4.2.13 Statistical analysis

Please refer to Section 3.2.13.

4.3 Results

4.3.1 Interferon beta 1a exhibits significant antiproliferative activity

Interferons exhibit several immunomodulatory properties including antiproliferative activity. The studies also indicate interferons have an impact on different phases of mitotic cycle in particular causing G1 phase arrest (Tani et al. 1998).

The overall aim was to investigate the antigenic determinant of IFN-β-1a Rebif™ on healthy donors PBMCs. However, the biologic effect of IFN-β on T-cells (antiproliferative) could prevent achieving this aim. In the first instance, we wished to assess the extent of the antiproliferative effect of IFN-β. We therefore used in vitro tetanus toxoid-stimulated T-cell proliferation assay to measure the antiproliferative effects. In this experiment the PBMCs are stimulated using fixed amount of Tetanus toxoid 0.5 µg/ml to elicit proliferation and different concentrations of IFN-β-1a Rebif™ ranging from 30 – 0.01 ng/ml was added to investigate the antigenic determinant of IFN-β-1a Rebif™ (refer Figure 4.3). The TT is used as routine positive control antigen for T-cell assays and exhibits both humoral and cellular responses in healthy controls. In this experiment we found IFN-β-1a caused antiproliferative effects in dose dependent manner in presence of TT and % of inhibition consisted of approx. 15 – 58 % for all the conditions respectively. The statistical differences were determined using ANOVA with Dunnett's post hoc test. The 3H-Thymidine (3HT) was added to the culture on day 5 and

the proliferation was measured on day 6. Our data suggest IFN-β-1a Rebif™ exhibited significant dose dependent antiproliferative response at all the tested doses.

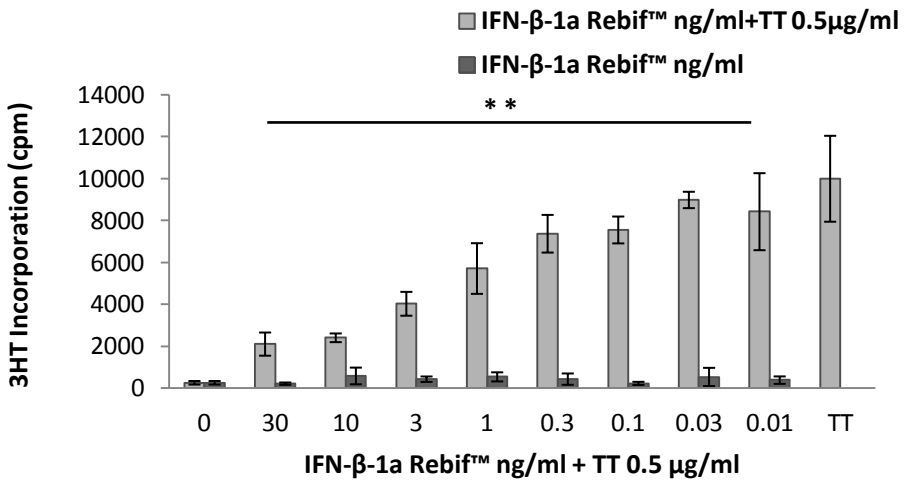


Figure 4.3: IFN-β-1a exhibits significant antiproliferative activity. The Figure 4.3 represents dose dependent proliferative responses of three healthy control PBMCs to research grade IFN-β-1a in presence of Tetanus toxoid. The statistical differences were determined using ANOVA with Dunnett's post hoc test. Tetanus toxoid at 5 µg/ml was used a positive control. The different concentration of IFN-β is indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation. The graph is representative of three independent experiments.

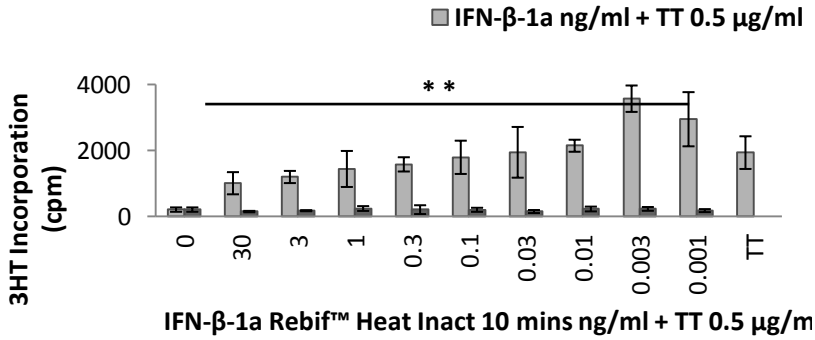
4.3.2 Heat inactivation for 30 minutes at 100 °C ablates antiproliferative effects of Interferon beta 1a Rebif™

Heat inactivation of IFN-β-1a Rebif™ was performed to inhibit the antiproliferative effects on PBMCs whilst retaining the antigenic properties. In our previous experiments (refer Figure 4.3) IFN-β-1a was shown to exhibit antiproliferative properties. Hence, it was not possible to stimulate the PBMCs directly. However, heat inactivation was performed to investigate the relative antigenicity of IFN-β-1a molecule.

In order to determine the ideal time interval for heat inactivation, IFN-β-1a Rebif™ was placed at 100 ^{o}C at 10, 20 and 30 minutes. The heat inactivated IFN-β-1a Rebif™ was used to measure the T-cell proliferation. The PBMCs from a healthy control was co-cultured with different concentrations 30 – 0.001 ng/ml of heat inactivated IFN-β-1a Rebif™ and with fixed amount of Tetanus toxoid 0.5 µg/ml. In Figure 4.4a, the response to 10 minutes heat

inactivated IFN-β-1a Rebif™ exhibited similar response to Figure 4.3 in dose dependent manner in presence of TT and % of inhibition consisted of approx. 20 – 80 % for all the conditions respectively. The maximum proliferation was observed at 0.003 ng/ml of heat inactivated IFN-β-1a Rebif™ plus 0.5 µg/ml TT. Therefore, suggesting the heat inactivation was not effective and has to be performed for longer time interval. The statistical differences were determined using ANOVA with Dunnett's post hoc test. In Figure 4.4b and c the response to 20 and 30 minutes heat inactivated IFN-β-1a Rebif™ exhibited a variable antiproliferative activity. The 20 minutes heat inactivation exhibited approx. 30 – 80 % of inhibition for all the conditions respectively. The 30 minutes heat inactivation exhibited approx. 30 – 80 % of inhibition for all the conditions respectively. However, 30 minutes of heat inactivation of IFN-β-1a Rebif™ ablated the antiproliferative effects on PBMCs and was chosen ideal for establishing T-cell cloning experiments. In all of the experiments 3HT was added to the culture on day 5 and the proliferation was measured on day 6. Our data suggest heat inactivation of IFN-β-1a Rebif™ for 30 minutes at 100 °C ablates the antiproliferative effects on healthy donors PBMCs.

A



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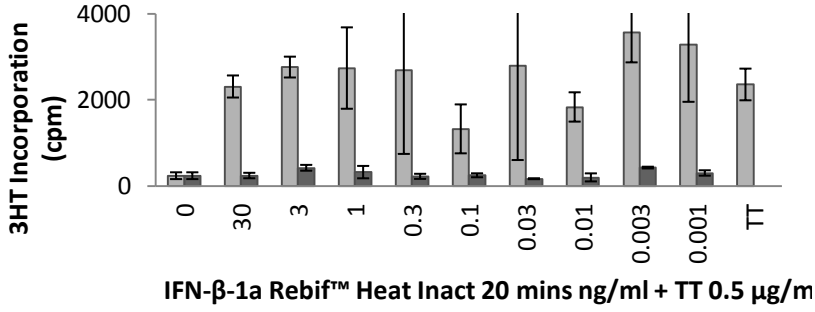

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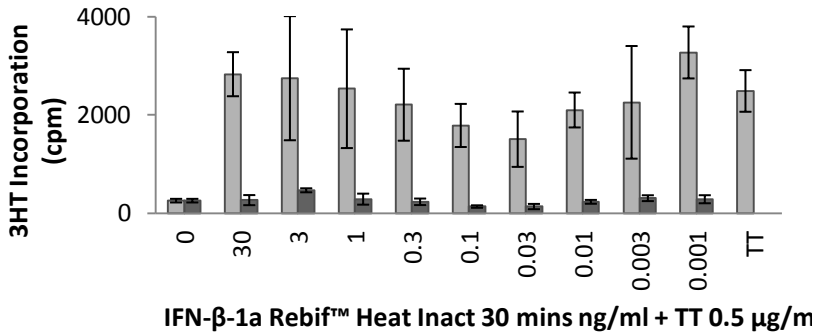


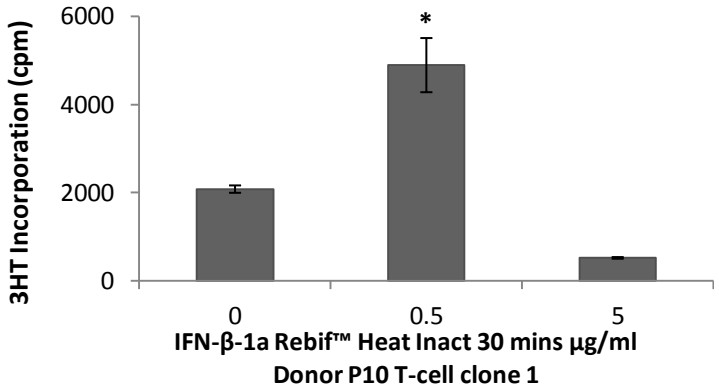
Figure 4.4: Heat inactivated IFN-β-1a Rebif™ exhibit variable antiproliferative activity. The Figure 4.3a, b and c represents the antiproliferative responses of a healthy control PBMCs to IFN-β-1a Rebif™ and proliferative responses to Tetanus toxoid in combination with 10 minutes, 20 minutes and 30 minutes heat inactivated IFN-β-1a Rebif™. The statistical differences were determined using ANOVA with Dunnett's post hoc test. Tetanus toxoid at 5 µg/ml was used a positive control. The different concentration of heat inactivated IFN-β-1a Rebif™ is indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation. Each graph is representative of three independent experiments.

4.3.3 Generation of heat inactivated Interferon beta 1a Rebif™ T-cell clones

In an attempt to understand the cellular basis of IFN-β-1a Rebif™ T-cell responses the anti-drug antibodies positive donors P3 and P10 were chosen for pilot T-cell cloning experiment. T-cell cloning provides the population of T-cells that derives from the same progenitor with unique phenotypic and functional characteristics. The obtained T-cell clone consists of cells with same phenotype and functions. The nature of T-cell response might provide useful insights of T-cells involvement in eliciting immune response. The T-cell cloning was executed using heat inactivated IFN-β-1a Rebif™ and no IFN-β-1a Rebif™ T-cell clones was generated for donor P3 but donor P10 provided two responsive T-cell clones (refer Figures 4.5a and b). The IFN-β-1a Rebif™ T-cell clone 1 and 2 for donor P10 provided maximum proliferation of PBMCs at 0.5 µg/ml to heat inactivated IFN-β-1a Rebif™. The obtained T-cell clones were expanded but failed to further respond and therefore dose dependent response to heat inactivated IFN-β-1a Rebif™ was not achieved. The obtained T-cell clones was tested using autologous B-LCLs as antigen presenting cells and 30 minutes heat inactivated IFN-β-1a Rebif™ was used. The data obtained suggests heat inactivated IFN-β-1a

Rebif™ T-cell clones can be generated and illustrates T-cell involvement in the formation of anti-drug antibodies.

A



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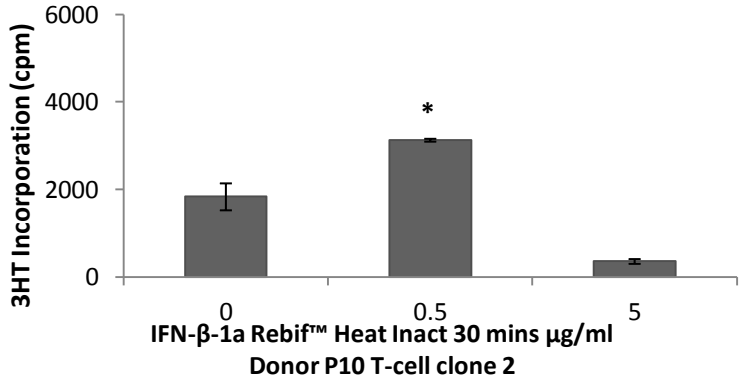


Figure 4.5: Heat inactivated IFN-β-1a Rebif™ specific T-cell clones. The Figures 4.5a and b represents the testing for 30 minutes heat inactivated IFN-β-1a Rebif™ specific T-cell clones obtained from donor P10. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The different concentration of heat inactivated IFN-β-1a Rebif™ is indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation.

4.3.4 Identification of Interferon beta 1a Rebif™ T-cell epitopes using in silico technologies

Understanding the relevance of T-cell activation in anti-drug antibodies remains crucial for predicting and assessing immunogenicity. T-cell epitope is an amino acid or set of amino acids that are capable of being recognised from one or more T-cell receptors. The T-cells

recognise the linear peptides bound to MHC molecules. Several methods have been used to detect T-cell epitopes. In this study we focused on MHC class II molecules. $CD4^+$ T-cells recognise the peptides presented through MHC class II molecules that are expressed on antigen presenting cells. The structural features of MHC class II molecules and the peptides can predispose the T-cell responses. Steinitz et al has identified Factor VIII immunogenic peptides containing $CD4^+$ T-cell epitopes in humanised animal models (Steinitz et al. 2012). $CD4^+$ T-cells epitopes are short linear peptides spanning approx. 11 – 20 amino acids long. Hence, identification of $CD4^+$ T-cells epitopes is therefore considered a rational approach for assessing immunogenicity of the protein therapeutics.

In silico technologies use computational algorithms to identify variant protein sequences that exhibits desired functional properties. The computer algorithms can also identify immunogenic T-cell epitopes. In our study in silico predictions were used to identify MHC class II IFN-β-1a Rebif™ T-cell epitopes using IEDB–AR (Zhang et al. 2008) and ProPred (Singh & Raghava 2001) in conjunction with the published articles (Barbosa et al. 2006) (refer Figures 4.6 and 4.7b). The screenshots represent the MHC class II IFN-β-1a Rebif™ binding sequence for HLA DRB1*1501 allele. However, the assessment of all the available HLA alleles in respective in silico prediction tools was carried out systematically. Low percentile rank in the IEDB–AR indicates good binders. In ProPred the blue colored amino acids indicate good binders whereas the red coloured amino acids suggest the possible promiscuous amino acids. The potential MHC class II immunogenic sequence of 36 amino acids "**HYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN**" within IFN-β-1a Rebif™ (position 130 – 166) was identified.

IEDB–AR MHC class II binding prediction results for IFN-β-1a Rebif™

Allele	#	Start	End	Sequence	Method used	Percentile Rank
HLA-DRB1*1501	1	120	134	LHLKRYYGRILHYLK	Consensus (*smm,nn,sturniolo*)	0.28
HLA-DRB1*1501	1	118	132	SSLHLKRYYGRILHY	Consensus (*smm,nn,sturniolo*)	0.29
HLA-DRB1*1501	1	119	133	SLHLKRYYGRILHYL	Consensus (*smm,nn,sturniolo*)	0.32
HLA-DRB1*1501	1	117	131	MSSLHLKRYYGRILH	Consensus (*smm,nn,sturniolo*)	0.38
HLA-DRB1*1501	1	116	130	LMSSLHLKRYYGRIL	Consensus (*smm,nn,sturniolo*)	0.41
HLA-DRB1*1501	1	145	159	IVRVEILRNFYFINR	Consensus (*smm,nn,sturniolo*)	0.48
HLA-DRB1*1501	1	146	160	VRVEILRNFYFINRL	Consensus (*smm,nn,sturniolo*)	0.49
HLA-DRB1*1501	1	121	135	HLKRYYGRILHYLKA	Consensus (*smm,nn,sturniolo*)	0.51
HLA-DRB1*1501	1	122	136	LKRYYGRILHYLKAK	Consensus (*smm,nn,sturniolo*)	0.56
HLA-DRB1*1501	1	151	165	LRNFYFINRLTGYLR	Consensus (*smm,nn,sturniolo*)	1.55
HLA-DRB1*1501	1	124	138	RYYGRILHYLKAKEY	Consensus (*smm,nn,sturniolo*)	1.62
HLA-DRB1*1501	1	142	156	AWTIVRVEILRNFYF	Consensus (*smm,nn,sturniolo*)	1.74
HLA-DRB1*1501	1	143	157	WTIVRVEILRNFYFI	Consensus (*smm,nn,sturniolo*)	1.74
HLA-DRB1*1501	1	144	158	TIVRVEILRNFYFIN	Consensus (*smm,nn,sturniolo*)	1.74
HLA-DRB1*1501	1	112	126	TRGKLMSSLHLKRYY	Consensus (*smm,nn,sturniolo*)	1.8
HLA-DRB1*1501	1	147	161	RVEILRNFYFINRLT	Consensus (*smm,nn,sturniolo*)	1.93
HLA-DRB1*1501	1	148	162	VEILRNFYFINRLTG	Consensus (*smm,nn,sturniolo*)	1.98
HLA-DRB1*1501	1	125	139	YYGRILHYLKAKEYS	Consensus (*smm,nn,sturniolo*)	2.09
HLA-DRB1*1501	1	126	140	YGRILHYLKAKEYSH	Consensus (*smm,nn,sturniolo*)	2.17
HLA-DRB1*1501	1	127	141	GRILHYLKAKEYSHC	Consensus (*smm,nn,sturniolo*)	2.56
HLA-DRB1*1501	1	152	166	RNFYFINRLTGYLRN	Consensus (*smm,nn,sturniolo*)	2.58
HLA-DRB1*1501	1	18	32	QKLLWQLNGRLEYCL	Consensus (*smm,nn,sturniolo*)	2.92
HLA-DRB1*1501	1	111	125	FTRGKLMSSLHLKRY	Consensus (*smm,nn,sturniolo*)	2.95
HLA-DRB1*1501	1	16	30	QCQKLLWQLNGRLEY	Consensus (*smm,nn,sturniolo*)	3.26
HLA-DRB1*1501	1	15	29	FQCQKLLWQLNGRLE	Consensus (*smm,nn,sturniolo*)	3.31
HLA-DRB1*1501	1	17	31	CQKLLWQLNGRLEYC	Consensus (*smm,nn,sturniolo*)	3.38
HLA-DRB1*1501	1	114	128	GKLMSSLHLKRYYGR	Consensus (*smm,nn,sturniolo*)	3.38
HLA-DRB1*1501	1	19	33	KLLWQLNGRLEYCLK	Consensus (*smm,nn,sturniolo*)	3.43
HLA-DRB1*1501	1	20	34	LLWQLNGRLEYCLKD	Consensus (*smm,nn,sturniolo*)	3.43

Figure 4.6: Screenshot of IEDB–AR MHC class II prediction tools. The Figure represents the IEDB–AR MHC class II binding results for IFN-β-1a Rebif™ molecule exclusively for HLA DRB1*1501 allele. Low Percentile Rank in the table indicates good binders.

ProPred MHC class II binding prediction results for IFN-β-1a Rebif™

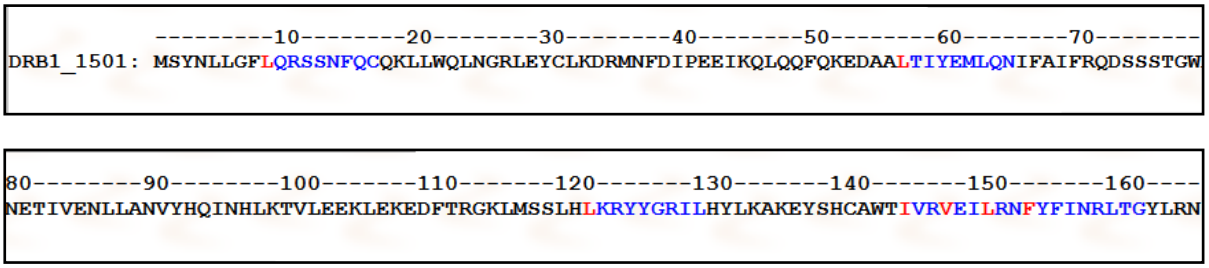


Figure 4.7: Screenshots of ProPred MHC class II binding prediction tools. The Figure represents the ProPred MHC class II binding results for IFN-β-1a Rebif™ molecule exclusively for HLA DRB1*1501 allele. The amino acids in blue colour indicate good binders and amino acids in red indicate the possible promiscuous amino acids.

4.3.5 T helper cells responses to anti-Interferon beta 1a Rebif™ derived peptides with predicted immunogenic potential

In order to assess the T-cell involvement in anti-IFN-β-1a Rebif™ antibody response, antigen specific responding T-cells in PBMCs, from anti-IFN-β-1a Rebif™ antibody positive donors, negative donors and healthy donors was investigated. The overlapping (17mer, 5 offset) IFN-β-1a peptides from previously identified MHC class II immunogenic sequence were synthesised.

Peptide 1	HYLKAKEYSHCAWTIVR	17mer	5 offset
Peptide 2	KEYSHCAWTIVRVEILR	17mer	5 offset
Peptide 3	CAWTIVRVEILRNFYFI	17mer	5 offset
Peptide 4	VRVEILRNFYFINRLTG	17mer	5 offset
Peptide 5	LRNFYFINRLTGYLRN	16mer	5 offset

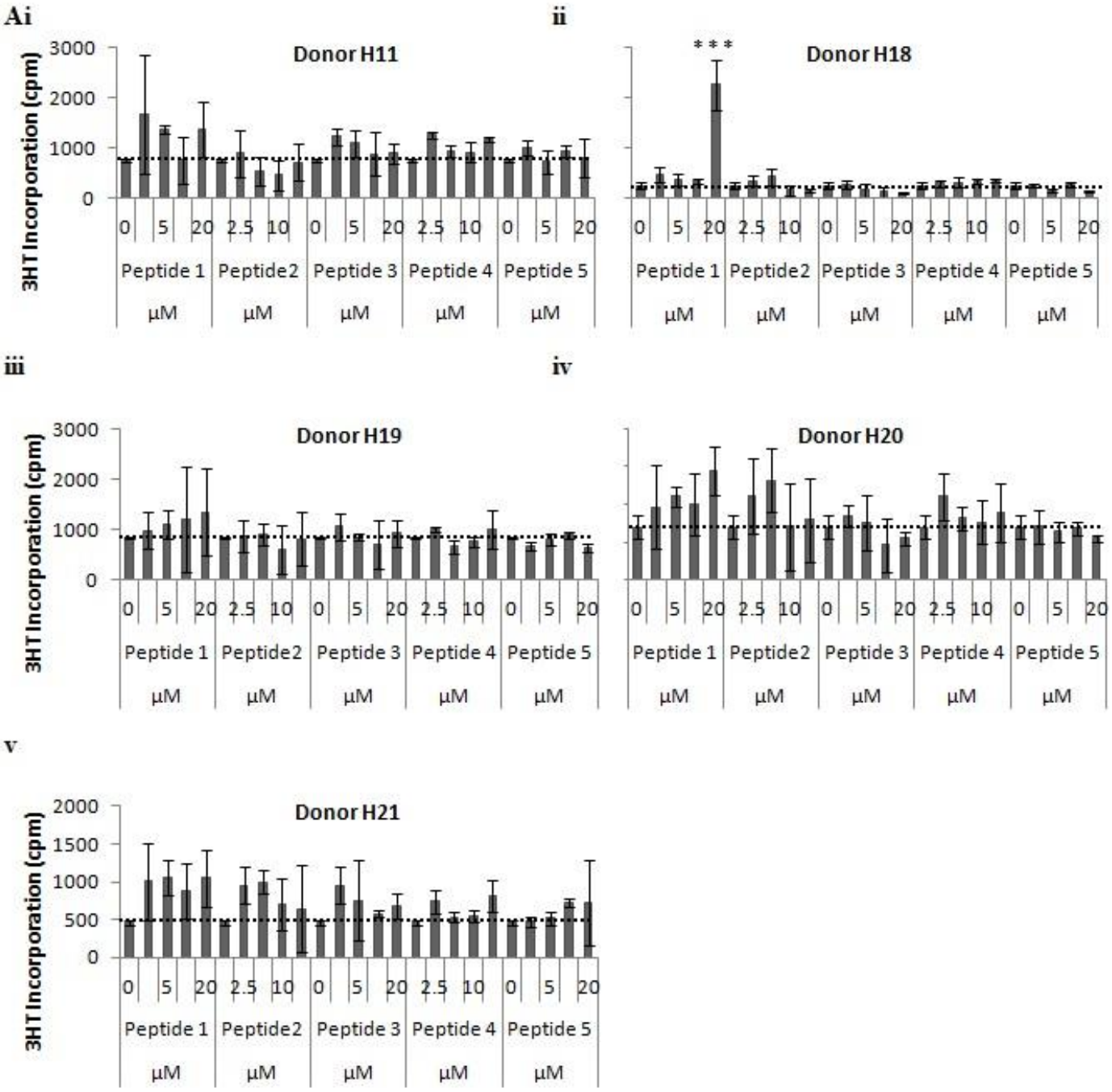
The synthesised IFN-β-1a Rebif™ peptides were dissolved in 50 % acetic acid in R9 medium as per instructions. Hence, the solubilisation and sequence of IFN-β-1a Rebif™ peptides were examined using LC–MS before executing T-cell proliferation assays (refer Appendix 8) for ion current chromatograms of identified IFN-β-1a Rebif™ peptides).

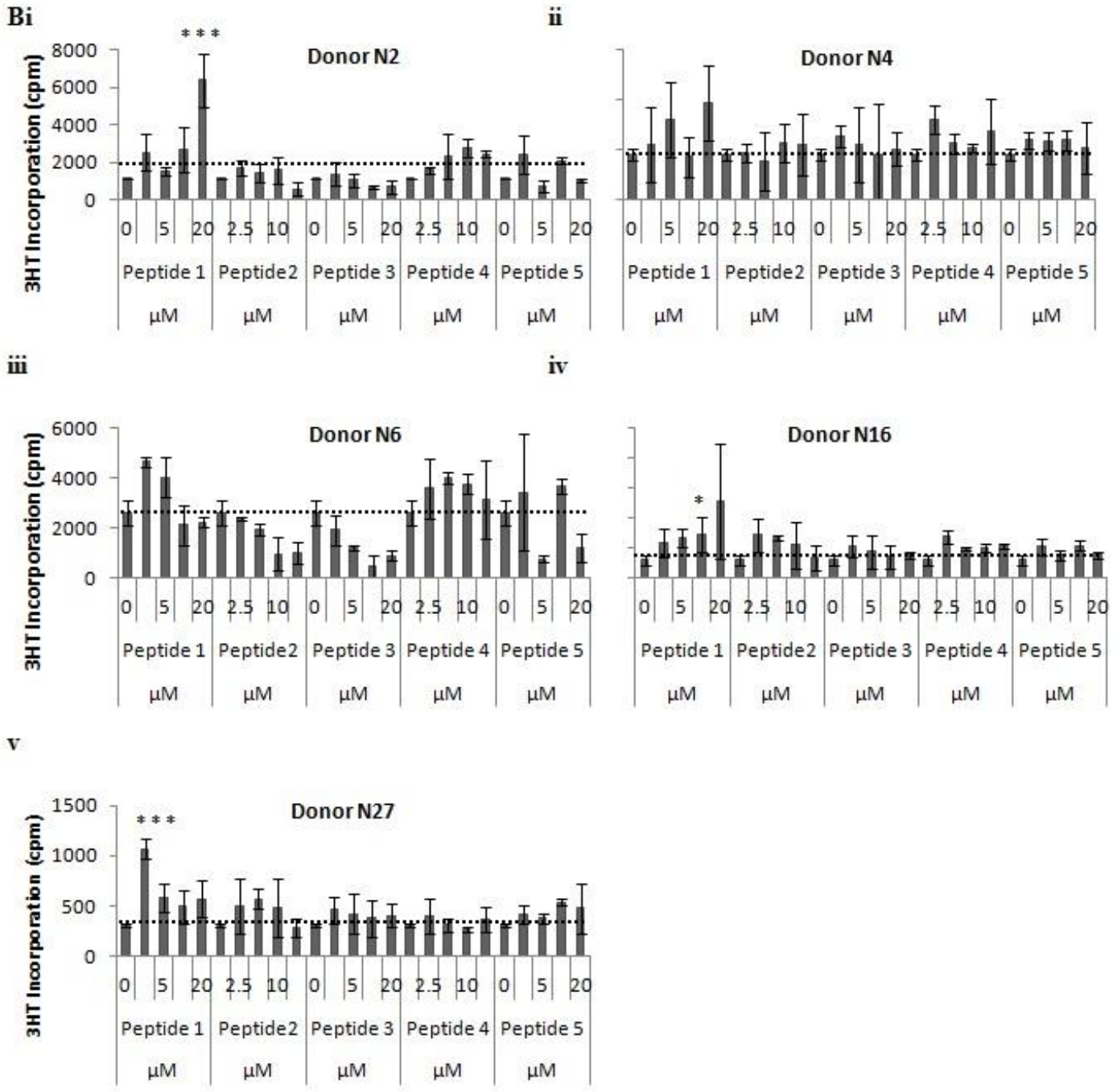
In order to test for the reactivity against IFN-β-1a Rebif™ by *in vivo* primed T-cells, we used overlapping 17mer 5 offset peptides which were predicted to be immunogenic. The PBMCs isolated from donors were then incubated with these peptides and T-cell reactivity was determined using T-cell proliferation assay (measured by 3HT incorporation). No significant dose dependent T-cell activation was observed with PBMCs from anti-IFN-β-1a Rebif™ antibody negative donors N2, N4, N6, N16, N27 and in healthy donors H11, H18, H19, H20,

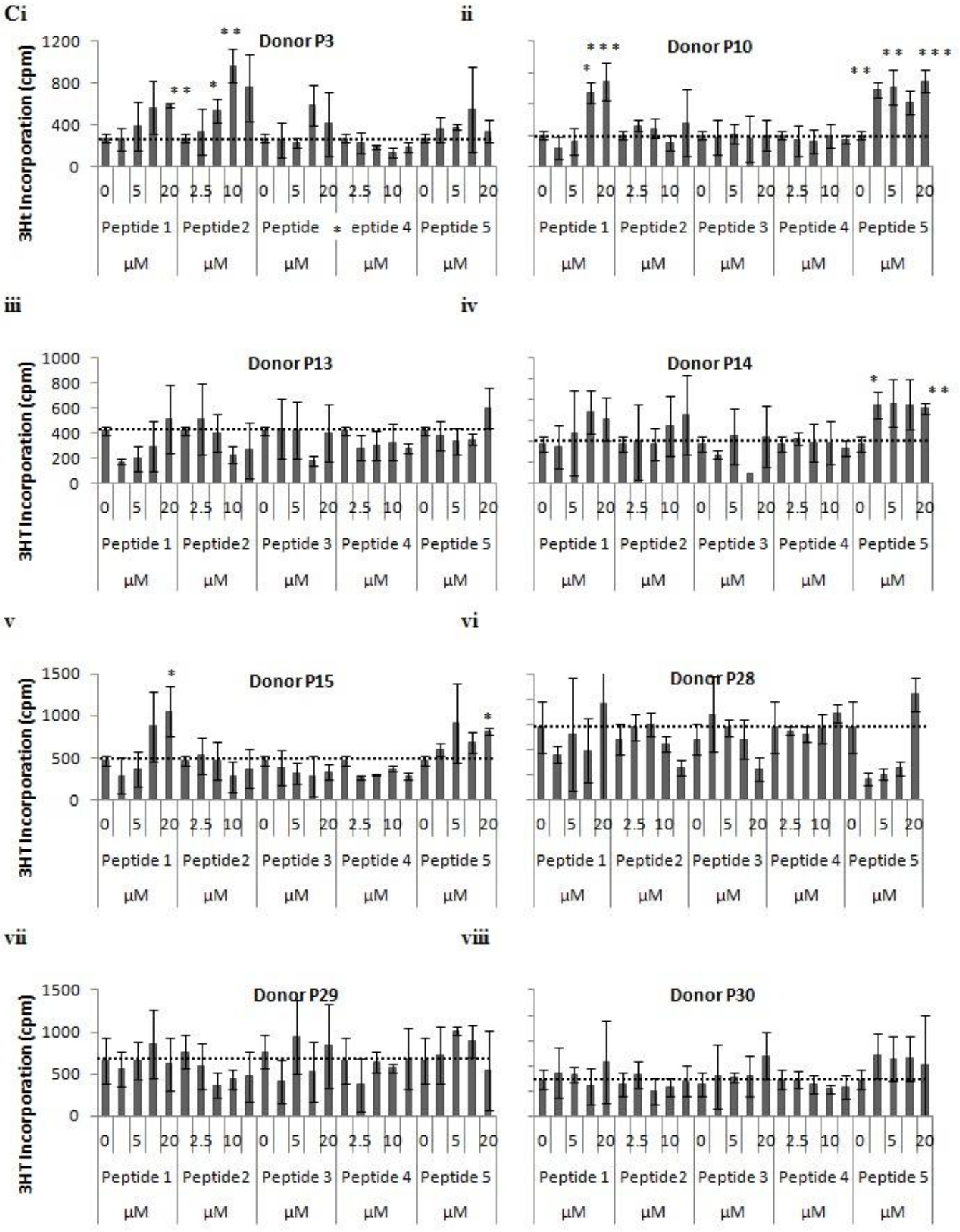
H21 (refer Figure 4.8). However, the donors N2, N16 and N27 exhibited significant proliferation of PBMCs to IFN-β-1a Rebif™ peptide 1 at 20 µM, 10 µM and 2.5 µM respectively and donor N2 exhibited significant proliferation of PBMCs at IFN-β-1a Rebif™ peptide 4 at 20 µM (refer Figures 4.8Bi, iv and v). Similarly, a healthy donor H18 exhibited significant proliferation of PBMCs to IFN-β-1a Rebif™ peptide 1 at 20 µM (refer Figure 4.8Ai). In contrast, PBMCs from anti-IFN-β-1a Rebif™ antibodies positive donors exhibited variable T-cell activation in presence of IFN-β-1a Rebif™ peptide 1 (donors P3 at 20 µM, P10 at 10 µM and 20 µM and P15 at 20 µM), peptide 2 (donor P3 at 5 µM, 10 µM and 20 µM) and peptide 5 (donors P10 at 2.5 µM, 5 µM, 10 µM and 20 µM, P14 at 2.5 µM and 20 µM and P15 at 20 µM) respectively (refer Figures 4.8Ci, ii, iv and v). However, in donors P28, P29 and P30 no significant T-cell activation was observed (refer Figure 4.8). In all the datasets the incorporated 3HT was measured using beta particles as cpm. The cpm > negative control was considered of IFN-β-1a Rebif™ peptide specific. The dotted line on the graphs represents the minimum threshold for considering peptide specific T-cell activation. The stimulation index was calculated and represents the ratio of cpm between stimulated and negative control wells. The SI ≥ 1 was accepted as a cut-off for peptide induced proliferation (refer Figure 4.8D for healthy donors, Figure 4.8E for anti-IFN-β-1a Rebif™ antibodies negative donors and Figure 4.8F for anti-IFN-β-1a Rebif™ antibodies positive donors). Our data suggest the identified IFN-β-1a Rebif™ peptides are potentially immunogenic in anti-IFN-β-1a Rebif™ antibodies positive donors P3, P10, P14 and P15. However, T-cell activation was also observed in anti-IFN-β-1a Rebif™ antibodies negative donors N2, N16 and N27 and in a healthy donor H18.

The ELISpots assays are well established for detection of T-cell activation against peptides (Mosmann et al. 1990). ELISpots can detect number of IFN-γ secreting T-cells. In order to further assess the T-cell involvement in anti-IFN-β-1a Rebif™ antibody response and to test the reactivity against IFN-β-1a Rebif™ by in vivo primed T-cells, PBMCs isolated from anti-IFN-β-1a Rebif™ antibody positive donors P3, P10, P14 and P15 were incubated with the identified 17mer 5 offset IFN-β-1a Rebif™ peptides 1 and 5. The T-cell reactivity (measured by IFN-γ production) was determined with an ELISpot assay (refer Figures 4.9 and 4.10). The Figures 4.9a, b, c, d and 4.10a, b, c and d represents IFN-γ secretion to IFN-β-1a Rebif™ peptide 1 and 5 at 0 µM, 2.5 µM, 5 µM, 10 µM and 20 µM in anti-IFN-β-1a Rebif™ antibodies positive donors P3, P10, P14 and P15 respectively. The Figure 4.9e and 4.10f represents the quantification of IFN-γ spots in 500,000 PBMCs stimulated with IFN-β-1a

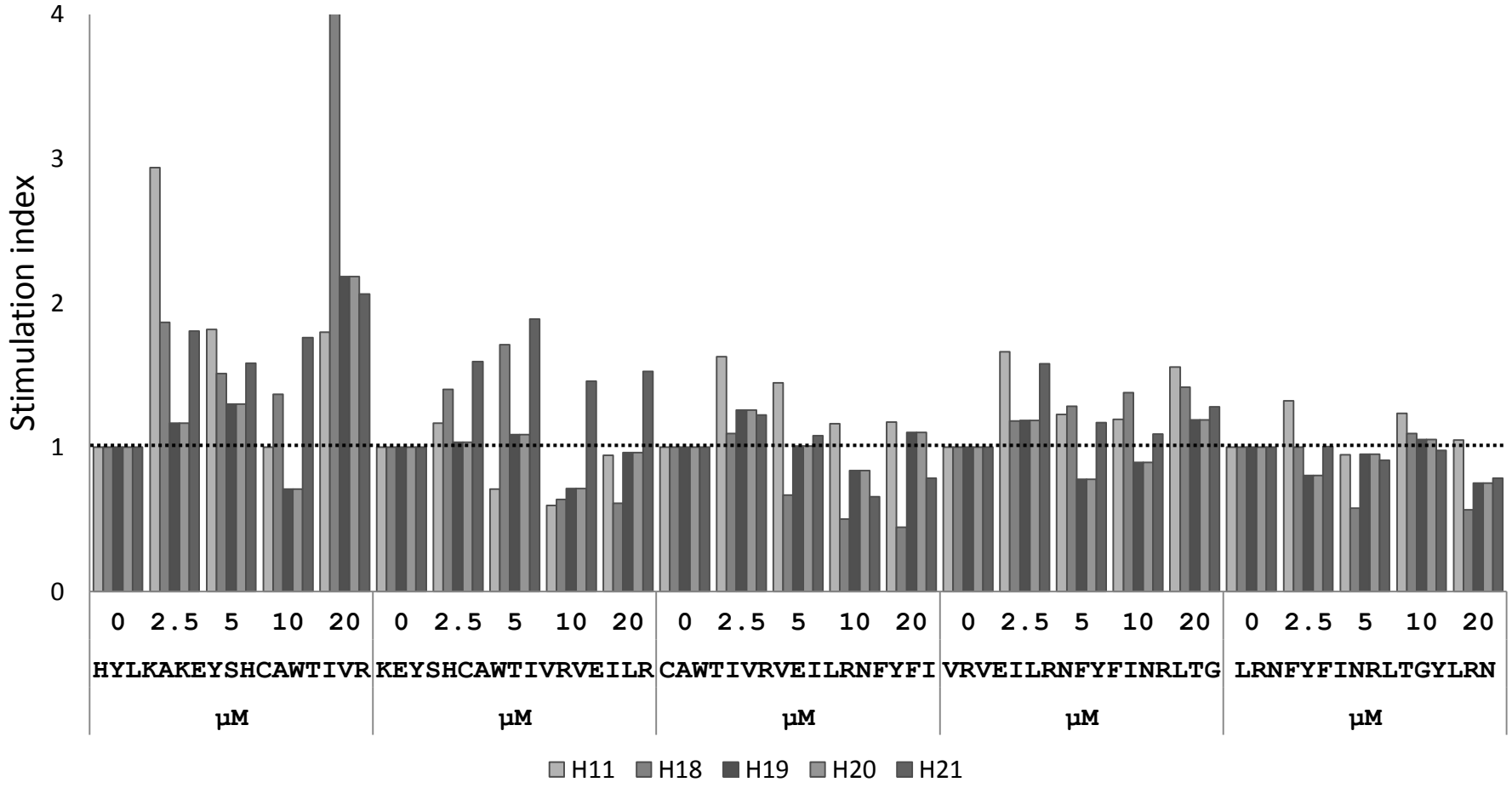
Rebif™ peptide 1 and 5 at different concentrations. The donor P3 (at 10 µM and 20 µM) exhibited significant IFN-γ spots to IFN-β-1a Rebif™ peptide 1. The donors P3 (at 10 µM and 20 µM) and P10 (at 5 µM, 10 µM and 20 µM) exhibited significant IFN-γ spots to IFN-β-1a Rebif™ peptide 3. The donors P13, P28, P29 and P30 was excluded because no T-cell activation was seen in the previous T-cell proliferation assay (refer Figure 4.8c i – viii). Our data suggests the identified IFN-β-1a Rebif™ peptides are potentially immunogenic in antiIFN-β-1a Rebif™ antibodies positive donors P3 and P10 for IFN-β-1a Rebif™ peptides 1 and 5 respectively.



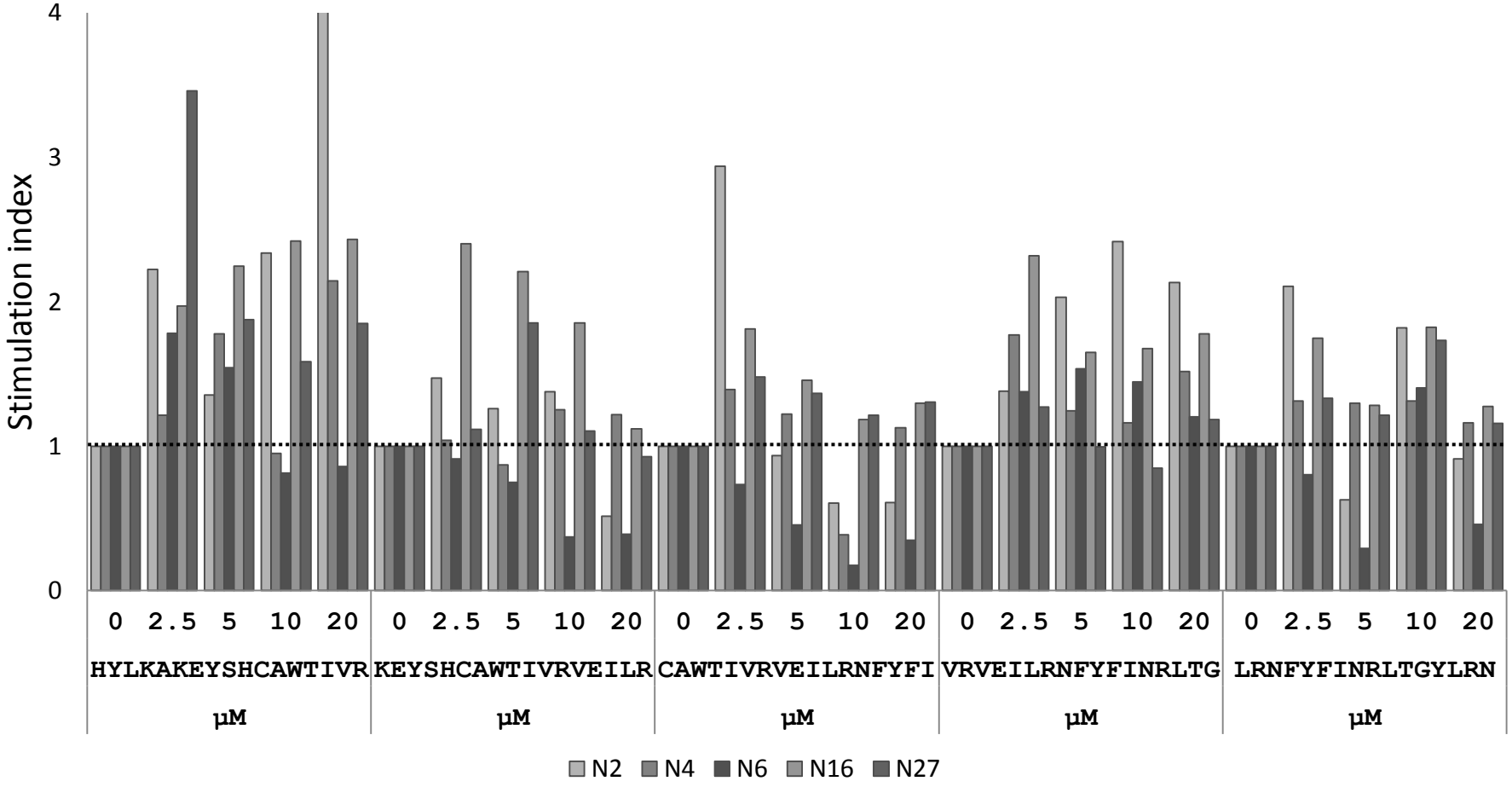

Bi
ii
iii
iv
v
Donor N2
Donor N4
Donor N6
Donor N16
Donor N27
3HT Incorporation (cpm)
* * *
*
0
2000
4000
6000
8000
1500
1000
500
0 5 20 2.5 10
Peptide 1
Peptide2
Peptide 3
Peptide 4
Peptide 5
µM


Ci
Donor P3
3Ht Incorporation (cpm)
1200
800
400
0
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide * eptide 4 Peptide 5
µM µM µM µM µM
ii
Donor P10
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
iii
Donor P13
3HT Incorporation (cpm)
1000
800
600
400
200
0
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
iv
Donor P14
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
v
Donor P15
3HT Incorporation (cpm)
1500
1000
500
0
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
vi
Donor P28
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
vii
Donor P29
3HT Incorporation (cpm)
1500
1000
500
0
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM
viii
Donor P30
0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20 2.5 10 0 5 20
Peptide 1 Peptide2 Peptide 3 Peptide 4 Peptide 5
µM µM µM µM µM

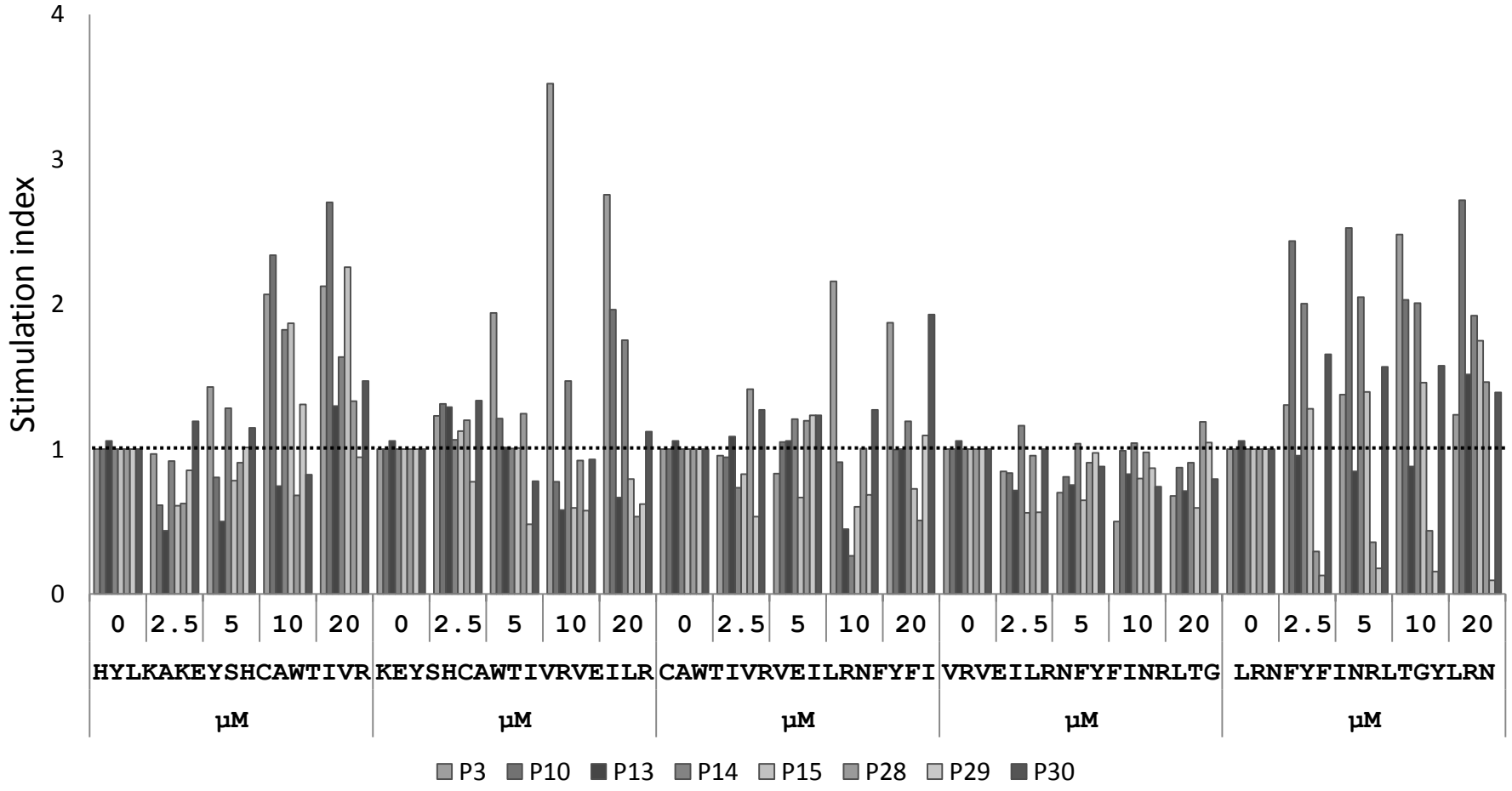
D


Stimulation index
4
3
2
1
0
0 2.5 5 10 20
HYLKAKEYSHCAWTIVR
µM
KEYSHCAWTIVRVEILR
CAWTIVRVEILRNFYFI
VRVEILRNFYFINRLTG
LRNFYFINRLTGYLRN
H11 H18 H19 H20 H21

E


Stimulation index
4
3
2
1
0
0 2.5 5 10 20
HYLKAKEYSHCAWTIVR
KEYSHCAWTIVRVEILR
CAWTIVRVEILRNFYFI
VRVEILRNFYFINRLTG
LRNFYFINRLTGYLRN
µM
N2 N4 N6 N16 N27

F


Stimulation index
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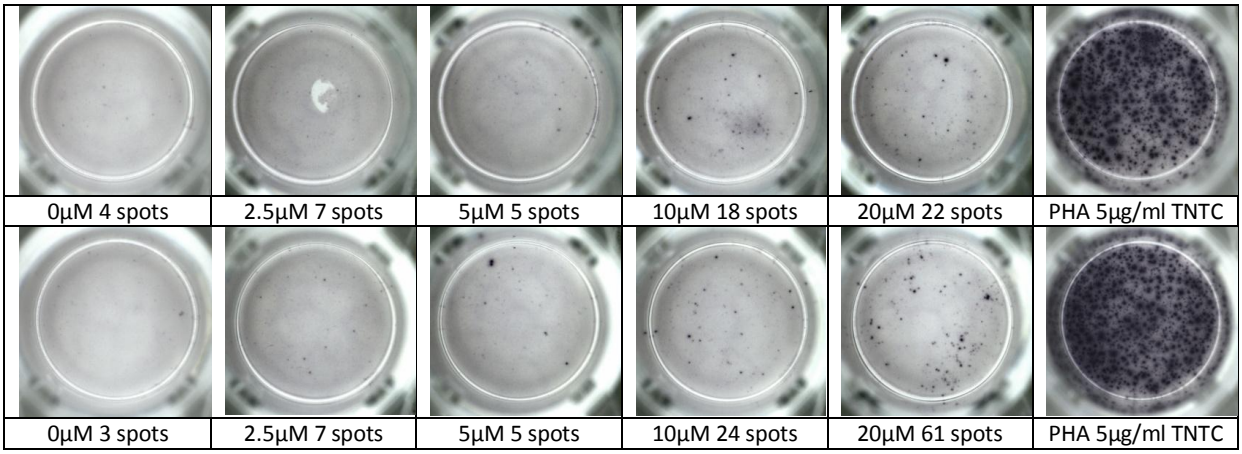
3
2
1
0
0 2.5 5 10 20 0 2.5 5 10 20 0 2.5 5 10 20 0 2.5 5 10 20 0 2.5 5 10 20
HYLKAKEYSHCAWTIVR KEYSHCAWTIVRVEILR CAWTIVRVEILRNFYFI VRVEILRNFYFINRLTG LRNFYFINRLTGYLRN
µM µM µM µM µM
P3 P10 P13 P14 P15 P28 P29 P30

Figure 4.8: T-cell epitope mapping in IFN-β-1a Rebif™ molecule. The Figures 4.8a (i – v), 4.6b (i – v), 4.6c (i – viii), 4.6d, 5.6e and 4.6f represents in vitro stimulation of PBMCs using IFN-β-1a Rebif™ peptides in five healthy donors H11, H18, H19, H20 and H21, five anti-IFN-β-1a Rebif™ antibody negative donors N2, N4, N6, N16 and N27 and eight anti-IFN-β-1a Rebif™ antibody positive donors P3, P10, P13, P14, P15, P28, P29 and P30. The IFN-β-1a Rebif™ peptide 1 elicited T-cell activation in donors P3, P10 and P15, peptide 2 elicited T-cell activation in donor P3 and peptide 5 elicited T-cell activation in donors P10, P14 and P15. The dotted line on the graphs represents the minimum threshold for considering positivity of IFN-β-1a Rebif™ peptides specific T-cell activation. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™ peptides is indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation. Each graph is representative of three independent experiments.

A

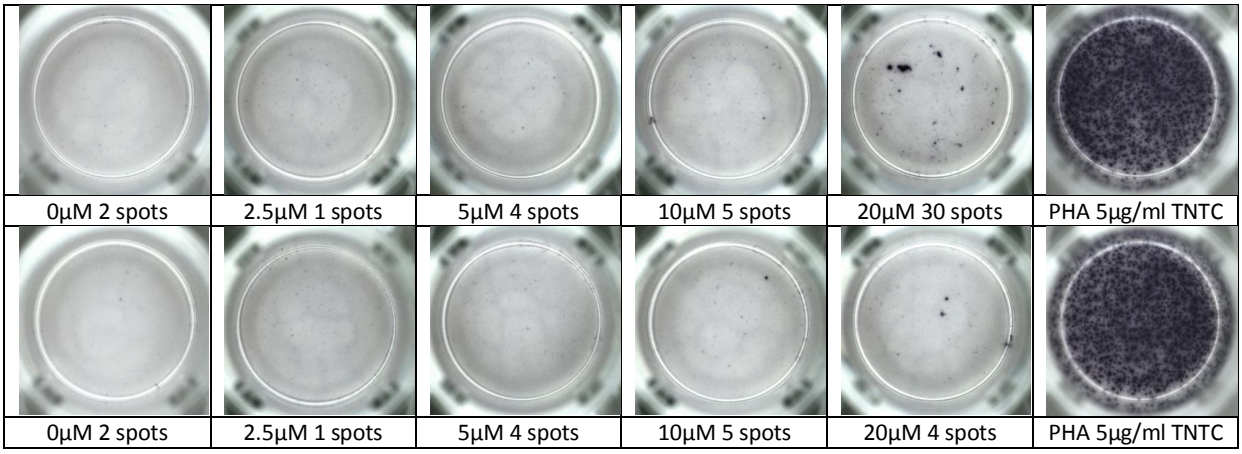
Donor P3



0µM 4 spots	2.5µM 7 spots	5µM 5 spots	10µM 18 spots	20µM 22 spots	PHA 5µg/ml TNTC
0µM 3 spots	2.5µM 7 spots	5µM 5 spots	10µM 24 spots	20µM 61 spots	PHA 5µg/ml TNTC

B

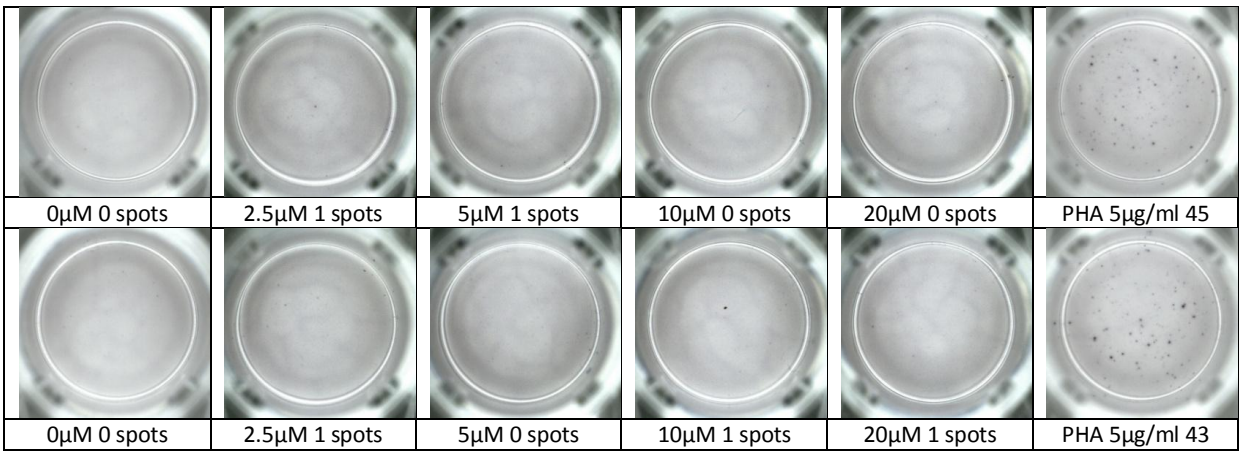
Donor P10



0µM 2 spots	2.5µM 1 spots	5µM 4 spots	10µM 5 spots	20µM 30 spots	PHA 5µg/ml TNTC
0µM 2 spots	2.5µM 1 spots	5µM 4 spots	10µM 5 spots	20µM 4 spots	PHA 5µg/ml TNTC

C

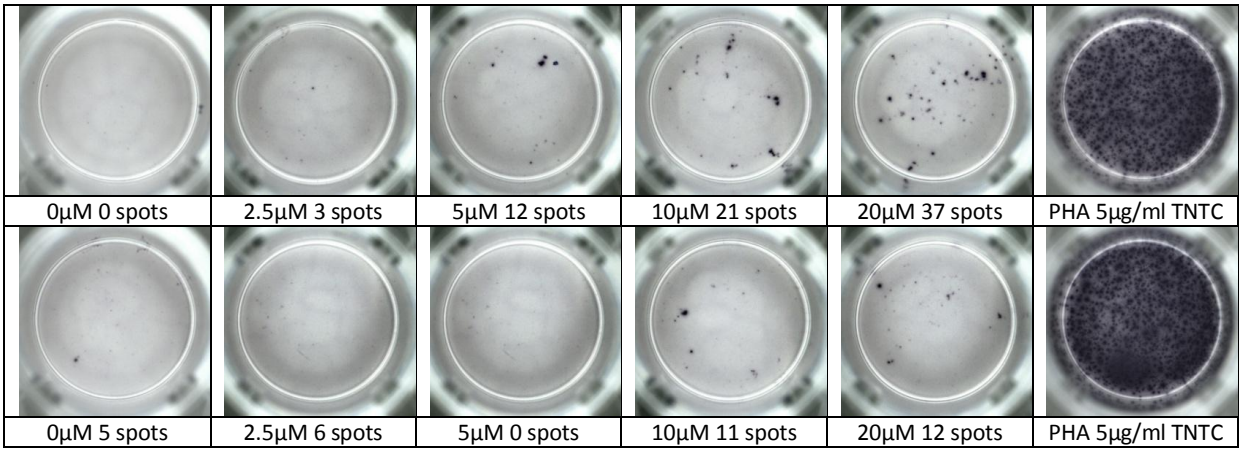
Donor P14



0µM 0 spots	2.5µM 1 spots	5µM 1 spots	10µM 0 spots	20µM 0 spots	PHA 5µg/ml 45
0µM 0 spots	2.5µM 1 spots	5µM 0 spots	10µM 1 spots	20µM 1 spots	PHA 5µg/ml 43

D

Donor P15



E

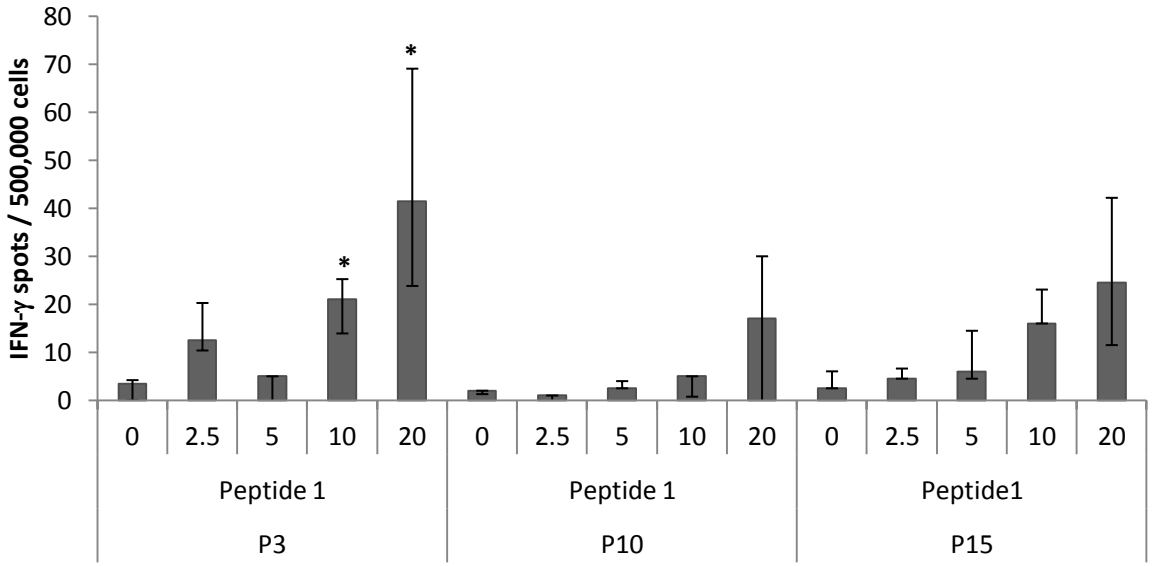
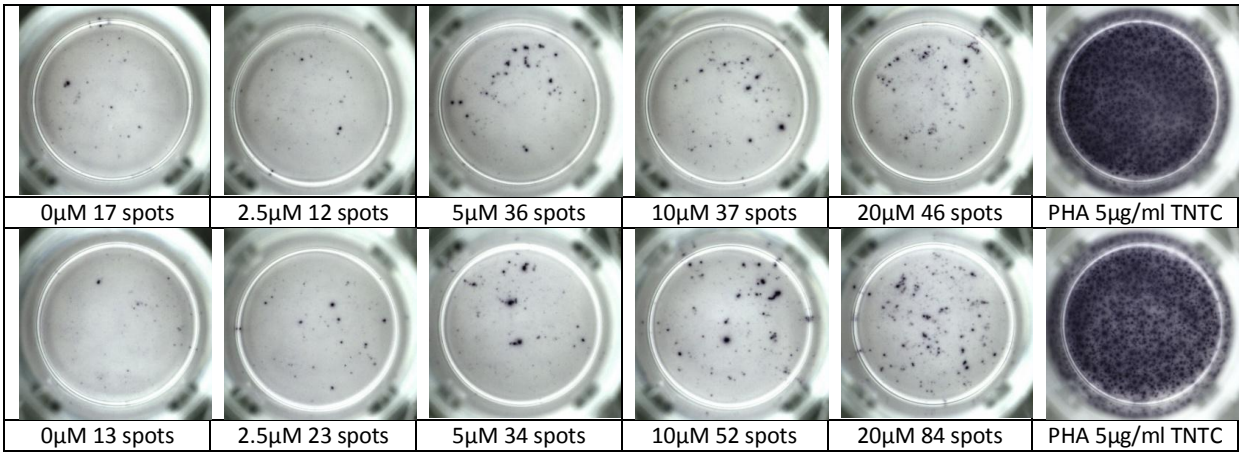


Figure 4.9: IFN-β-1a Rebif™ epitope mapping using an ELISpot assay. The PBMCs from anti-IFN-β-1a Rebif™ antibody positive donors P3, P10, P14 and P15 was stimulated for IFN-γ production in presence of identified 17mer 5 offset IFN-β-1a Rebif™ peptide 1. The Figures 4.9a, b, c and d represents IFN-γ secretion in PBMCs from donors P3, P10, P14 and P15 in presence of IFN-β-1a Rebif™ peptide 1. The Figure 4.9d represents IFN-γ spots/500, 000 cells in donors P3, P10 and P15. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™ peptide 1 is indicated on the X-axis and IFN-γ spots is shown on the Y-axis.

A

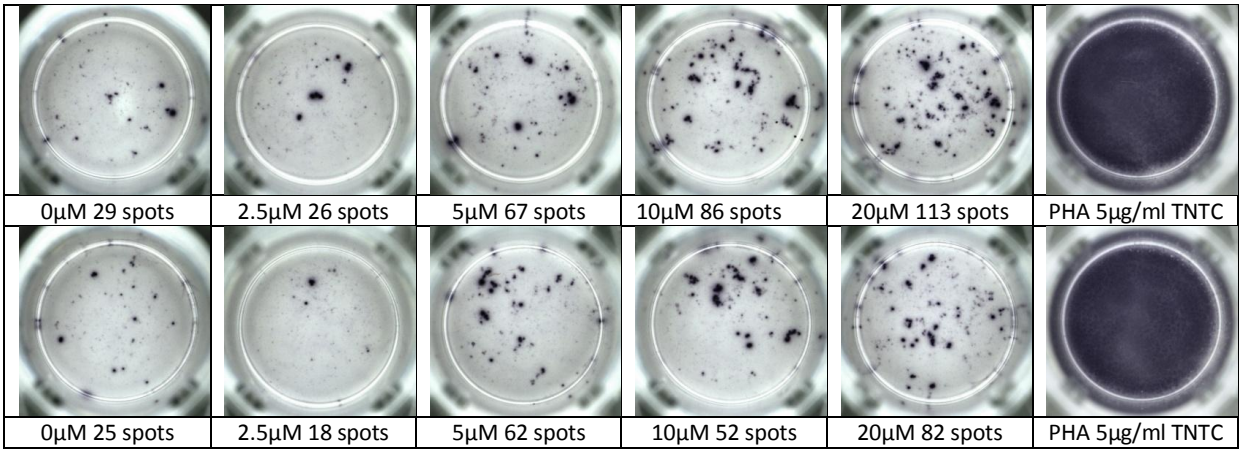
Donor P3



0µM 17 spots	2.5µM 12 spots	5µM 36 spots	10µM 37 spots	20µM 46 spots	PHA 5µg/ml TNTC
0µM 13 spots	2.5µM 23 spots	5µM 34 spots	10µM 52 spots	20µM 84 spots	PHA 5µg/ml TNTC

B

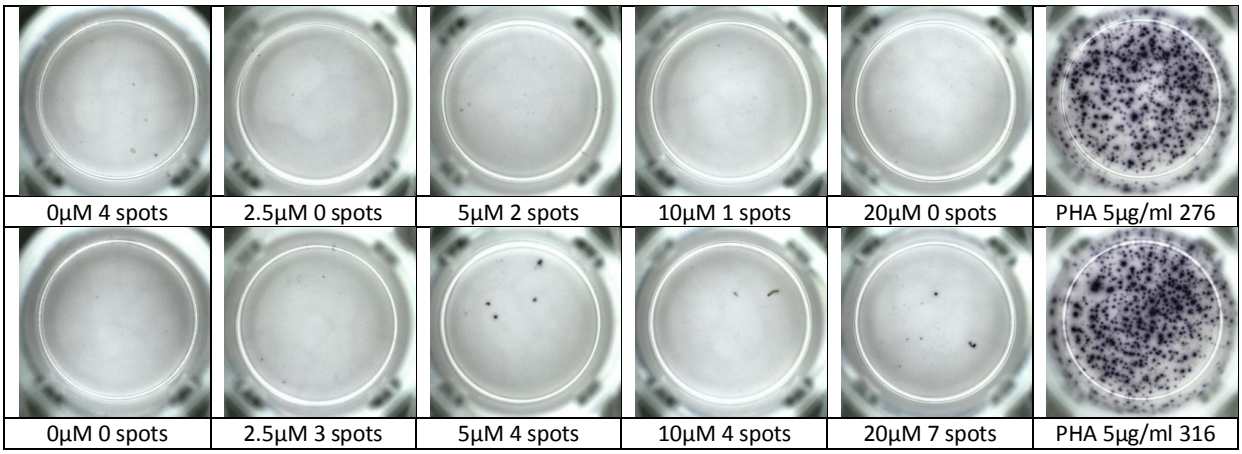
Donor P10



0µM 29 spots	2.5µM 26 spots	5µM 67 spots	10µM 86 spots	20µM 113 spots	PHA 5µg/ml TNTC
0µM 25 spots	2.5µM 18 spots	5µM 62 spots	10µM 52 spots	20µM 82 spots	PHA 5µg/ml TNTC

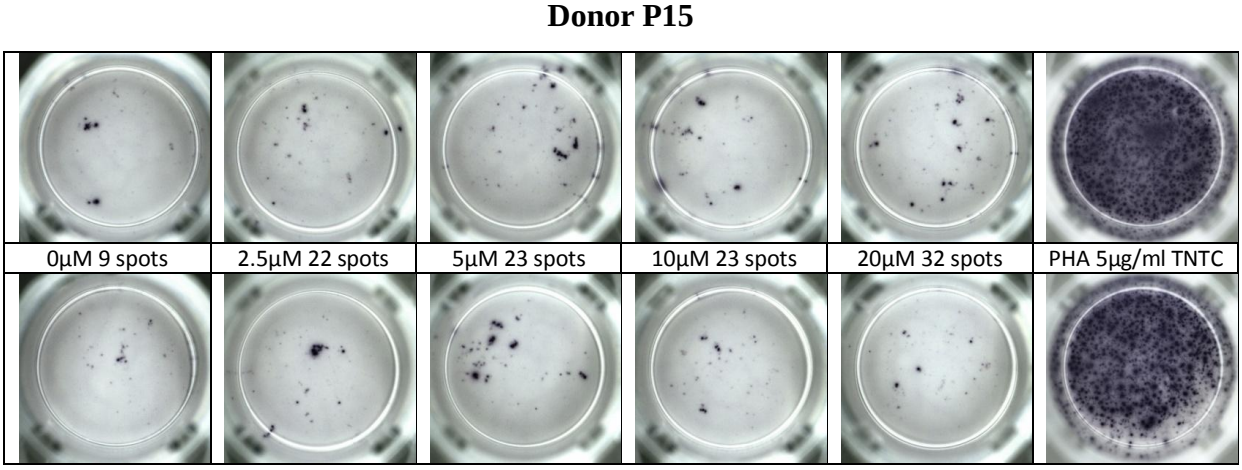
C

Donor P14



0µM 4 spots	2.5µM 0 spots	5µM 2 spots	10µM 1 spots	20µM 0 spots	PHA 5µg/ml 276
0µM 0 spots	2.5µM 3 spots	5µM 4 spots	10µM 4 spots	20µM 7 spots	PHA 5µg/ml 316

D



E

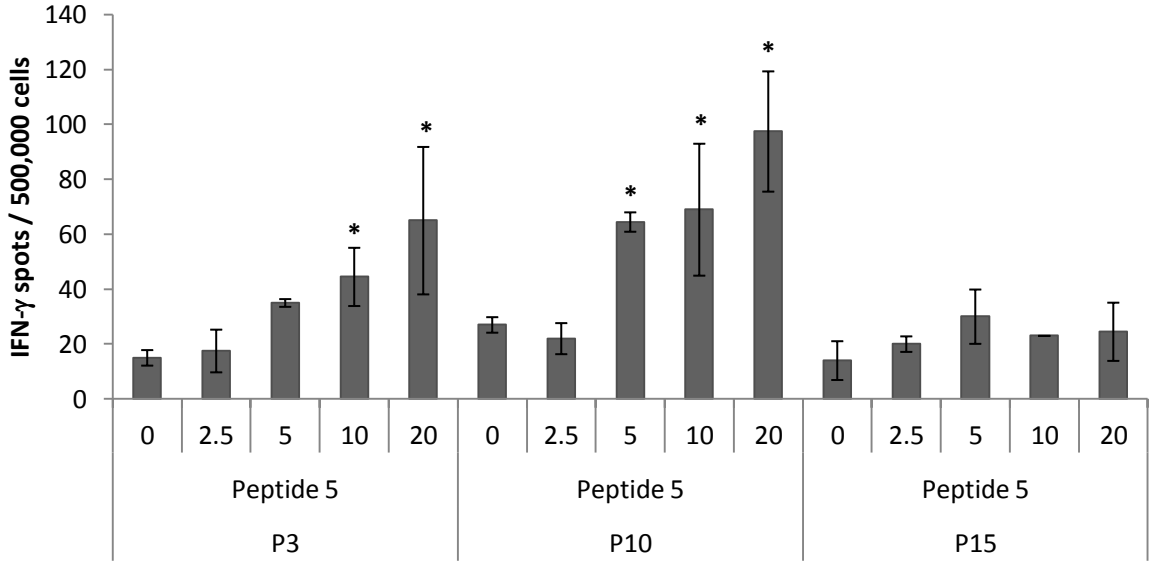
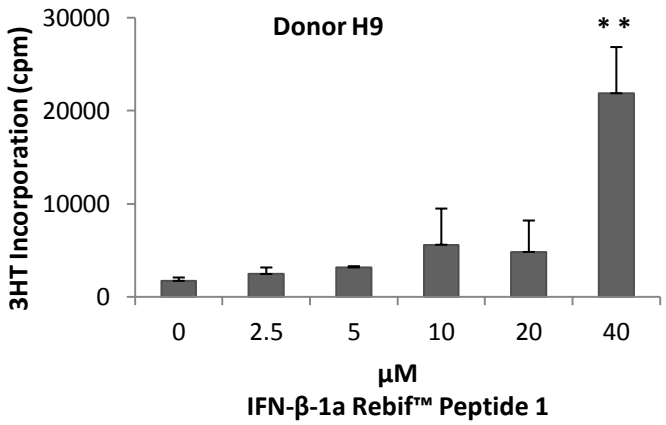


Figure 4.10: IFN-β-1a Rebif™ epitope mapping using an ELISpot assay. The PBMCs from anti-IFN-β-1a Rebif™ antibody positive donors P3, P10, P14 and P15 was stimulated for IFN-γ production in presence of identified 17mer 5 offset IFN-β-1a Rebif™ peptide 5. The Figures 4.10a, b, c and d represents IFN-γ secretion in PBMCs from donors P3, P10, P14 and P15 in presence of IFN-β-1a Rebif™ peptide 5. The Figure 4.10d represents IFN-γ spots/500, 000 cells in donors P3, P10 and P15. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™ peptide 5 is indicated on the X-axis and IFN-γ spots is shown on the Y-axis.

Whilst performing T-cell proliferation assays in healthy controls we observed incidental T-cell activation to IFN-β-1a Rebif™ peptide 1 in donor H18 (refer Figure 4.8Ai). T-cell

proliferation was also tested in healthy controls PBMCs H9, H22, H23, H24 and H25. The donors H9 (at 40 µM) and H25 (at 20 µM and 40 µM) exhibited significant proliferation of PBMCs to IFN-β-1a Rebif™ peptide 1 (refer Figure 4.11a and b). However, no T-cell activation was observed in donors H22, H23 and H25. The maximum stimulation for donor H9 and H25 was observed at 40 µM. Our data provide evidence for existence of potential IFN-β-1a Rebif™ T-cell epitopes in non-primed healthy donors.

A



B

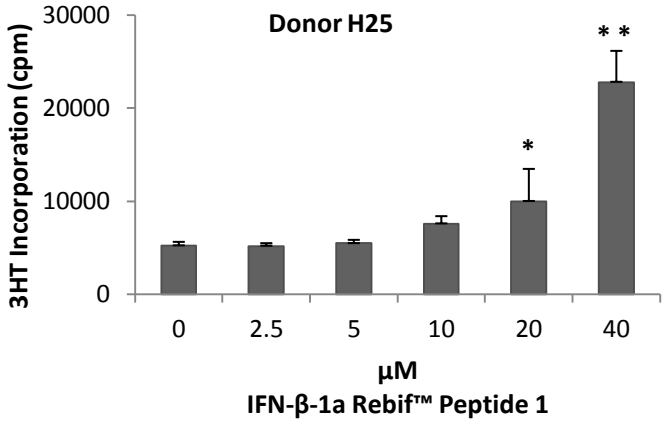


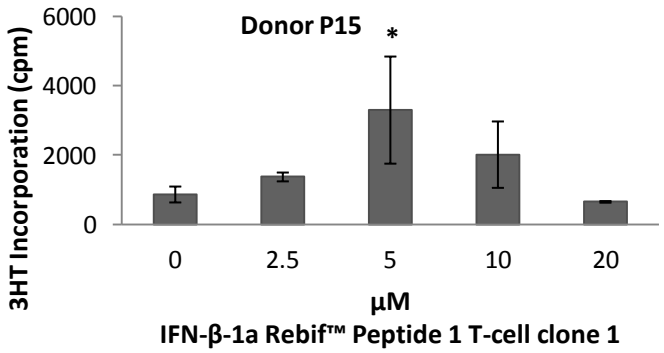
Figure 4.11: IFN-β-1a Rebif™ molecule T-cell epitope mapping in healthy donors. The Figure 4.11 represents in vitro stimulation of PBMCs using IFN-β-1a Rebif™ peptide 1 in healthy donors H9 and H25. The IFN-β-1a Rebif™ peptide 1 elicited dose dependent T-cell activation in donors H9 and H25. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™

peptide 1 is indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation.

4.3.6 Generation of Interferon beta 1a Rebif™ peptide responsive T-cell clones

In an attempt to understand the cellular basis of IFN-β-1a Rebif™ peptide 1 and 5 T-cell responses the anti-drug antibodies positive donor P15 was chosen for pilot T-cell cloning experiments. T-cell cloning provides the population of T-cells that derives from the same progenitor with unique phenotypic and functional characteristics. The nature of T-cell response might provide useful insights of T-cells involvement in eliciting immune response against IFN-β-1a Rebif™ peptides. The choice of using donor P15 for T-cell cloning was based up on the T-cell proliferation data, IFN-γ production to IFN-β-1a Rebif™ peptides and also this donor exhibited highest amount of IFN-β-1a Rebif™ anti-drug antibodies. Hence, the donor P15 PBMCs was stimulated with 20 µM of each IFN-β-1a Rebif™ peptide 1 and 5 and T-cell cloning experiment was performed. For IFN-β-1a Rebif™ peptide 1 two responsive T-cell clones and peptide 5 one responsive clone were generated (refer Figure 4.12a, b and c). The IFN-β-1a Rebif™ peptide 1 T-cell clones 1 and 2 and IFN-β-1a Rebif™ peptide 2 T-cell clone 1 provided maximum proliferation of PBMCs at 5 µM of respective IFN-β-1a Rebif™ peptides. The obtained T-cell clones were expanded but failed to further respond. The obtained T-cell clones were tested using autologous B-LCLs as antigen presenting cells and IFN-β-1a Rebif™ peptide 1 and 5. The data obtained suggests IFN-β-1a Rebif™ peptide specific T-cell clones can be generated and illustrates potential T-cell involvement in the formation of anti-drug antibodies.

A



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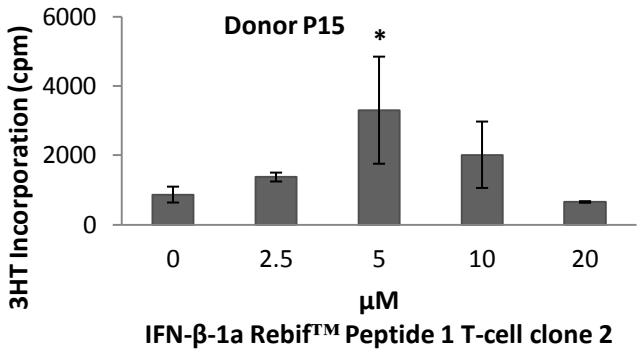

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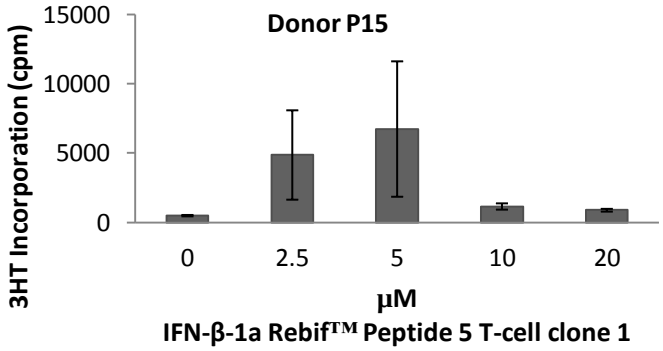


Figure 4.12: IFN-β-1a Rebif™ peptides specific T-cell clones. The Figures 4.12a and b represents the testing for IFN-β-1a Rebif™ peptide no. 1 specific T-cell clones obtained from donor P15. The Figure 4.12c represents the testing of peptide no. 3 specific T-cell clone obtained from donor P15. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The different concentration of IFN-β-1a Rebif™ peptides no. 1 and 5 are indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in presence and absence of antigenic stimulation.

4.3.7 Identification of Interferon beta 1a Rebif™ T-cell epitopes in Human leukocyte antigens genotyped healthy donors

The development of anti-IFN-β-1a IgG antibodies has been found to correlate with development of antigen specific T-cell responses. Several studies have identified the link between the human leukocyte antigens (HLA) with activation of antigen specific T-cells. For examples abacavir hypersensitivity was associated with HLA B*5701 (Mallal et al. 2002),

carbamazepine hypersensitivity in Caucasians and Japanese was associated with HLA A*3101 (McCormack et al. 2011; Ozeki et al. 2011), carbamazepine provoked Stevens Johnson syndrome in Han Chinese was associated with HLA B*1502 (Chung et al. 2004) and recently flucloxacillin induced liver injury was associated with HLA B*5701 (Monshi et al. 2013).

In order to investigate the HLA association with T helper cell activation to IFN-β-1a Rebif™ and development of IFN-β-1a Rebif™ anti-drug antibodies, the IFN-β-1a Rebif™ peptides identified earlier was examined for presence of T-cell epitopes using 30 HLA genotyped healthy donors. The proliferative response was measured using 3HT incorporation. The SI of ≥ 2 was collated as positive responses to that peptide from that healthy donor (refer Figure 4.13). Out of 30 donors tested for IFN-β-1a Rebif™ peptides – 3 donors at 2.5 µM, 6 donors at 5 µM, 2 donors at 10 µM and 2 donors at 20 µM elicited T helper cell activation to IFN-β-1a Rebif™ peptide 1 (refer Figure 4.13a), 7 donors at 2.5 µM, 4 donors at 5 µM, 6 donors at 10 µM and 4 donors at 20 µM elicited T helper cell activation to IFN-β-1a Rebif™ peptide 2 (refer Figure 4.13b), 10 donors at 2.5 µM, 10 donors at 5 µM, 5 donors at 10 µM and 16 donors at 20 µM elicited T helper cell activation to IFN-β-1a Rebif™ peptide 3 (refer Figure 4.13c), 4 donors at 2.5 µM, 1 donor at 5 µM, 1 donor at 10 µM and 1 donor at 20 µM elicited T helper cell activation to IFN-β-1a Rebif™ peptide 4 (refer Figure 4.13d) and 3 donors at 2.5 µM, 6 donors at 5 µM, 4 donors at 10 µM and 6 donors at 20 µM elicited T helper cell activation to IFN-β-1a Rebif™ peptide 5 (refer Figure 4.13d). Overall the maximum T helper cell activation was observed at IFN-β-1a Rebif™ peptide 3. However, the dose dependent T helper cell activation was observed in healthy donor 20 with HLA DRB1*1501 for IFN-β-1a Rebif™ peptides 1 and 5, healthy donor 24 with HLA DRB1*0101 for IFN-β-1a Rebif™ peptides 2 and 3, healthy donor 27 with HLA DRB1*1502 for IFN-β-1a Rebif™ peptide 2, healthy donor 28 with HLA DRB1*0301 for IFN-β-1a Rebif™ peptide 3, healthy donor 29 with HLA DRB1*1501 for IFN-β-1a Rebif™ peptide 3, healthy donor 32 with HLA DRB1*0101 for IFN-β-1a Rebif™ peptide 4, healthy donor 36 with HLA DRB1*1502 for IFN-β-1a Rebif™ peptide 3, healthy donors 37, 38, 39 with HLA DRB1*1501 for IFN-β-1a Rebif™ peptide 3 respectively. Our data indicates the proliferative response to IFN-β-1a Rebif™ peptide 3 was associated with the presence of the HLA haplotype DRB1*1501 (refer Figure 4.14).

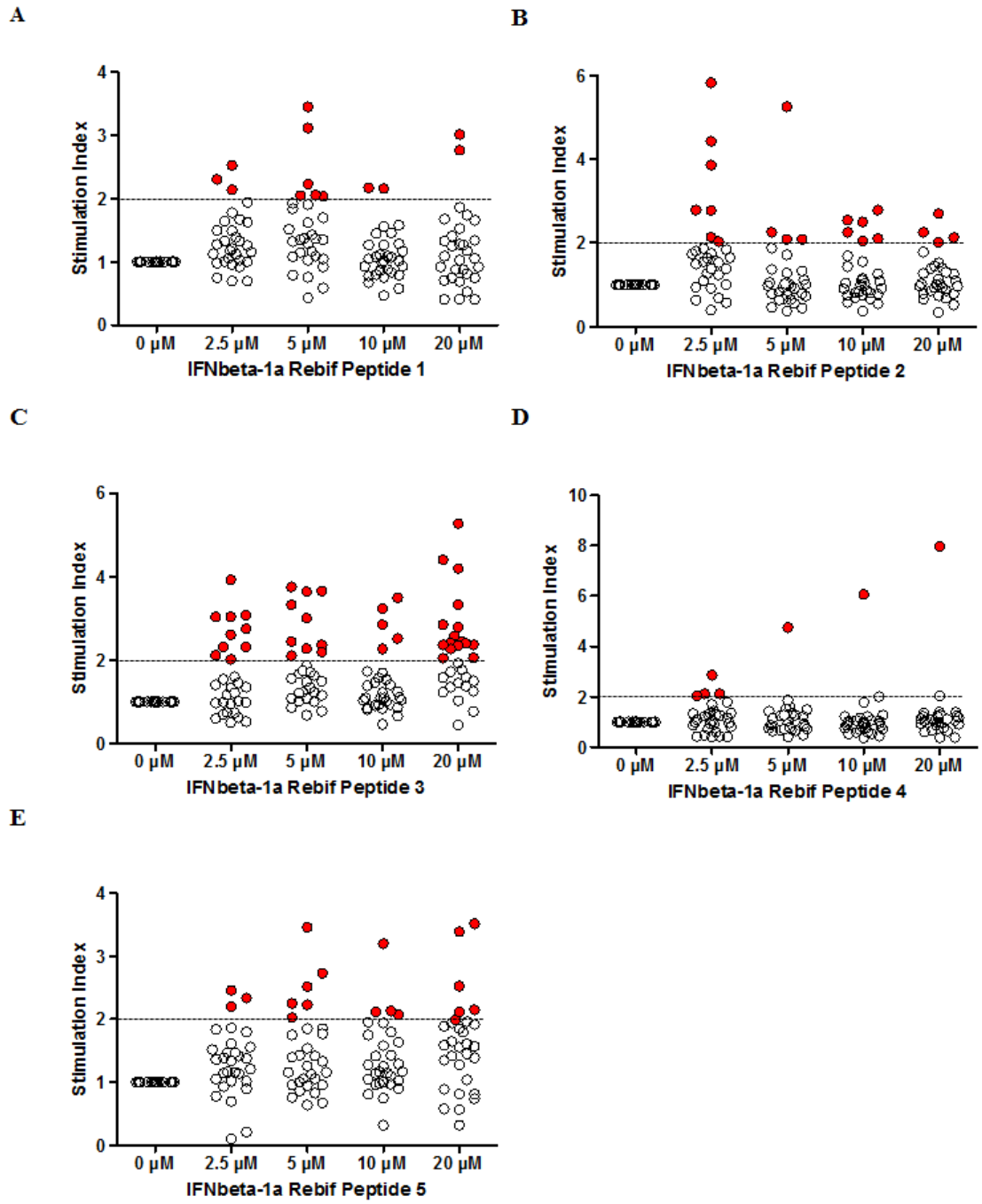


Figure 4.13: IFN-β-1a Rebif™ molecule T-cell epitope mapping. The Figure 4.13a, b, c, d and e represents in vitro stimulation of PBMCs and frequency of responses using identified IFN-β-1a Rebif™ peptides in 30 different HLA genotyped healthy donors. The IFN-β-1a Rebif™ peptides elicited differential T-cell activation in several healthy donors (indicated in red coloured circles). The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™ peptides is indicated on the X-axis and Stimulation index (SI) is shown on the Y-axis. The SI represents the ratio of cpm between stimulated and negative control wells. The dotted line on the graphs represents the minimum threshold for considering IFN-β-1a Rebif™ peptides specific T-cell activation.

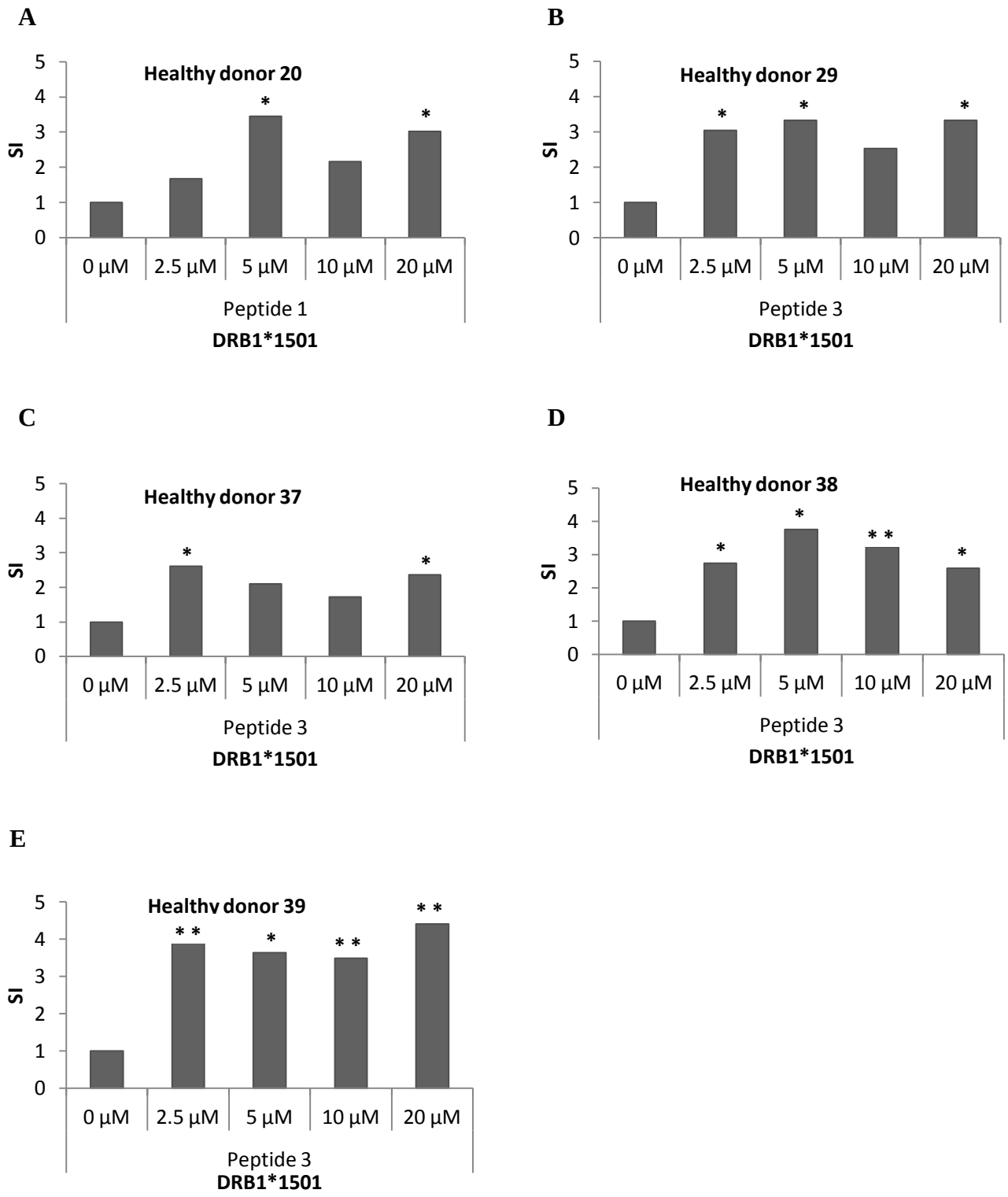


Figure 4.14: T-cell activation to IFN-β-1a Rebif™ peptides in HLA-DRB1*1501 healthy donors. The Figure 4.14a, b, c, d and e represents in vitro stimulation of PBMCs using IFN-β-1a Rebif™ peptide 1 and 3 in healthy donors 20, 29, 37, 38 and 39. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The phytohaemagglutinin at 5 µg/ml was used as a positive control. The different concentration of IFN-β-1a Rebif™ peptides is indicated on the X-axis and Stimulation index (SI) is shown on the Y-axis.

4.3.8 Regulatory T-cells in anti-Interferon beta 1a Rebif™ antibodies development

Regulatory T-cells (Tregs) are essential for control of adaptive immune responses and also constitutes tolerance to both self and non-self antigens (Sakaguchi et al. 2008). Tregs mediates regulation of B cells and promote tolerance against self antigens through contact dependent suppression or secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. The studies suggests the role of Tregs in regulation of autoantibody responses through B cells either activating B cells directly or through T helper cells and antigen presenting cells (Bystry et al. 2001; Ludwig-Portugall et al. 2009; Ludwig-Portugall et al. 2008).

In this set of experiments an attempt has been made to investigate the role of Tregs in regulation of anti-IFN-β-1a Rebif™ antibodies in MS patients. Tregs can be identified by the presence of cell surface markers, $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$. The intracellular marker FoxP3 belongs to fork-head family of transcriptional factors and also considered as a master regulator of Tregs development and function (Hori, Nomura & Sakaguchi 2003; Khattri et al. 2003). The gating strategy for identification of Tregs in peripheral blood is shown in Figure 4.15). In this study we first investigated the frequency of Tregs in PBMCs of IFN-β-1a Rebif™ neutralising antibodies positive, negative and healthy donors (refer Figure 4.16). The Figures 4.16a and b represents percentage population of $CD4^{+}$ T-cells, the medians (25th, 75th percentiles) include 38.25 (29.21, 51.13) and 46.40 (37.67, 54.02) for two groups and 38.25 (29.21, 51.13), 43.47 (37.71, 53.88) and 51.75 (37.53, 55.01) for three groups respectively. The Figure 4.16c and d represents percentage population of $CD4^{+}CD25^{high}$ T-cells, the medians (25th, 75th percentiles) include 4.430 (2.525, 6.190) and 3.450 (2.800, 5.000) for two groups and 4.430 (2.525, 6.190), 3.350 (2.270, 5.265) and 3.970 (2.808, 5.318) for three groups respectively. The Figures 4.16e and f represents percentage population of $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ T-cells, the medians (25th, 75th percentiles) include 2.870 (1.250, 4.560) and 2.440 (1.790, 3.380) for two groups and 2.870 (1.250, 4.560), 2.575 (2.158, 3.478) and 2.335 (1.408, 2.938) for three groups respectively. Our data suggests no significant differences in the frequency of Tregs in PBMCs of IFN-β-1a Rebif™ neutralising antibodies positive, negative and healthy donors respectively.

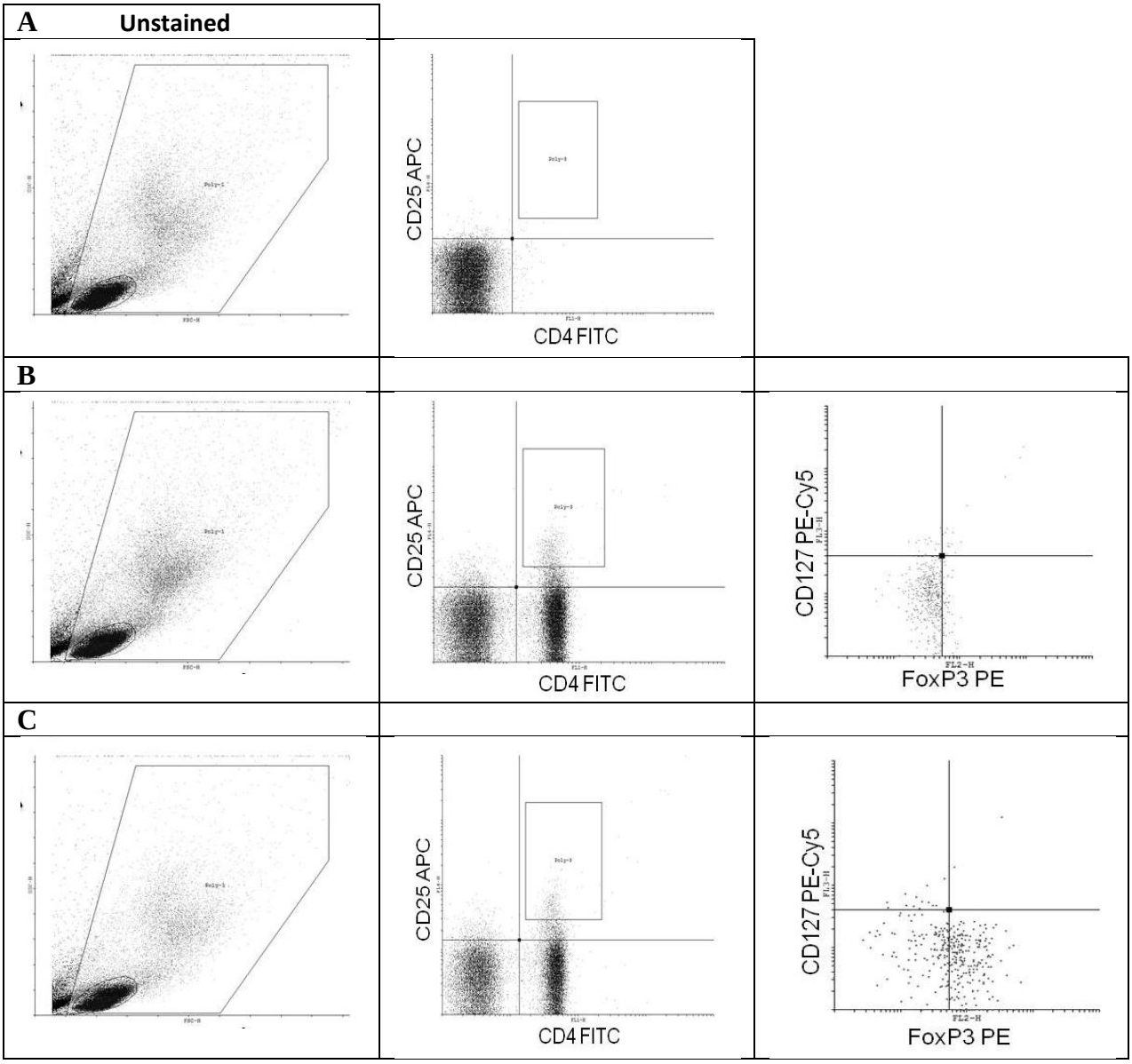


Figure 4.15: Global immunophenotyping of Tregs. The representation of gating strategy adopted for identification of Tregs in PBMCs using FACS. The Figure 4.15a represents dot plot for unstained cells as background control. The Figure 4.15b represents dot plot for cells stained with FITC conjugated anti-CD4, APC conjugated anti-CD25 and PE-Cy5 conjugated anti-CD127 antibodies. The Figure 4.15c represents dot blot for cells stained for FITC conjugated anti-CD4, APC conjugated anti-CD25, PE-Cy5 conjugated anti-CD127 and PE conjugated anti-FoxP3 antibodies. Isotype control antibodies were used for all cell surface staining. The dot plots were obtained using Cyflogic™, a FACS data analysis tool.

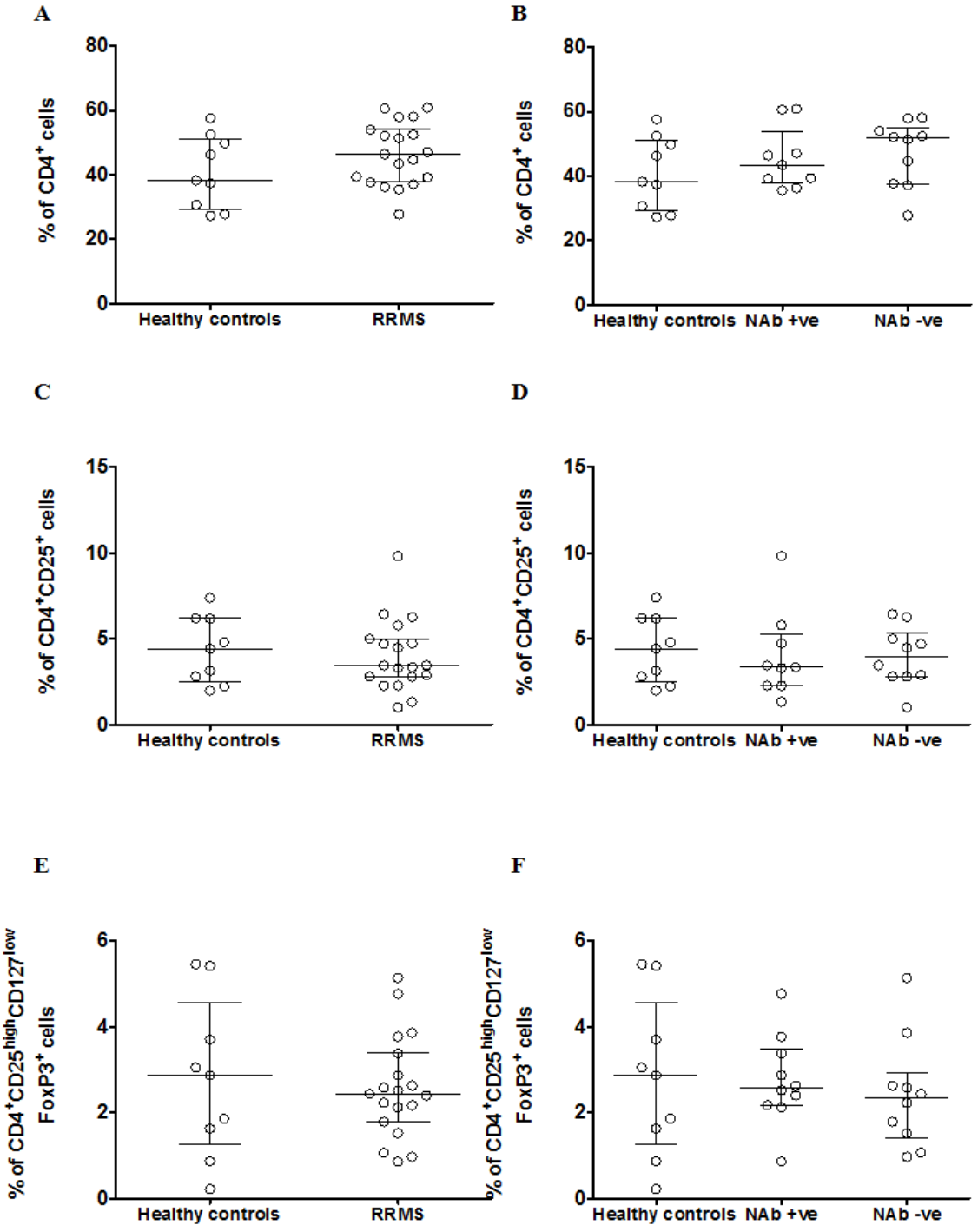


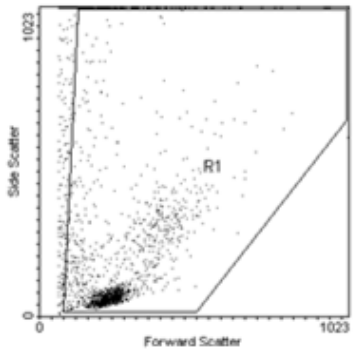
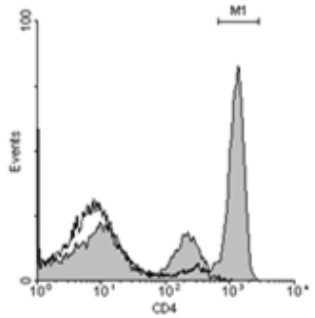
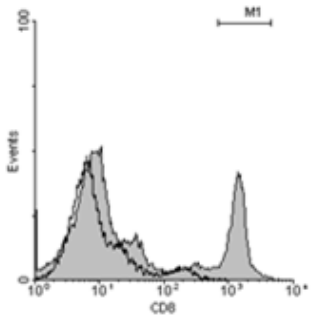
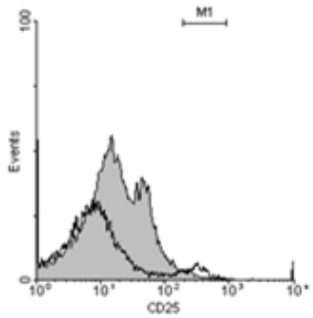
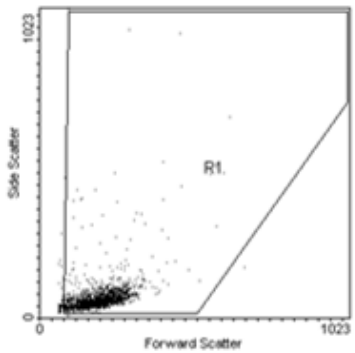
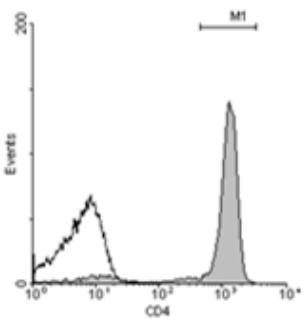
Figure 4.16: Frequency of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs. Comparison of percentage of $CD4^+$ T-cells (refer Figures 4.16a and b), $CD4^+CD25^{high}$ T-cells (refer Figures 4.16c and d) and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs population (refers Figures 4.16e and f) in IFN-β-1a Rebif™ neutralising antibodies positive, negative and in healthy donors in the peripheral blood. Each open circle represents an individual percentage of defined T-cell population. Bars represent the IQR (25th and 75th percentiles) from the median (horizontal line). Statistical differences were not observed between differences in any groups and were determined using Mann Whitney U test for two non-parametric groups and Kruskall-Wallis test for more than two nonparametric groups.

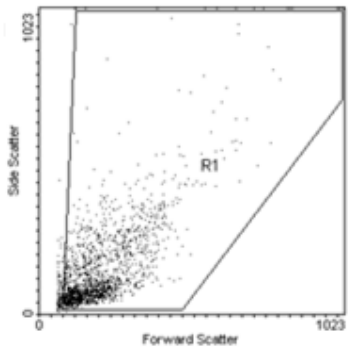
4.3.9 Evaluation of $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ regulatory T-cells suppressive function

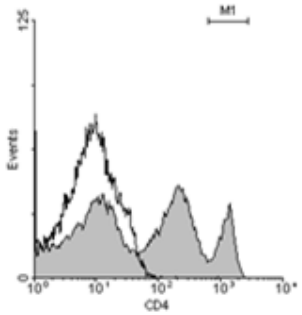
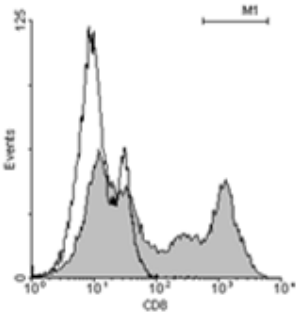
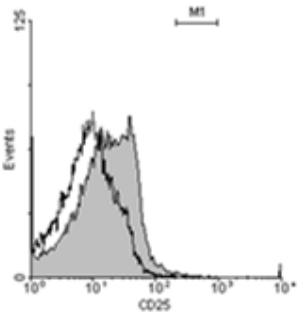
In order to investigate whether there are differences in Tregs function between the different patient groups, Tregs suppression assay was used. The CD4+$CD25^{high}$ Tregs were isolated and Tregs negative fraction $CD4^{+}CD25^{low}$ T-cells were used as responder T-cells (Tresp). The cells were stimulated using phytohaemagglutinin, irradiated non $CD4^{+}$ T-cells were used as feeder cells and different ratio of Tregs were added to the culture and the suppression was measured using 3HT incorporation. The obtained cells fractions were surface stained for % of purity and were measured using FACS (refer Figures 4.17a, b and c). The ideal concentration for polyclonal stimulation phytohaemagglutinin was optimised at 20 µg/ml and for antigenic stimulation Tetanus toxoid was optimised at 2 µg/ml and was determined using Tregs suppression assay. The maximum stimulation was observed for phytohaemagglutinin at 20 µg/ml and for Tetanus toxoid at 2 µg/ml (refer Figures 4.18a and b). The functional activity of Tregs were measured in healthy donors H9, H11 and H18, IFN-β-1a Rebif™ positive donors P3 and P15 and negative donors N2, N4 and N12 using Tregs suppression assays (refer Figures 4.19). The no. of Tregs required to suppress Tresp was calculated which is illustrated in Figure 4.19j. Bars represent the IQR (25th and 75th percentiles) from the median (horizontal line). The medians (25th, 75th percentiles) include 16062 (14404, 17720), 12216 (8592, 15840) and 15274 (11554, 16495) for three groups respectively. Our data suggests no significant differences in the Tregs functions of IFN-β-1a Rebif™ neutralising antibodies positive, negative and healthy donors respectively.

However, during Tregs isolation we incidentally identified significantly difference in $CD8^{+}$ T-cells in healthy donors and IFN-β-1a Rebif™ neutralising antibodies positive and negative donors. Bars represent the IQR (25th and 75th percentiles) from the median (horizontal line). The medians (25th and 75th percentiles) include 21.35 (17.23, 27.72) and 10.77 (6.660, 15.11) for two groups and 21.35 (17.23, 27.72), 7.620 (4.940, 10.77) and 15.36 (14.34, 16.24) for three groups respectively.

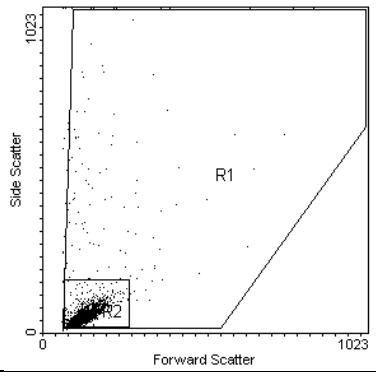
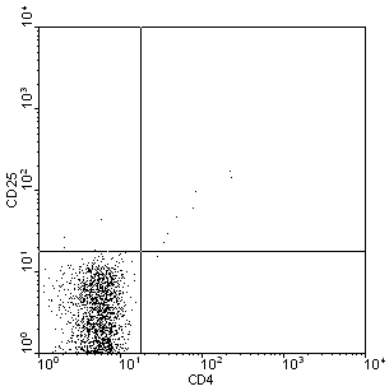
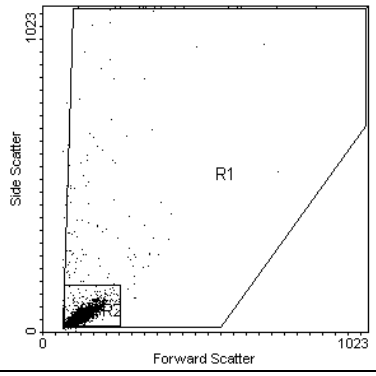
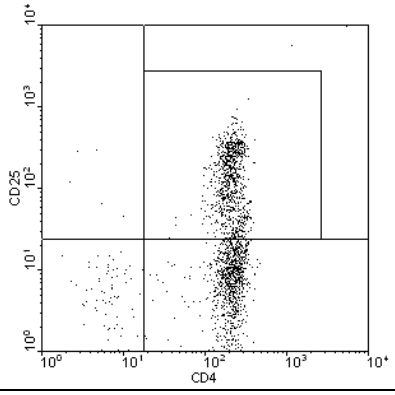
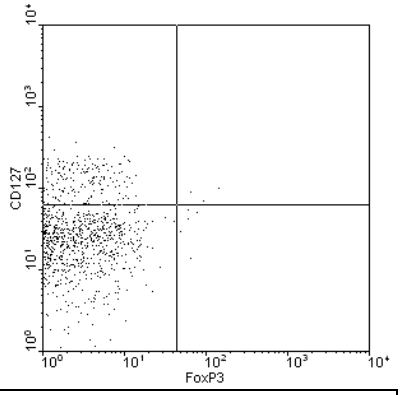
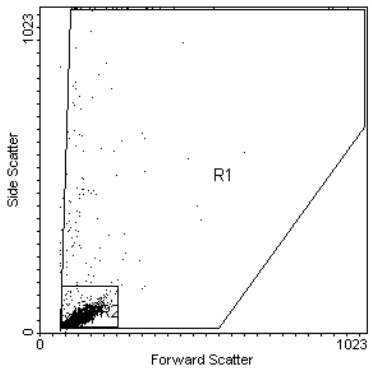
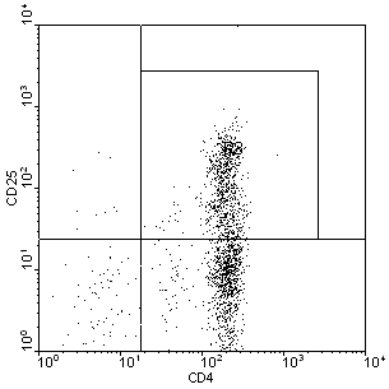
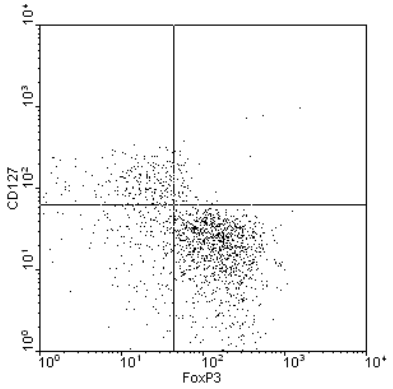
A

Total PBMC – Unstained		
		
CD4-PE 38.24 %	**CD8-FITC 20.46 %**	**CD25-PE 1.68 %**
		
CD4 cells – Unstained	**CD4-PE 82.80 %**	
		

Non CD4 cells – Unstained


CD4-PE 11.20 %	CD8-FITC 26 %	CD25-PE 0.66 %
		

B

Unstained cells –Total PBMC **R1 events 98.45 %**	**R2 events 92.08 %**	
		
Negative control – Total PBMC **R1 events 97.52 %**	**CD4-FITC and CD25-APC** **R2 events = 92.46 %** **CD4 single positive** **events = 50.62 % of R2** **CD4 positive CD25 positive** **events = 44.12 % of R2**	**Gated on R3** **(No FoxP3 staining)**
		
CD4 postive CD25 positive cells **R1 events 97.44 %**	**CD4-FITC and CD25-APC** **R2 events = 92.48 %** **CD4 single positive** **events = 54.54 % of R2** **CD4 positive CD25 positive** **events = 42.26 % of R2**	**Gated on R3** **FoxP3-PE events = 70.46 %** **of R3**
		

C

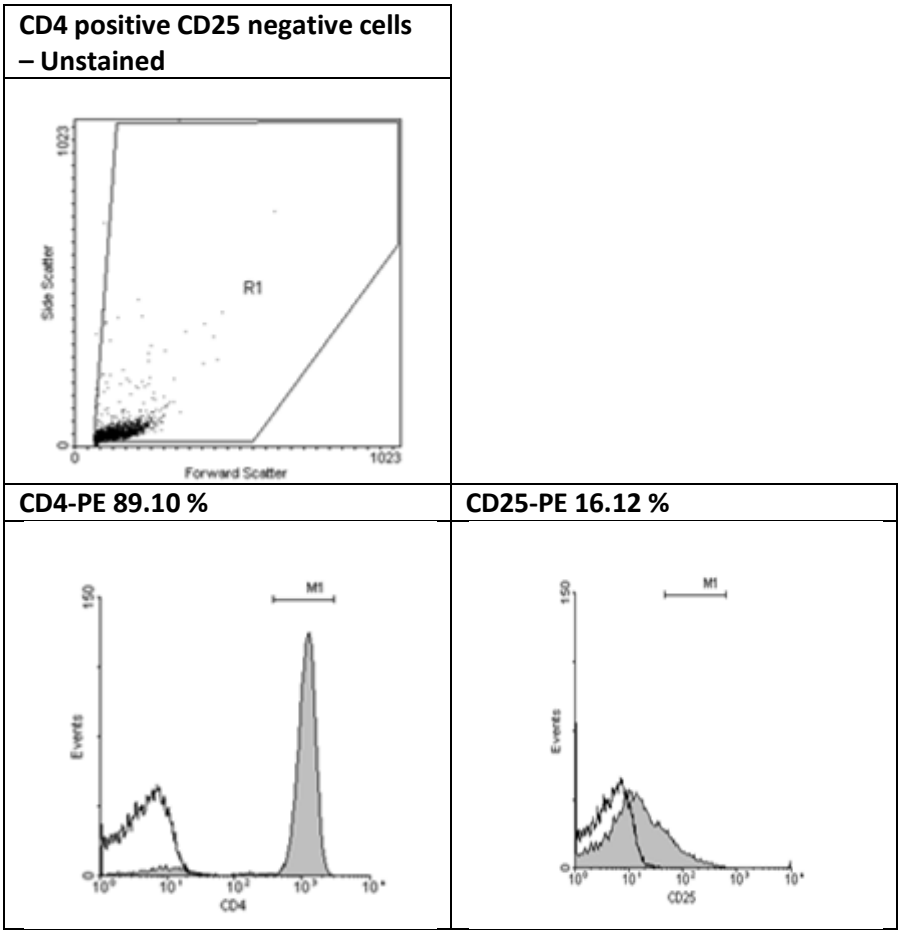
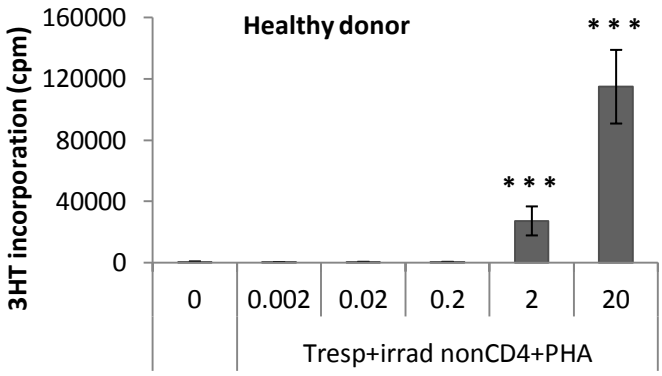


Figure 4.17: Assessing purity of Tregs. In order to ensure the quality of cells used for Tregs suppression assay, the identity and purity analysis was performed using cell surface staining. The Figure 4.17a represents the dot plot for unstained cells as background control. The panel also includes the histograms for PBMCs stained for PE conjugated anti-CD4, FITC conjugated anti-CD8 and PE conjugated anti-CD25 antibodies. The baseline percentages of cells were obtained at this stage. The isolated $CD4^+$ T-cells were stained for PE conjugated anti-CD4 and non $CD4^+$ T-cells were stained for PE conjugated anti-CD4, FITC conjugated anti-CD8 and PE conjugated anti-CD25 antibodies. The Figure 4.17b represents the dot plot for unstained cells as background control. The panel also includes the dot plots for PBMCs stained for FITC conjugated anti-CD4 and APC conjugated anti-CD25 antibodies. The isolated $CD4^+CD25^{high}$ Tregs are stained for FITC conjugated anti-CD4, APC conjugated anti-CD25, PE-Cy5 conjugated anti-CD127 and PE conjugated anti-FoxP3 antibodies. The Figure 4.17c represents dot plot for isolated $CD4^+CD25^{low}$ Tresp cells and are stained for PE conjugated anti-CD4 and PE conjugated anti-CD25 antibodies. Isotype control antibodies

were used for all cell surface staining. The dot plot was obtained using WinMDI 2.8, a FACS data analysis tool.

A



B

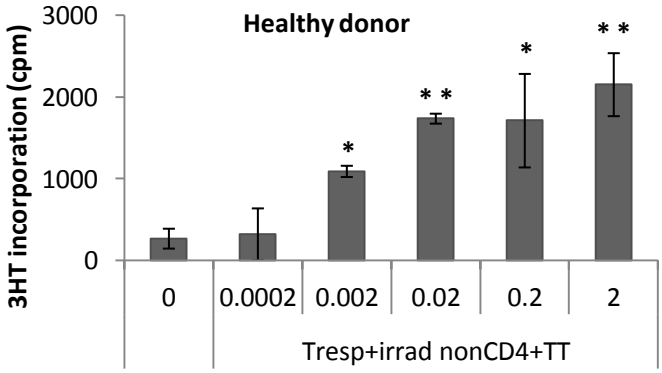
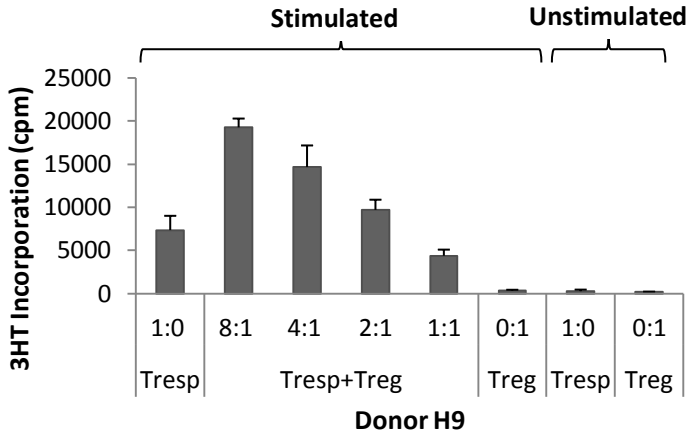
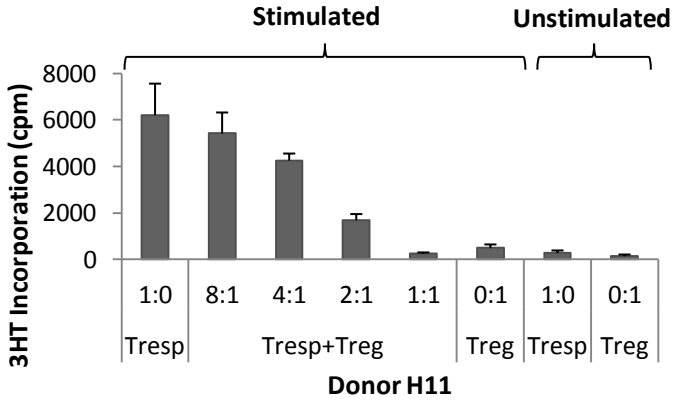


Figure 4.18: Validation of Treg suppression assay. The Figure 4.18a represents the proliferation of a healthy donor PBMCs to polyclonal stimulation phytohaemagglutinin. The $CD4^{+}CD25^{low}$ T-cells were co cultured with irradiated non $CD4^{+}$ T-cells at different concentrations of phytohaemagglutinin. The 3HT was added to the culture on day 3 and the proliferation was measured on day 4. The Figure 4.18b represents the proliferation of a healthy donor PBMCs to antigen stimulation Tetanus toxoid. The $CD4^{+}CD25^{low}$ T-cells were co cultured with irradiated non $CD4^{+}$ T-cells at different concentrations of Tetanus toxoid. The 3HT was added to the culture on day 5 and the proliferation was measured on day 6. The statistical differences were determined using ANOVA with Dunnett's post hoc test when compared to no stimulation. The different concentrations of phytohaemagglutinin and Tetanus toxoid are indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results are shown are mean cpm ± SD in presence and absence of phytohaemagglutinin and Tetanus toxoid.

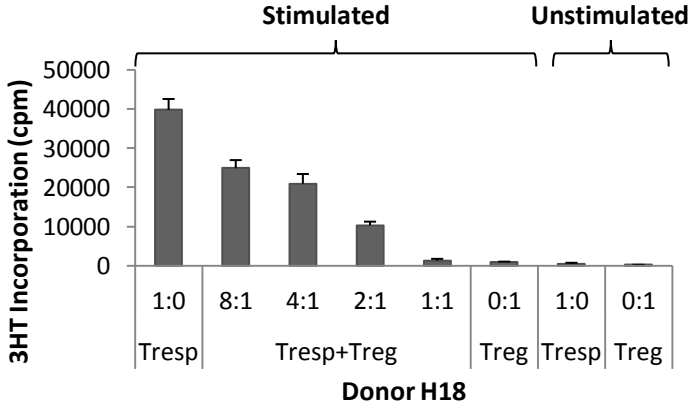
A


Stimulated
Unstimulated
3HT Incorporation (cpm)
25000
20000
15000
10000
5000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor H9

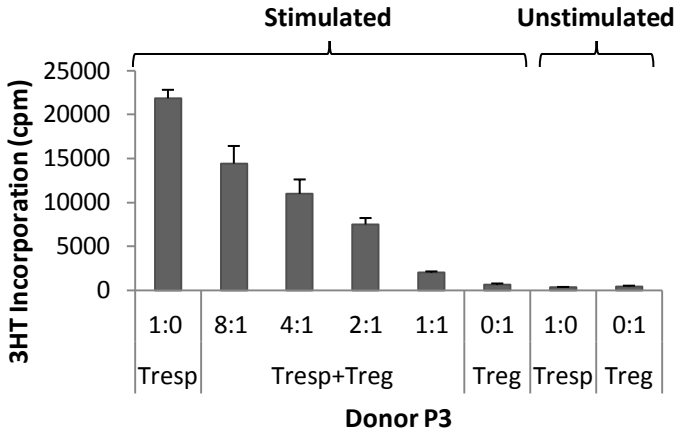
B


Stimulated
Unstimulated
3HT Incorporation (cpm)
8000
6000
4000
2000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor H11

C


Stimulated
Unstimulated
3HT Incorporation (cpm)
50000
40000
30000
20000
10000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor H18

D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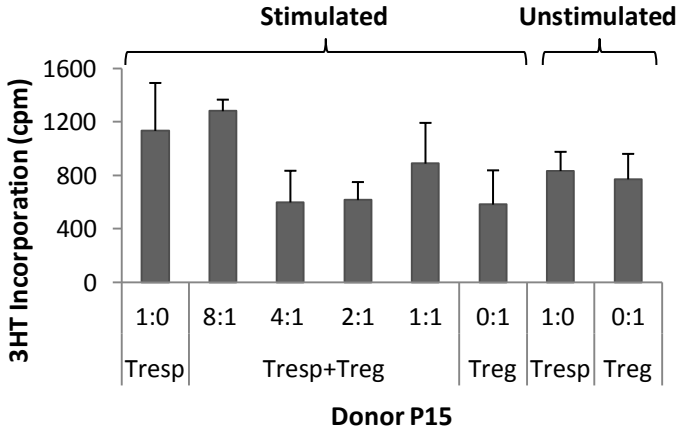
Unstimulated
3HT Incorporation (cpm)
25000
20000
15000
10000
5000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor P3

E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Unstimulated
3HT Incorporation (cpm)
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800
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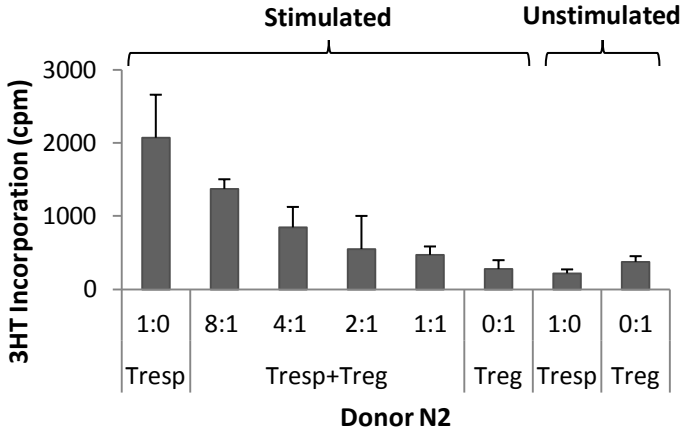
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor P15

F


Stimulated
Unstimulated
3HT Incorporation (cpm)
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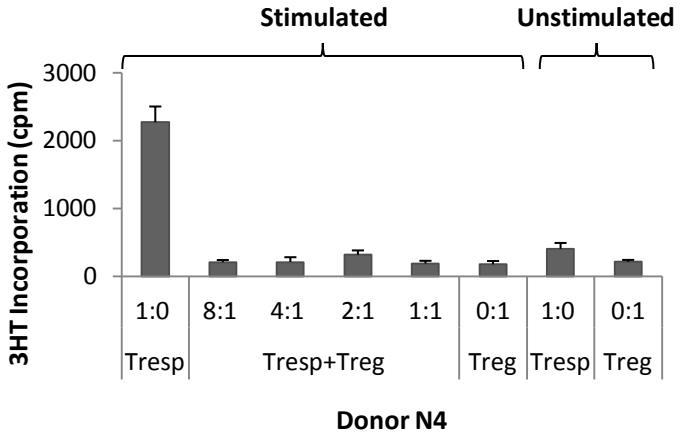
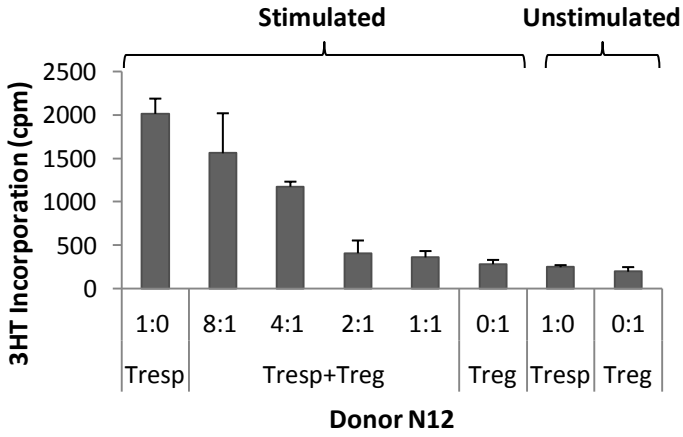
2000
1000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor N2

G


Stimulated
Unstimulated
3HT Incorporation (cpm)
3000
2000
1000
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor N4

H


Stimulated
Unstimulated
3HT Incorporation (cpm)
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2000
1500
1000
500
0
1:0
8:1
4:1
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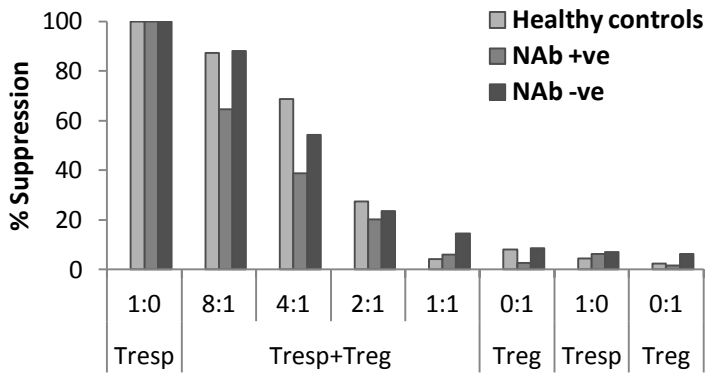
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg
Donor N12

I


Healthy controls
NAb +ve
NAb -ve
% Suppression
100
80
60
40
20
0
1:0
8:1
4:1
2:1
1:1
0:1
1:0
0:1
Tresp
Tresp+Treg
Treg
Tresp
Treg

J

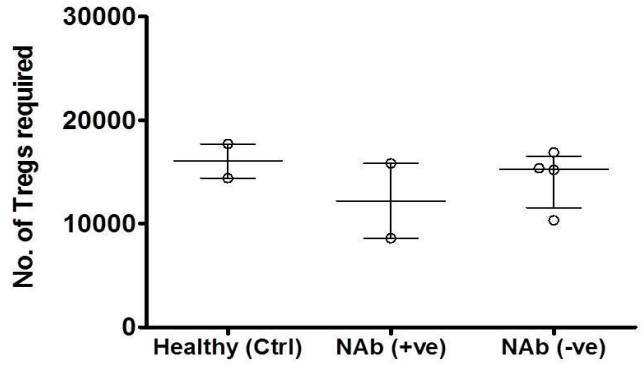


Figure 4.19: In vitro Tregs suppression assays. In order to determine the functional activity of Tregs in vitro Tregs suppression assay was used to investigate Tregs function in healthy donors H9, H11, H18 (refer Figures 4.19a, b, c), IFN-β-1a Rebif™ neutralising antibodies positive donors P3, P15 (refer Figures 4.19d, e) and negative donors N2, N4, N12 (refer Figures 4.19f, g, h). Isolated $CD4^{+}CD25^{high}$ Tregs were co cultured with $CD4^{+}CD25^{low}$ Tresp in different ratios. The phytohaemagglutinin at 20 µg/ml was used to stimulate the cells. The controls $CD4^{+}CD25^{high}$ Tregs and $CD4^{+}CD25^{low}$ Tresp were cultured without phytohaemagglutinin. The proliferation was determined by measuring 3HT incorporation. The different ratios of T-cell fractions are indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in the presence and absence of stimulation. The Figure 4.19i represents the % suppression of Tregs using phytohaemagglutinin in all the three groups. The Figure 4.19j represents the number of Tregs required to suppress 50 % of Tresp in vitro. Each open circle represents an individual no. of Tregs required to suppress Tresp in vitro. Statistical differences were not observed between differences in any groups and comparisons were determined using Kruskall-Wallis test for more than two nonparametric groups.

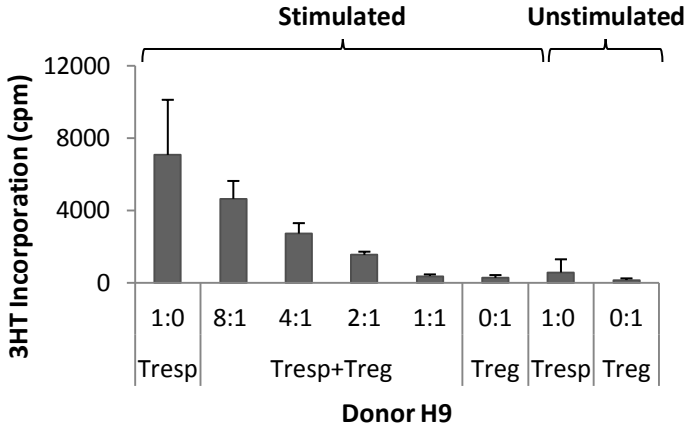


Figure 4.20: In vitro antigen specific Tregs suppression assay. In order to demonstrate the antigen specific Tregs suppression assay isolated $CD4^{+}CD25^{high}$ Tregs were co cultured with $CD4^{+}CD25^{low}$ Tresp in different ratios. In this experiment Tetanus toxoid 2 µg/ml was used to stimulate the cells. The proliferation was determined by measuring 3HT incorporation. The different ratios of T-cell fractions are indicated on the X-axis and 3HT incorporation (cpm) is shown on the Y-axis. Results shown are mean cpm ± SD in the presence and absence of antigenic stimulation.

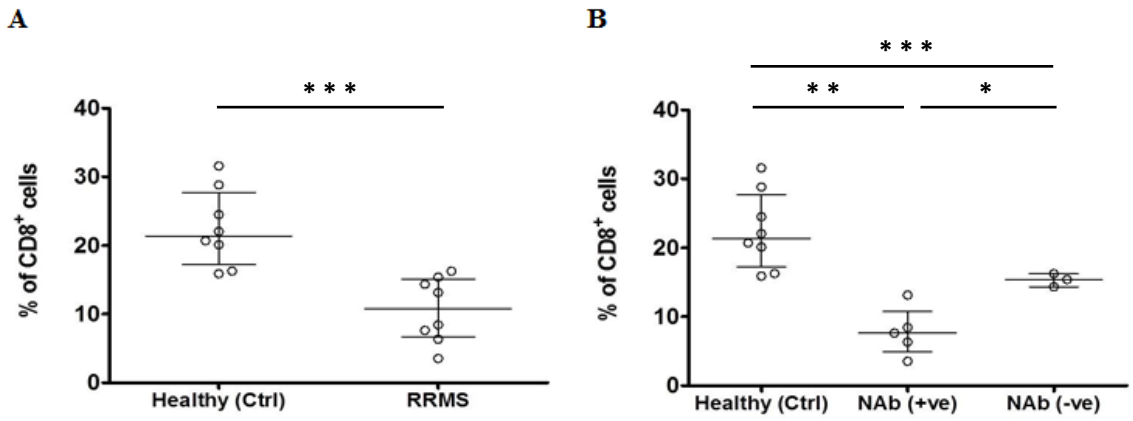


Figure 4.21: Frequencies of $CD8^{+}$ T-cells. The PBMCs were stained for $CD8^{+}$ T-cell surface marker whilst isolating Tregs. The Figures 4.21a and b represents the frequencies of $CD8^{+}$ T-cells in healthy donors and IFN-β-1a Rebif™ neutralising antibodies positive and negative donors in relapsing forms of multiple sclerosis. Each open circle represents an individual percentage of $CD8^{+}$ T-cells from the peripheral blood PBMCs. Statistically significant differences are shown in panels and comparisons were determined using Mann Whitney U test for two non-parametric groups and Kruskall-Wallis test for more than two nonparametric groups.

4.4 Discussion

The main area of interest was to identify T helper cell responses to IFN-β-1a Rebif™ and any particular peptide epitopes that are involved. Heat inactivation was chosen as a method to ablate the therapeutic protein IFN-β-1a Rebif™ because it may not be possible to stimulate PBMCs directly as the protein modifies the function of responding T-cells. IFN-β-1a Rebif™ exhibit anti-proliferative activities and using the protein directly on PBMCs will always suppress T-cell proliferative responses. Stickler et al measured T-cell activation in healthy donors using heat inactivated IFN-β. The Figure 4.3 demonstrates IFN-β-1a exhibits significant anti-proliferative function. Therefore, Stickler et al heat inactivation protocol for heat inactivating IFN-β-1a was pursued and found using 30 minutes of heat inactivation was sufficient to stimulate the PBMCs. In order to understand the cellular basis of heat inactivated IFN-β-1a Rebif™ T-cell responses the donors P3 and P10 PBMCs were attempted for T-cell cloning. The T-cell cloning will provide a platform to understand and analyse the functionalities of immune mechanisms for example mediated by T-cells, antigen presentation, the nature and mode of action of cytokines, the cell surface molecules and also provide insights into signalling molecules that orchestrate T-cell activation. However, may not represent the whole population of T-cells in *in vivo* settings. The antigen specific T-cells can be detected after in vitro stimulation with the antigen of interest. Steinitz KN et al generated Factor VIII specific T-cell clones to assess Factor VIII peptides specificity (Steinitz et al. 2012) in humanised mouse models. Furthermore, Jacquemin M et al have identified T-cell epitopes of Factor VIII recombinant protein using $CD4^+$ T-cell clones which are isolated from Haemophilia A patient (Jacquemin et al. 2003). Identification of $CD4^+$ T-cells epitopes is considered as a rational approach for assessing immunogenicity of the protein therapeutics. In our study the T-cell cloning experiment provided several T-cell lines and after initial stimulation with heat inactivated IFN-β-1a Rebif™ two responsive clones was generated. Unfortunately, after expansion of these responsive cells, the cells failed to respond further in a dose dependent manner indicating non specificity. The EBV-transformed lymphoblastoid cell line was used to stimulate the obtained IFN-β-1a Rebif™ T-cell lines. The evidence also indicates stimulation with protein loaded monocyte-derived dendritic cells is more effective in expanding antigen specific T-cell population (Ho et al. 2006). Such approaches could be adopted to increase the efficiency of generating T-cells clones. Further investigations into the obtained IFN-β-1a Rebif™ T-cell lines for example TcR vβ repertoire to determine T-cell clonality (Bonfigli et al. 2003; Gennery & Cant 2007; van den Beemd et al. 2000)could provide additional insights.

In order to further investigate the presence of IFN-β-1a Rebif™ T-cell epitopes in silico methods were utilised due to limited number of patient PBMCs. In silico predictions are of advantage to the extent especially in pharmaceutical industry but the servers also indicate for further in vitro assessments. The ideal situation is to examine IFN-β-1a Rebif™ overlapping peptides of the entire sequence for T-cell activation. In order to overcome the limited number of cells the PBMCs could have been stimulated with IFN-β-1a Rebif™ peptides pools. However, the PBMCs were stimulated using the identified IFN-β-1a Rebif™ 17mer 5 offset peptides because of limited number of PBMCs. The dataset indicates the different degree of T-cell activation and also compatible to the previously published data (refer Figures 4.8). Several T-cell activation assays are available to identify T-cell epitopes such as CFSE T-cell proliferation assays and this enables accurate measurements of proliferating CD^+ T-cells. ELISpots detects the secretion of cytokine at a single cell level and therefore using ELISpots for T-cell epitope identification can be more sensitive. In this study ELISpots for IFN-γ was used to validate the IFN-β-1a Rebif™ peptides identified (refer Figures 4.9 and 4.10). In order to further understand the cellular basis of IFN-β-1a Rebif™ peptides T-cells responses the donor P15 was used for T-cell cloning. The T-cell cloning experiment provided several T-cell lines and after initial stimulation with respective IFN-β-1a Rebif™ peptides three responsive clones was generated (refer Figures 4.12). The expansion of cells caused T-cell clones to become non-responsive. In our T-cell cloning experiments B-LCLs was used as antigen presenting cells to stimulate the cells obtained. In order to establish maximum stimulation DC T-cell assays are found to be beneficial, sensitive and versatile which measures if the protein can induce T-cell proliferation. For example, Ho et al generated high avidity T-cell clones against WT1 (tumour associated antigen) using protein loaded monocyte-derived dendritic cells (Ho et al. 2006).

The evidences also suggest link between the HLA and activation of antigen specific T-cells. Therefore, the identified IFN-β-1a Rebif™ peptides were tested in 50 randomly selected healthy donors of whom for 30 donors the HLA genotyped PBMCs were used from the cell archive. Using 30 randomly selected healthy donors represent or mimic the HLA allele frequencies found in global population. Initial stimulation using IFN-β-1a Rebif™ peptides revealed proliferative responses to IFN-β-1a Rebif™ peptide no. 1 (refer Figure 4.13). However, executing T-cell proliferation assays in 30 donors revealed proliferative responses to IFN-β-1a Rebif™ peptide 3 in HLA-DRB1*1501 haplotype (refer Figures 4.13 and 4.14). The HLA-DRB1*1501 represents an important locus for several diseases for example Type I

diabetes, rheumatoid arthritis and multiple sclerosis (Davies et al. 1994; Nepom & Erlich 1991; Todd et al. 1988). Studies on DRB1*1501 with antigenic peptides has been investigated for example using human MBP to study multiple sclerosis and concludes this allele is characterised by hydrophobic P4 pocket in presence of alanine at DRβ71 position (Smith et al. 1998). The major anchor residue for human MBP peptide (residue 85-99) was phenylalanine. Understanding and identification of the antigenic anchor residues could also reveal those residues that may be important for TCR contact. In our studies we speculate hydrophobic amino acids in IFN-β-1a Rebif™ peptide CAWTIVRVEILRNFYFI might participate in T-cell activation and these amino acids are in the predicted positions for HLA-DRB1*1501 binding. The hydrophobic amino acids in IFN-β-1a Rebif™ peptide sequence are coloured in red. Steinitz KN et al 2012 has also identified Factor VIII peptide regions presented by HLA-DRB1*1501 in humanised haemophilic mouse model. However, the identified IFN-β-1a Rebif™ peptide presented by HLA-DRB1*1501 by healthy donors can be cross examined using available in silico technologies. The clear understanding of peptide binding affinities to specific HLA haplotype can determine the molecular interactions involved in the aetiology of diseases and also assist in development of effective drugs. Chang et al reports identification of peptide binding repertoires can provide effective development of epitope-based vaccines (Chang et al. 1997). In our studies we identified HLA-DRB1*1501 association in IFN-β-1a Rebif™ peptide. However, the evidence provided here requires further characterisation. The possible studies include generation of IFN-β-1a Rebif™ peptide specific T-cell clones using HLA-DRB1*1501 PBMCs and characterising the obtained T-cell clones. The clones can also be used to define the functional epitopes on HLA-DRB1*1501 molecule (Goronzy, Weyand & Waase 1985). The potential association between HLA haplotype and T-cell response to IFN-β-1a Rebif™ peptide indicate a potential risk factor for development of antibodies against IFN-β-1a Rebif™. Interestingly, the identified HLA DRB1*1501 has been associated with increased susceptibility to multiple sclerosis (Alcina et al. 2012).

The frequency of circulating Treg cells subpopulation in relapsing forms of multiple sclerosis with IFN-β-1a Rebif™ treatment and in healthy donors was also evaluated. The Figures 4.16 suggest there was no significant difference in the global Tregs frequencies and function. Feger U et al has determined increased frequencies of Tregs in the CSF of multiple sclerosis patients (Feger et al. 2007). The evidence also suggests the deficit in Tregs suppressive function in multiple sclerosis patients (Frisullo et al. 2009). Namder et al suggests IFN-β

treatment in multiple sclerosis may restore Tregs function (Namdar et al. 2010). The data obtained in our studies indicate no significant difference in the Tregs function between IFN-β-1a Rebif™ neutralising antibodies positive, negative and in healthy donors. The use of IFN-β-1a Rebif™ or peptides in Tregs suppression assays to determine the antigen specific Treg function could provide more meaningful data. However, to achieve this, antigenic epitopes within IFN-β need to be determined. Venkan et al has shown the difference in naïve and memory Tregs in patients and in healthy controls. The naïve Tregs was defined as $CD4^{+}CD25^{high}CD127^{low}CD45RA^{+}$ (nTregs) and memory Tregs was defined as $CD4^{+}CD25^{high}CD127^{low}CD45RA^{-}$ (mTregs) (Venken et al. 2008). Constantino et al has shown no difference in Treg frequency and function from untreated patients and in healthy controls. The Tregs was defined as $CD4^{+}CD25^{high}$ T-cell population (Costantino, Baecher-Allan & Hafler 2008). Our results are in agreement with Constantino et al as possibly due to the fact that we focus on at $CD4^{+}CD25^{high}$ T-cell population. A finer resolution of Treg subpopulations might uncover any potential differences.

4.5 Conclusions

- Using T-cell epitope prediction tools (IEDB–AR and ProPred) and T-cell functional assays we identified an immunogenic sequence of 36 amno acids within IFN-β molecule (position 130-166).
- The data also suggest one IFN-β peptide within this sequence is a potential T-cell immunogenic epitope.
- Identified possible association of one of the IFN-β peptide with HLA haplyotype DRB1*1501.

Chapter 5: Final discussion

Unwanted immunogenic responses such as anti-drug antibodies to protein therapeutics can cause decrease in efficacy, hypersensitivity reactions, anaphylaxis, induction of autoimmunity and cross reactivity to endogenous proteins. Interferon beta (Rebif™, Avonex™, Betaseron/Betaferon™) is approved for chronic administration to relapsing forms of multiple sclerosis. Immunogenicity resulting from IFN-β-1a Rebif™ administration is significant complication associated with protein therapeutics. The mechanisms of development of anti-IFN-β antibodies are poorly understood. The overall aim of the work was to characterise the immunogenic responses to therapeutic IFN-β-1a Rebif™.

As part of the thesis work, a systematic review was conducted and findings from which suggest 1.22 – 19.44 % of patients developed neutralising antibodies to Avonex™, 15.20 – 33.25 % of patients developed neutralising antibodies to Rebif™ and 27.38 – 58.79 % of patients developed neutralising antibodies to Betaseron/Betaferon™. Overall the development of neutralising antibodies was less in IFN-β-1a Avonex™, moderate in IFN-β-1a Rebif™ and high in IFN-β-1b Betaseron/Betaferon™. Based on this review IFN-β-1a Avonex™ 30 µg IM should be the drug of choice for patients suffering from relapsing forms of multiple sclerosis. An attempt was made to capture all the factors affecting the development of neutralising antibodies. Therefore where necessary data are available the meta-analysis was executed. Our review managed to perform the comparison of neutralising antibodies against IFN-β-1a Avonex™ 30 µg and 60 µg, IFN-β-1a Rebif™ 22 µg and 44 µg and IFN-β-1a and IFN-β-1b. However, due of limited number of studies and variability in reporting we could not able to perform the relevant analysis for example frequency of administration for the contribution of development of neutralising antibodies.

In experimental chapters I have characterised anti-drug antibodies in a cohort of MS patients, have explored the neutralising capabilities of IgG subclass specific antibodies, tested the potential of ADAs to form immune complexes and activate complement, and have addressed the role of T-cells in development of anti-drug antibodies.

5.1 Do IgG subclass specific anti-drug antibody responses to Interfeon beta 1a Rebif™, have differential neutralising capabilities?

The work conducted was to characterise anti-IFN-β-1a Rebif™ antibodies against therapeutic IFN-β-1a Rebif™ in a cohort of IFN-β-1a Rebif™ treated patients and to determine the relative contribution of anti-IFN-β-1a Rebif™ antibodies in neutralising IFN-β bioactivity.

The analyses of plasma samples identified that ADAs were predominantly of IgG1 and IgG4 antibodies. IgG4 antibodies are implicated in greater neutralisation potential compared to other IgG subclasses. Out data showed IgG4 antibodies are likely to be the major contributor to the neutralisation by anti-IFN-β-1a Rebif™ antibodies. But, this need further investigation perhaps increasing sample size and investigating using purified forms of IgG4 antibodies for investigating differential neutralising capabilities are beneficial . The longitudinal studies are required to investigate the evolution of IgG4 antibodies.

The role of IgG1 antibodies also needs to be further explored. Clearly, IgG1 antibodies also contribute towards the neutralising potential. Hence, isolating IgG1 antibodies and investigating for neutralising potential could answer important questions relevant to immunogenicity. In order to further elucidate the evolution of IgG subclasses in anti-IFN-β antibodies, clearly defined longitudinal analysis are required which can provide insights into development of anti-drug antibodies.

The evidence suggests IgG subclasses patterns vary in IFN-β-1b treated compared to IFN-β-1a treated patients. In IFN-β-1b treated patients the IgG1 and IgG3 antibodies peaked within first 6 months followed by progressive decline, whilst IgG4 antibodies increased later after 24 months. In contrast, in IFN-β-1a treated patients IgG1, 2 and 4 peaked after 18 months thereafter with progressive decline whilst IgG4 antibodies begin to increase. IgG4 antibodies were prominent in neutralising antibodies positive patients. However, correlations on the implications of different IgG subclass antibodies with clinical outcomes are difficult to make. There is some evidence in the case of other biologics that neutralising antibodies may tend to disappear or be reduced on prolonged treatment (Hegen et al. 2012b; Pungor et al. 1998; Rice et al. 1999). However, in the case of IFN-β, anti-drug antibodies can persist for a long time even after cessation of treatment, which could still impact patient safety. In terms of persistence of different subclasses, IgG1, IgG2 and IgG4 antibodies exhibits 21 – 23 days of half life whereas IgG3 antibodies exhibit 7 – 8 days of half life. Because of long survival the IgG1, 2 & 4 antibodies may give rise to immune complex formation and persist in circulation for indefinite period of time (Suen et al. 2003). However, the importance of IFN-β specific IgG subclasses half-life in neutralisation potential remains to be investigated. The development of anti-drug antibodies against other biologics for example IFN-α, erythropoietin, Factor VIII, IX, insulin, GM-CSF, anti-TNF alpha has also resulted in compromised therapeutic efficacy. The IgG subclass profiling for IFN-α indicated all four IgG subclasses were involved IgG1 35 %, IgG2 10 %, IgG3 27 % and IgG4 28 % (Gregorek

et al. 2000). The detailed investigation of IgG subclass profiling for anti-erythropoietin antibodies indicate anti-EPO IgG1 antibodies in all clinical patients but neutralising antibodies were associated with anti-EPO IgG4 antibodies (Barger et al. 2012). The distinct characteristics of IgG subclass profiling for anti-factor VIII antibodies indicate both IgG1 and IgG4 antibodies in patients with FVIII inhibitors however IgG4 antibodies was absent in healthy controls (Whelan et al. 2013). Orstavik et al reports anti-factor IX antibodies contain all the IgG subclasses except IgG3 antibodies (Orstavik 1990). The IgG subclass response in anti-insulin antibodies involves IgG1, IgG3 and IgG4 and the anti-insulin antibodies also vary with type of diabetes. The evidence suggests the variations in IgG subclass profiles exist depending on the protein therapeutic. Sometimes development of anti-drug antibodies caused life threatening conditions.

5.2 Do neutralising antibodies neutralise both exogenous and endogenous Interferon beta?

Investigation of neutralising IFN-β-1a Rebif™ antibodies on endogenous IFN-β revealed varying degrees of neutralisation of endogenous IFN-β. High titre neutralising antibodies can persist for years and may impede IFN-β signalling and impacts the immune system. Potential consequences include susceptible to viral infections. It has been indicated neutralisation of endogenous proteins may potentially impact fetal development when such a response is generated during pregnancy or whilst breast feeding (Guidance document on immunogenicity assessment for proteins drugs issued from U.S Department of Health and Human Services http://www.fda.gov/downloads/Drugs/.../Guidances/UCM338856.pdf assessed February 2013). Furthermore, the evidence also indicates red cell aplasia due to anti-erythropoitin antibodies (Casadevall et al. 2002), thrombocytopenia due to anti-thrombopoietin antibodies (Li et al. 2001b) are a result of cross reactivity to endogenous protein counterpart.

5.3 Does Interferon beta 1a Rebif™ specific anti-drug antibodies form immune complexes with Interferon beta 1a Rebif™ and can such complexes activate the classical complement pathway?

Our data demonstrated the IFN-β-1a Rebif™ specific anti-drug antibodies formed immune complexes with IFN-β-1a Rebif™ and activated complements. Delayed hypersensitivity reactions are those mediated through immune complexes and T-cells (Hunley et al. 2004). The clinical signs and symptoms of immune complex deposition include flu like symptoms, rash, arthralgia, myalgia, proteinuria, hematuria and so on. There is some evidence in the

form of a case report on IFN-β-1b treated patient developing severe vasculitis like skin reaction (delayed severe cutaneous reaction) after 9 months of treatment (Cohen, Greenberger & Saini 1998). Some of the signs are suggestive of immune complex formation has also been shown in patients undergoing tolerance induction treatment using Factor IX and alpha-glucosidase (Hunley et al. 2004). Immune complexes can activate complement through classical pathway. The evidence indicates development of anti-drug antibodies can lead to complement activation with subsequent production of anaphylatoxins such as C3a and C5a and release of mediators from mast cells (Vultaggio, Maggi & Matucci 2011).

The release of C3a and C5a are implicated in enhancing antigen processing and presentation and also in maintenance of tolerance (Hegen et al. 2012b; Strainic et al. 2008; Weaver et al. 2010). The evidence suggests C3a and C5a enhance function of DCs and generate T helper 1 cells. The role of C3a and C5a in immunogenicity needs to be fully explored. The activated complements C3a and C5a influence antibody responses through modulating DCs, T-cells and B-cells function. The antigen presenting cells express complement receptors, the complement regulatory proteins for optimal maturation and T-cell activation (Hashimoto et al. 2010; Kerekes et al. 2001; Weaver et al. 2010; Zhou et al. 2006). Primary APC T-cell assays in conjunction with targeting of complement receptors (by genetic and pharmacological means) need to be performed to assess the role of complements in T-cell activation (Strainic et al. 2008).

5.4 Could antibody-binding sites on Interferon beta 1a Rebif™ be identified?

An attempt was made to investigate the potential antibody linear epitopes. The multipin peptide technology revealed significant binding residues 1–32, 40–62, 70–86, 106–112 and 136–166 on IFN-β-1a Rebif™ molecule and the in vitro competitive binding assay indicated no linear binding epitopes on IFN-β-1a Rebif™ molecule. The identification of antibody epitopes can potentially lead to strategies to avoid immunogenicity. It is conceivable that reengineering of IFN-β-1a Rebif™ molecule such as substitutions or deletions of amino acids within predicted epitope regions might reduce the incidence of development of anti-drug antibodies. However, such a reengineering should not result in compromise in IFNb functional or receptor binding activities. Formation of neoepitopes through reengineering is also a possibility and needs to be taken into consideration.

5.5 Could T helper cells specific for Interferon beta 1a Rebif™ epitopes be identified in anti-drug antibodies positive patients?

In order to understand the role of T helper cells in immunogenicity of IFN-β, we identified T-cell epitopes using T-cell prediction tools and overlapping (17mer, 5 offset) IFN-β-1a Rebif™ peptides in cells from patients, healthy donors and HLA genotyped cell archive from healthy donors.

T-cell prediction tools IEDB–AR and ProPred was used to predict MHC class II bindig sequence of 36 amino acids within IFN-β-1a Rebif™ (position 130 – 166). The overlapping (17mer, 5 offset) IFN-β-1a Rebif™ peptides were synthesised. The reactivities of in vivo primed cells using T-cell proliferation assays and IFN-γ ELISpots suggest the identified IFN-β-1a Rebif™ peptides are relatively immunogenic in some ADA positive donors. However, T-cell activation was also observed in some ADA negative and healthy cells. Such T-cell responses in healthy subjects have also been observed in the case of other biologics, for example EPO (Barger et al. 2012).

In order to address the HLA association with T helper cell activation to IFN-β-1a Rebif™ and development of anti-drug antibodies, the identified IFN-β-1a Rebif™ peptides was investigated for the presence of T-cell epitopes in 30 HLA genotyped healthy donors. The data indicates IFN-β-1a Rebif™ peptide 3 was associated with the presence of HLA DRB1*1501 allele. Several associations between the specific HLA and diseases (for e.g. HLA-DRB1*0401 is associated with Type 1 Diabetes, HLA-DRB1*1501 is associated with multiple sclerosis, HLA-DRB1*5701 and DRB1*3501 is associated with HIV infections), specific HLA and activation of T-cells (for e.g. HLA B*5701 is associated with abacavir hypersensitivity, HLA A*3101 is associated with carbamazepine hypersensitivity, HLA B*1502 is associated with carbamazepine provoked Stevens Johnson syndrome, HLA B*5701 is associated with flucloxacillin induced liver injury) and specific HLA and immunogenicity against biologics (for e.g. HLA-DRB1*0401, HLA-DRB1*1601, HLA-DRB1*0408, HLA-DRB1*0701, HLA-DR2, HLA-DQ6, DQB1*0602, HLA-DR15 is associated with formation of anti-IFN-β antibodies, HLA-DQA1*0102, HLA-DR4.1, DQ4, DQA1*0301, HLA-DRA-DRB1*1104 is associated with formation of anti-Factor VIII antibodies, HLA-DRB1*09-DQB1*0309 is associated with anti-erythropoietin antibodies) have been discovered. It is possible that stratifying individuals for treatment according to HLA can reduce risk for development of anti-drug antibodies against biologics. Additionally, strategies that can be employed to decrease immunogenicity is by eliminating T-cell epitopes (Jones et al. 2009). Depleting T-cell epitopes from therapeutic proteins has been confirmed to reduce immunogenicity and several deimmunised proteins are in clinical trials for example

antibody drug conjugates such as Brentuximab vedotin SGN-35 for Hodgkin lymphoma, Inotuzumab ozogamicin for non-Hodgkin lymphoma and Trastuzumab-DM1 for breast cancer (Alley, Okeley & Senter 2010; Baker & Jones 2007).

5.6 Does a difference in regulatory T-cells frequencies and subsequent immunosuppressive function lead to Interferon beta 1a Rebif™ immunogenicity?

In order to investigate the role of Tregs in immunogenicity the frequency of Tregs in PBMCs of IFN-β-1a Rebif™ neutralising antibodies positive, negative and healthy donors were investigated. The data suggest no significance in global Tregs. The functionalities of Tregs were assessed using Tregs suppression assay and no significant differences were found. Our Tregs project has number of limitations. Tregs from PBMCs were isolated and it was challenging to retain the functionalities of isolated Tregs. Furthermore, phytohaemagglutinin was used to stimulate Tregs in Tregs suppression assay. The use of antigenic stimulation for example using antigenic IFN-β-1a Rebif™ or peptides to stimulate the Tregs is more appropriate to understand the role of Tregs in immunogenicity. There is a potential strategy that can induce tolerance to biologics by expanding Tregs. Expanding induced Tregs ex vivo and injecting the same can be an option for inducing donor specific tolerance (Nesburn et al. 2007). The studies on mice with interferons have shown anti-IFN IgG antibodies are inhibited by oramucosal administration (Meriter et al. 2001). Nagal – Anderson C et al concludes the induction of tolerance in oromucosal administration is dependent on tolerising dendritic cells and Tregs (Nagler-Anderson & Shi 2001).

Furthermore, T-cell epitopes which are associated with Tregs and termed as Tregitopes are being investigated for suppressing immunogenicity (Nakatsui, Wong & Groot 2008).

5.7 Concluding remarks

In conclusion, the work described in this thesis has sought to understand immunogenicity to IFN-β-1a Rebif™ in relapsing forms of multiple sclerosis through characterisation of humoral and cellular immune responses to IFN-β-1a Rebif™.

The work detailed in this thesis has provided new information on the subclass profile of IFN-β ADAs, the potential involvement of T helper cells and possible antibody epitopes within IFN-β. Future studies should be aimed at providing finer detail on the evolution of the ADA response and identify strategies to remove immunogenic determinants from IFN-β and other biologics.

Appendices

Appendix 1 List of Biologics

Biologic	Type & Mechanism of action	Indications	Immune related adverse events
Abatacept	Fusion protein (CTLA4-Fc-IgG1) which binds to CD80 and/or CD86 to prevent interaction with CD28 and so inhibits selective T-cell co-stimulation	RA, JIA	No boxed or product label warning issued
Abciximab	Is a glycoprotein (GP) IIb/IIIa receptor inhibitor that binds to GP IIb/IIIa receptor on the platelet, blocking interaction of fibrinogen with GP IIb/IIIa receptor which is a final pathway for aggregation of platelets	Used as adjunct to percutaneous coronary intervention (a procedure to open the blocked arteries in the heart)	Anaphylaxis (Guzzo & Nichols 1999) Warning for thrombocytopenia on product label
Adalimumab	Human mAb which binds to TNF and blocks its interaction with its receptors	RA, JIA, PsA, AS, CD	Boxed warning for serious infections (tuberculosis and invasive fungal infections) and malignancy (lymphoma and leukaemia) Immunogenicity reported (Bartelds et al. 2011) Warnings for cytopenias: pancytopaenia, lymphopaenia and thrombocytopaenia on product label

Aflibercept	Fusion protein (VGEF receptors 1 and 2 extracellular domains:Fc-IgG1) which inhibits the binding and activation of cognate VEGF receptors	Neovascular AMD, macular edema following CRVO	Warnings for neutropenia and neutropenic complications on product label
Agalsidase beta	rh α-galactosidase enzyme that catalyze the hydrolysis of glycosphigolipids	Fabry disease	Warnings for anaphylactic and allergic reactions on product label
Aldesleukin	rhIL-2 which mimics endogenous IL-2	Metastatic renal cell carcinoma	Boxed warning for capillary leak syndrome and infections
Alefacept	Fusion protein (soluble LFA-Fc-IgG1) that binds to CD2 and inhibits the interaction between CD2 and LFA3 during lymphocyte activation	Adult moderate to severe chronic plague psoriasis	Infections and malignancy reported in post marketing studies
Alemtuzumab	Humanised mAb that binds to CD52 on leukocytes and initiates antibody-dependent cellular-mediated lysis	B-CLL	Boxed warnings for cytopenias, infusion reactions and infections. Autoimmunity (Investigators et al. 2008) and immunogenicity (Somerfield et al. 2010) is also reported
Abciximab	Lysosomal glycogen-specific enzyme and provides exogenous source of α-glucosidase	Pompe disease	Boxed warning for anaphylaxis and allergic reactions

Anakinra	rh IL-1RA derived from E Coli binds to IL-1Rα and prevents the interaction between IL-1 and IL-1R1	RA	No boxed or product label warning issued
Asparaginase erwinia chrysanthemi	Asparagine specific enzyme which inhibits asparagine synthetase activity	ALL who have developed hypersensitivity to E Coliderived asparaginase	Warnings for serious hypersensitivity reactions on product label
Basiliximab	Chimeric mouse human mAb that binds to IL-2Rα and prevents the interaction between IL-2 and IL-2R	Renal transplant rejection	Warnings for hypersensitivity and anaphylaxis on product label Immunogenicity reported (Baudouin et al. 2003)
Becaplermin	rh platelet derived growth factor (GF) and promote chemotactic recruitment and proliferation of cells involved in wound repair	Lower extremity diabetic neuropathic ulcers	Warnings for increased rate of mortality secondary to malignancy on product label
Bevacizumab	rh monoclonal IgG1 antibody that binds to human vascular endothelial growth factor (VEGF) preventing interaction of VGEF to its receptors on the endothelial cells	mCRC, non-small cell lung cancer, Gliobalstoma, mRCC	Warnings for arterial and venous thromboembolic events on product label
Belatacept	Fusion protein (CTLA-4-Fc-IgG1) that binds to CD80 and/or CD86 to prevent the interaction with CD28 and therefore inhibits selective T-cell co-stimulation	Renal transplant rejection	Boxed warning for post-transplant lymphoproliferative disease (PTLD), malignancy and infections

Belimumab	Human mAb that inhibits BLyS/BAFF	SLE	No boxed or product label warning issued Hypersensitivity and anaphylaxis reported in post marketing studies
Brentuximab vedotin	Chimeric mouse-human mAb that forms antibody-drug conjugate CD30-specific mAb conjugated to monomethyl auristatin (MMAE) which induce cell cycle arrest and cause apoptosis	Hodgkin lymphoma, and systemic anaplastic large cell lymphoma	Boxed warning for PML Warnings for neutropenia and infection on product label (Gopal et al. 2012)
Canakinumab	Human mAb which binds to human IL-1β to prevent the interaction with IL-1R	CAPS, FCAS, MWS	Warnings for risk of infection on product label
Catumaxomab	Rat-mouse hybrid mAb bispecific antibody which binds to CD3-EpCAM and forms the bridge between cancer cells and T-cells	Cancer and malignant ascites	Boxed warnings for CRS and or SIRS Immunogenicity reported (Ruf et al. 2010)
Capromab pendetide	Murine mAb IgG1k directed against prostrate specific membrane antigen (PMSA)	Tissue diagnosis of prostate cancer	Warnings for risk of anaphylaxis and allergic reactions on product label

Certolizumab pegol	Pegylated Fab' humanised mAb that binds to TNF and blocks its interaction with TNFR	CD, RA	Boxed warnings for serious infections and malignancy Warnings for significant cytopenia such as leukopenia, pancytopenia and thrombocytopenia on product label Severe skin reactions reported in post marketing studies
Cetuximab	EGFR antagonist and binds to EGFR and competitively inhibits binding of EGF and other ligands such as TGF-α (Kirkpatrick P et al 2004)	Head and neck cancer, colorectal cancer	Warnings for serious infusion reactions on product label
Collagenase clostridium histolyticum	Proteinases that can hydrolyse collagen resulting in lysis of collagen deposits.	Dupuytren's disease	Warnings for injection site reactions on product label
Daclizumab (withdrawn due to non-safety reasons)	Humainsed mAb binds to IL-2 receptor and prevents the interaction between IL-2 and IL-2R	Renal transplant rejection	No boxed or product label warning issued

Denileukin diftitox	Fusion protein (IL-2 and Diptheria toxin) which binds to CD25 of IL-2R and triggers toxin-induced cell death	T-cell lymphoma	Boxed warnings for severe infusion reactions and capillary leak syndrome
Denosumab	Human mAb prevents RANKL binding to receptors that inhibits osteoclast formation, function and survival	Osteoporosis	No boxed or product label warning issued Hypersensitivity reactions reported in post marketing studies
Dornase alfa	rh DNase which cleaves extracellular DNA reduces the viscocity of purulent lung secretions	CF	No boxed or product label warning issued
Ecallantide	Plasma kallikrein inhibitor and binds to plasma kallikrein and blocks the binding site inhibiting conversion of kininogen to bradykinin.	Acute attacks of hereditary angioedema	Boxed warnings for anaphylaxis
Eculizumab	Humanised mAb that binds to complement complex C5 and inhibits its cleavage to C5a and C5b and prevents the formation of terminal complement complex C5b-9	PNH, aHUS	Boxed warnings for serious meningococcal infections
Epoetin alfa	Erythropoiesis-stimulating agent that mimics endogenous erythopoietin	Treatment of anaemia due to CKD and chemotherapy in patients with cancer	Warnings for immunogenicity on product label

Etanercept	Fusion protein (TNFR2-IgG1) which binds to TNF and blocks its interaction with TNFR	RA, JIA, AS, Psoriasis	Boxed warnings for serious infections and malignancy
Filgrastim	Recombinat methionyl human G-CSF that mimics endogenous G-CSF	AML, bone marrow transplant in cancer, blood progenitor cell collection and therapy, chronic neuropenia	No boxed or product label warning issued Risk of splenic rupture and ARDS indicated in product label
Galsulfase	Hydrolytic lysosomal glycosaminoglycan (GAG)-specific enzyme replacement	Mucopolysaccharidosis VI (MPS VI; Maroteaux-Lamy syndrome)	Risk of infusion reactions indicated on product label
Golimumab	Human mAb binds to TNF and blocks its interaction with TNFR	RA, psoriatic arthritis and AS	Boxed warnings for serious infections and malignancy
Glucarpidase	Recombinant bacterial enzyme which causes hydrolyses of carboxyl-terminal glutamate residue from folic acid and methotrexate and converts methotrexate to inactive metabolites.	Toxic plasma methotrexate concentrations in impaired renal function patients.	Risk of serious allergic reactions indicated on product label
Ibritumomab tiuxetan	Mouse mAb, the Fab segment of the antibody targets CD20 on B-cells, allowing covalently linked radioactive yttrium, which emits a β particle to destroy the cell	Follicular NHL	Boxed warnings for acute infusion reactions, severe cutaneous and muco-cutaneous reactions and prolonged and severe cytopenia Anaphylaxis and immunogenicity reported (Jankowitz, Joyce & Jacobs 2008)

Idursulfase	Recombinant human iduronate-2-sulfatase and provides exogenous substitute	Hunter syndrome (Mucopolysaccharidosis II)	Boxed warning for anaphylaxis
Infliximab	Chimeric mouse human mAb which binds to TNF and blocks its interaction with TNFR	CD, paediatric CD, UC, paediatric UC, RA, AS	Boxed warnings for serious infections and malignancy PML reported (Kumar, Bouldin & Berger 2010) Immunogenicity reported (Baert et al. 2003) Severe thrombocytopenia reported (Mocciaro et al. 2013)
Interferon alpha	rh IFN alpha that mimics endogenous interferon alpha	Chronic hepatitis C, hairy cell leukemia, hairy cell leukemia, AIDS-related Kaposi's sarcoma, CML	Boxed warnings for autoimmune reactions and infections Risk of cytopenia indicated in product label Immunogenicity reported (Strayer & Carter 2012)
Interferon beta-1a	hu IFN-β-1a that mimics endogenous interferon beta	RRMS	No boxed or product label warnings Anaphylaxis reported post marketing studies Immunogenicity reported (Strayer & Carter 2012)

Interferon beta-1b	hu IFN-β-1b that mimics endogenous interferon beta	RRMS	No boxed warnings Risk of lymphopenia indicated on product label Immunogenicity reported (Strayer DR et al 2012)
Interferon gamma	rh IFN gamma that mimics endogenous interferon gamma	Chronic granulomatous disease and osteopetrosis	No boxed or product label warnings issued
Ipilimumab	Human mAb which binds to CTLA-4 to block the interaction of CTLA4 with its ligands CD80 and CD86	Unresectable and/or metastatic melanoma	Boxed warning for immune-mediated adverse reactions
Laronidase	Endogenous enzyme that replaces the missing or deficient lysosomal enzyme in MPS-1, alpha-L-iduronidase.	Hurler and Hurler-Scheie forms of MPS-1	Boxed warning for anaphylaxis
Muromonab-CD3	Mouse mAb which kills CD3 positive cells by inducing antibody-dependent cell mediated toxicity and complement-dependent cytotoxicity	Organ transplant rejection	Boxed warnings for CRS and anaphylactic reactions Warnings for infections and malignancy on product label Immunogenicity reported (Jaffers GJ et al 1986)

Natalizumab	Humanised mAb that binds to α4-subunit of α4β1 and α4β7 integrins and inhibits α4 integrin-mediated adhesion of leukocytes (except for neutrophils) to their counter-receptors	RRMS and CD	Boxed warnings for serious infections in particular PML Immunogenicity reported (Sorensen et al. 2011)
Nofetumomab	Fab fragment of IgG2a murine mAb used for diagnostic evaluation of fresh human tumors	Detection of untreated small cell lung cancer	Warnings for allergic and anaphylaxis reactions on product label
Ofatumumab	Human mAb that targets CD20 and facilitates cell lysis due to complement-dependent cell-mediated cytotoxicity and antibody-dependant cell-mediated cytotoxicity of B-cells	CLL	No boxed or product label warnings issues Infections reported (Coiffier et al. 2008)
Omalizumab	Humanised mAb IgG1□ which binds to IgE and prevents IgE-mediated activation of mast cells and basophils	Asthma	Boxed warnings for anaphylaxis parasitic (Helminth) infections reported (Cruz et al. 2007)
Oprelvekin	Recombinant IL-11 a thrombotic GF which stimulates megakaryocytopoiesis and thrombopoiesis	Severe thrombocytopenia in adults	Boxed warnings for allergic reactions including anaphylaxis
Panitumumab	Epidermal growth factor receptor antagonist that inhibits the binding of ligands for the receptors	mCRC	Boxed warnings for dermatologic toxicity and infusion reactions

Palifermin	Mucocutaneous epithelial human GH and enhance the growth of epithelial cells	Severe oral mucositis in patients with hematologic malignancies	No boxed or product label warnings issues
Palivizumab	Humanised mAb with anti-RSV activity	Serious lower respiratory tract disease in children	No boxed warnings Warnings for anaphylaxis and serious hypersensitivity reactions including immunogenicity on product label
PEGinterferon alfa-2a	rh IFN alpha that mimics endogenous interferon alpha	Chronic hepatitis C	Boxed warnings issued for autoimmune reactions and infections. Risk of cytopenia indicated in product label
PEGinterferon alfa-2b	rh IFN alpha that mimics endogenous interferon alpha	Chronic hepatitis C	Boxed warnings issued for autoimmune reactions and infections. Risk of cytopenia indicated in product label
PEGloticase	PEGylated recombinant uricase which catalyze the oxidation of uric acid to allantoin and therefore decreases the serum uric acid	Chronic gout in adults	Boxed warnings issued for anaphylaxis and infusion reactions

Pertuzumab	HER2 receptor antagonist and inhibits ligand-initiated intracellular signalling which results in cell growth arrest and apoptosis	HER2-positive metastatic breast cancer	Risk of infusion associated reactions, hypersensitivity reactions or anaphylaxis are indicated on product label
Rasburicase	Recombinant urate-oxidase which catalyze the enzymatic oxidation of soluble uric acid into inactive and more soluble metabolite	Management of plasma Uric acid levels in children and adults suffering from Leukemia, lumphoma and solid tumour malignancies.	Boxed warning issued for anaphylaxis
Ranibizumab	VGEF inhibitor and binds to biologically active VGEF110 hence reducing endothelial cells proliferation, vascular leakage and new blood vessel formation	Neovascular AMD, macular edema following RVO and DME	No boxed or product label warnings issues
Rilonacept	Fusion protein IL-IR-Fc-IgG1 which binds to IL-1β and neutralises its activity by blocking its intersection with IL-1R	CAPS	No boxed or product label warnings issues
Rituximab	Chimeric mouse human mAb that binds to CD20 on B-cells and triggers B-cell lysis by complement-dependent and antibody-dependent cell-mediated cytotoxicity.	NHL, CLL, RA, Wegener's granulomatosis and microscopic polyangiitis	Boxed warnings for acute infusion reactions and/or CRS, TLS, severe mucocutaneous reactions and PML
Romiplostim	TPO receptor agonist that activates TPO receptor and increases the platelet production	Thrombocytopenia in patients with chronic ITP	No boxed or product label warnings issues

Sargramostin	rh GM-CSF that mimics endogenous GM-CSF	Cancer, bone marrow transplant and neutropenia	No boxed or product label warnings issues
Tenecteplase	Recombinant tPA binds to fibrin rich clot and cleaves the bonding and hence exerts the thrombolytic effect	Acute MI and lysis of intracoronary emboli	No boxed or product label warnings issues
Tositumomab	Mouse mAb which binds to CD20 positive cells and cause cell death through ionising radiation when it is radiolabelled and also induces complement-dependent and antibody-dependent cell-mediated cytotoxicity	Non-Hodgkin's lymphoma (CD20 positive, follicular)	Boxed warnings for severe allergic reactions and/or anaphylaxis, adn prolonged and severe cytopenia Malignancy warning on product label Immunogenicity reported (Buchegger et al. 2006)
Trastuzumab	Is a mediator of antibody-dependent cellular cytotoxicity and inhibit the proliferation of human tumour cells that overexpress HER2	HER2 overexpressing breast cancer, HER2-overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma	Boxed warnings for infusion reactions Hypersensitivity and infections reported on post marketing studies
Ustekinumab	Human mAb which blocks the function of IL-12 and IL-23 by binding to the p40 subunit of these cytokines	Psoriasis	No boxed or product label warnings issued Allergy and/or hypersensitivity reported in post marketing studies

Appendix Table 1: The comprehensive list of FDA and EU approved biologics products. Abbreviations: AE – Adverse events; aHUS – Atypical haemolytic-uremic syndrome; AIDS – Acquired immune deficiency syndrome; ALL – Acute lymphoblastic leukemia; AMD – Acute myeloid leukemia; AMD – Age related macular degeneration; AS – Alkylosing spondylitis; BAFF – B-cell activating factor; B-CLL – B-cell chronic lymphocytic leukemia; CAPS – Cryopyrin associated periodic syndrome; CD – Crohn's disease; CD – Cluster of differentiation; CF – Cystic fibrosis; CKD – Chronic kidney disease; CML – Chronic myeloid leukaemia; CRVO – Central retinal vein occlusion; CTLA – Cytotoxic T-lymphocyte antigen; DME – Diabetic macular edema; DNA – Deoxy ribonucleic acid; EGFR – Endothelial growth factor receptor; EpCAM – Epithelial cell adhesion molecule; FCAS – Familial cold autoinflammatory syndrome; GF – Growth factor; GM-CSF – Granulocyte macrophage colony-stimulating factor; HER – Human epidermal growth factor receptor; IFN – Interferon; IL – Interleukin; JIA – Juvenile idiopathic arthritis; LFA – Lymphocyte function associated protein; MAb – Monoclonal antibody; mCRC – metastatic colorectal carcinoma; MI – Myocardial infarction; MMP – Matrix metalloproteinase; MPS – Mucopolysaccharidosis; mRCC – metastatic renal carcinoma; MWS – Muckle-Wells syndrome; NHL – Non-Hodgkin lymphoma; PEG – Polyethylene glycol; PML – Progressive multifocal leukoencephalopathy; PNH – Paroxysmal nocturnal hemoglobinuria; PsA – Psoriatic arthritis; RA – Rheumatoid arthritis; RANKL – Receptor activator of nuclear factor kappa B ligand; rh – recombinant; r-methu G-CSF – recombinant methionyl human granulocyte colony-stimulating factor; RRMS – Relapsing remitting multiple sclerosis; RSV – Respiratory syncytical virus; RVO – Retinal vein occlusion; SJIA – Systemic juvenile idiopathic arthritis; SLE – Systemic lupus erythematosus; TLS – Tumor lysis sundrome; TNF – Tumor necrosis factor; TNFR – Tumor necrosis factor receptor; TPO – Thrombopoietin; tPA – Tissue plasminogen activator; VEGF – Vascular endothelial growth factor.

Appendix 2 Search Strategies

MEDLINE on Ovid

Search: ((((((((((((((((((("Multiple Sclerosis, Relapsing-Remitting"[Mesh]) OR "Demyelinating Diseases"[Mesh:noexp]) OR "Demyelinating Autoimmune Diseases, CNS"[Mesh:noexp])) OR "relapsing remitting multiple sclerosis"[Title/Abstract]) OR "multiple sclerosis"[Title/Abstract]) OR "remitting relapsing multiple sclerosis"[Title/Abstract]) OR "acute relapsing multiple sclerosis"[Title/Abstract]) OR "demyelinating disease"[Title/Abstract]) OR "demyelinating disorders"[Title/Abstract]))))) OR (((("Encephalomyelitis"[Mesh:noexp]) OR "Encephalomyelitis, Acute Disseminated"[Mesh]) OR "Optic Neuritis"[Mesh])) OR ((((("encephalomyelitis adem"[Title/Abstract]) OR "encephalomyelitis"[Title/Abstract]) OR "optic neuritis"[Title/Abstract]) OR "transverse myelitis"[Title/Abstract])) OR ("Myelitis, Transverse"[Mesh:noexp])))))) AND ((((((((((((((((("Interferon Type I"[Mesh]) OR "Interferon-beta"[Mesh]) OR "interferon beta 1a"[Supplementary Concept]) OR "interferon beta-1b"[Supplementary Concept])) OR "avonex"[Title/Abstract]) OR "rebif"[Title/Abstract]) OR "betaferon"[Title/Abstract]) OR "betaseron"[Title/Abstract]) OR "extavia"[Title/Abstract]) OR Beta-Seron[Title/Abstract])) AND ((((("Antibodies/blood"[Mesh:noexp]) OR "Immunoassay"[Mesh:noexp]) OR ("Interferon-beta/immunology"[Mesh]) AND ("Interferon-beta/antagonists and inhibitors"[Mesh] OR "Interferon-beta/therapeutic use"[Mesh])) OR "Neutralization Tests"[Mesh])))))) OR "immunogenicity/antibody"[Title/Abstract] OR "immunogenicity"[Title/Abstract])) AND (((((("Antibodies/blood"[Mesh:noexp]) OR "Immunoassay"[Mesh:noexp]) OR ("Interferon-beta/immunology"[Mesh]) AND ("Interferon-beta/antagonists and inhibitors"[Mesh] OR "Interferon-beta/therapeutic use"[Mesh])) OR "Neutralization Tests"[Mesh])))) AND (((((((((("Randomized Controlled Trial"[Publication Type]) OR "Controlled Clinical Trial"[Publication Type])) OR ((((((((("Randomized Controlled Trial"[Publication Type]) OR "Controlled Clinical Trial"[Publication Type])) OR "drug therapy"[Title/Abstract]) OR "randomized"[Title/Abstract]) OR "randomised"[Title/Abstract]) OR "randomly"[Title/Abstract]) OR "placebo"[Title/Abstract]) OR "trials"[Title/Abstract]) AND "groups"[Title/Abstract])))) OR "longitudinal study"[Title/Abstract])) OR ("Longitudinal Studies"[Mesh]))

EMBASE on Ovid

#43 #18 AND #28 AND #33 AND #42 AND [humans]/lim

#42 #34 OR #35 OR #36 OR #37 OR #38 OR #39 OR #40 OR #41

#41 'longitudinal study':ab,ti

#40 'longitudinal study'/exp

#39 'trials':ab,ti

#38 'placebo':ab,ti

#37 'randomly':ab,ti

#36 'randomised':ti

#35 'controlled clinical trial'/exp

#34 'randomized controlled trial'/exp

#33 #29 OR #31 OR #32

#32 'neutralizing antibodies':ab,ti

#31 'nabs':ab,ti

#29 'neutralizing antibody'/exp

#28 #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27

#27 'extavia':ab,ti

#26 'betaseron':ab,ti

#25 'betaferon':ab,ti

#24 'rebif':ab,ti

#23 'avonex':ab,ti

#22 'interferon beta 1b'/mj

#21 'interferon beta 1a'/mj

#20 'interferon beta'/mj

#19 'interferon type i'/mj

#18 #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17

#17 'myelitis transverse'/mj

#16 'transverse myelitis':ab,ti

#15 'optic neuritis':ab,ti

#14 'encephalomyelitis':ab,ti

#13 'encephalomyelitis adem':ab,ti

#12 'optic neuritis'/mj

#11 'encephalomyelitis acute disseminated'/exp

#10 'encephalomyelitis'/mj

#9 'demyelinating disorders':ab,ti

#8 'demyelinating disease':ab,ti

#7 'acute relapsing multiple sclerosis':ab,ti

#6 'multiple sclerosis':ab,ti

#5 'relapsing remitting multiple sclerosis':ab,ti

#4 'demyelinating autoimmune diseases, cns'/mj

#3 'demyelinating diseases'/mj

#2 'multiple sclerosis relapsing remitting'/exp

CENTRAL on The Cochrane Library

Search: ((((((((((((((((((("Multiple Sclerosis, Relapsing-Remitting"[Mesh]) OR "Demyelinating Diseases"[Mesh:noexp]) OR "Demyelinating Autoimmune Diseases, CNS"[Mesh:noexp])) OR "relapsing remitting multiple sclerosis"[Title/Abstract]) OR "multiple sclerosis"[Title/Abstract]) OR "remitting relapsing multiple sclerosis"[Title/Abstract]) OR "acute relapsing multiple sclerosis"[Title/Abstract]) OR "demyelinating disease"[Title/Abstract]) OR "demyelinating disorders"[Title/Abstract]))))) OR ((("Encephalomyelitis"[Mesh:noexp]) OR "Encephalomyelitis, Acute Disseminated"[Mesh]) OR "Optic Neuritis"[Mesh])) OR ((((("encephalomyelitis adem"[Title/Abstract]) OR "encephalomyelitis"[Title/Abstract]) OR "optic neuritis"[Title/Abstract]) OR "transverse myelitis"[Title/Abstract])) OR ("Myelitis, Transverse"[Mesh:noexp])))))) AND ((((((((((((((((("Interferon Type I"[Mesh]) OR "Interferon-beta"[Mesh]) OR "interferon beta 1a"[Supplementary Concept]) OR "interferon beta-1b"[Supplementary Concept])) OR "avonex"[Title/Abstract]) OR "rebif"[Title/Abstract]) OR "betaferon"[Title/Abstract]) OR "betaseron"[Title/Abstract]) OR "extavia"[Title/Abstract]) OR Beta-Seron[Title/Abstract])) AND ((((("Antibodies/blood"[Mesh:noexp]) OR "Immunoassay"[Mesh:noexp]) OR ("Interferon-beta/immunology"[Mesh]) AND ("Interferon-beta/antagonists and inhibitors"[Mesh] OR "Interferon-beta/therapeutic use"[Mesh])) OR "Neutralization Tests"[Mesh])))))) OR "immunogenicity/antibody"[Title/Abstract] OR "immunogenicity"[Title/Abstract])) AND (((((("Antibodies/blood"[Mesh:noexp]) OR "Immunoassay"[Mesh:noexp]) OR ("Interferon-beta/immunology"[Mesh]) AND ("Interferon-beta/antagonists and inhibitors"[Mesh] OR "Interferon-beta/therapeutic use"[Mesh])) OR "Neutralization Tests"[Mesh]))) AND (((((((((("Randomized Controlled Trial"[Publication Type]) OR "Controlled Clinical Trial"[Publication Type])) OR ((((((((("Randomized Controlled Trial"[Publication Type])

OR "Controlled Clinical Trial"[Publication Type])) OR "drug therapy"[Title/Abstract]) OR "randomized"[Title/Abstract]) OR "randomised"[Title/Abstract]) OR "randomly"[Title/Abstract]) OR "placebo"[Title/Abstract]) OR "trials"[Title/Abstract]) AND "groups"[Title/Abstract])))) OR "longitudinal study"[Title/Abstract])) OR ("Longitudinal Studies"[Mesh]))

Clinicaltrials.gov

Search: Neutralising antibodies and multiple sclerosis

Appendix 3 Study Eligibility Screening Form

Study ID:	**Reviewer:**	**Date:**
Question 1: Did some or all of the participants received IFN-β Avonex™ or Rebif™ or Betaferon/Betaseron™ or Extavia™?	If no: EXCLUDE If yes: go to **Question 2** If unsure: go to **Question 2**	
Question 2: Are some or all of the participants Adults >18 years undergoing treatment for multiple sclerosis and fulfilling Poser (Poser CM et al 1983) or McDonald criteria (McDonald WI et al 2001) or defined by the study authors?	If no: EXCLUDE If yes: go to **Question 3** If unsure: go to **Question 3**	
Question 3: Does the paper report a randomised controlled trial or a non-randomised study which included >20 patients?	If no: EXCLUDE If yes: INCLUDE In unsure: INCLUDE	

Appendix 4 Data collection form for randomised studies

Paper ID:	**Reviewer:**	**Date:**
Study Characteristics		
Number of participants		
Number of participants in each treatment group		
Year completed		
Setting		
Inclusion criteria		
Definition of neutralising antibody positivity		
Length of follow up		
Participants		
Age		
Gender		
Underlying disease		
Indication for IFN-β treatment		
Treatment		
No. of treatment groups		
Avonex™ and/or Rebif™ and/or Betaferon/Betaseron™ and/or Extavia ™		
Dosage		
Route of administration		
Frequency of treatment		
Outcomes		
Status of neutralising antibodies		
Method used to detect neutralising antibodies		
Additional data relating to neutralising antibodies		
Additional data on neutralising antibodies		

Appendix 5 Data collection form for non-randomised studies

Paper ID:	**Reviewer:**	**Date:**
Study Characteristics		
Number of participants		
If there were two treatment groups, number of participants in each treatment group		
Year completed		
Setting		
Inclusion criteria		
Definition of neutralising antibody positivity		
Length of follow up		
Participants		
Age		
Gender		
Underlying disease		
Indication for IFN-β treatment		
Treatment		
Avonex™ and/or Rebif™ and/or Betaferon/Betaseron™ and/or Extavia™		
Dosage		
Route of administration		
Frequency of treatment		
Outcomes		
Status of neutralising antibodies		
Method used to detect neutralising antibodies		
Additional data relating to neutralising antibodies		
Additional data on neutralising antibodies		

Appendix 6 Elements adapted from McHarm Scale

1. Was NAb positivity pre-defined using standardised or precise definitions?
2. Was the mode of NAb data collection specified as active?
3. Did the study specify the baseline, timing and frequency of the NAb data collection?
4. Did the authors use standard scales(s) or checklist(s) for NAb data collection?
5. Is there a possibility of selective outcome reporting?

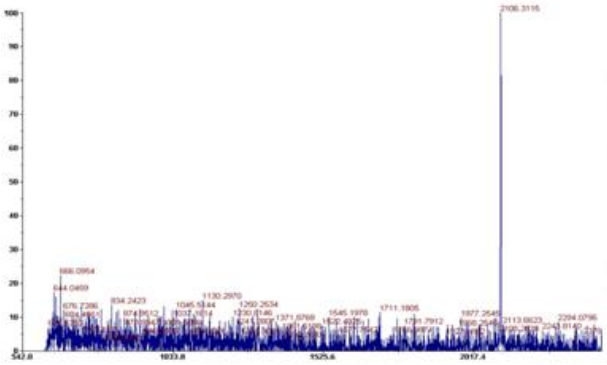
Appendix 7

	NIBSC Hertfordshire					CDSS Liverpool		
Samples	IFN-β-1a Rebif™ antibodies detected	NAbs titres	Screening	Screening	NAbs titres	IFN-β-1a Rebif™ antibodies detected		Neutralisation assay
	Neutralisation assay	EC50	Binding assay	Neutralisation assay	EC50	IFN-β-1a Rebif™ antibodies detected		MxA
	MxA assay	MxA assay	ECL assay	ECL assay	ECL assay	ELISA	ng/ml	Immunoblot
P1	Yes	200	Yes	Yes	25	Discontinued		
P3	Yes	>1000	Yes	Yes	240	Yes	1186	Yes 100%
P7	Yes	<10	No	No		No	50	No
P10	Yes	>1000	Yes	Yes	700	Yes	1094	Yes 100%
P13	Yes	>1000	Yes	Yes	380	Yes	1137	Yes 100%
P14	Yes	>1000	Yes	Yes	1000	Yes	1013	Yes 100%
P15	Yes	>1000	Yes	Yes	780	Yes	1217	Yes 100%
P28	Yes	850	Yes	Yes	35	Yes	213	Yes >75%
P29	Yes	80	Yes	Yes	8	Yes	782	Yes <10%
P30	Sample not available for analysis					Yes	725	Yes >75%

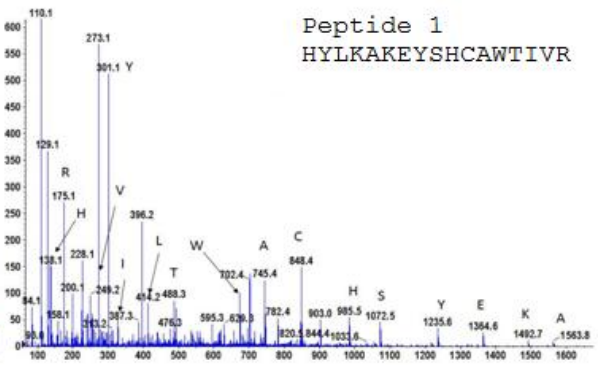
Appendix Table 2: Comparision of NIBSC, Hertfordshire and CDSS, Liverpool binding and neutralising antibodies data.

Appendix 8

A



B



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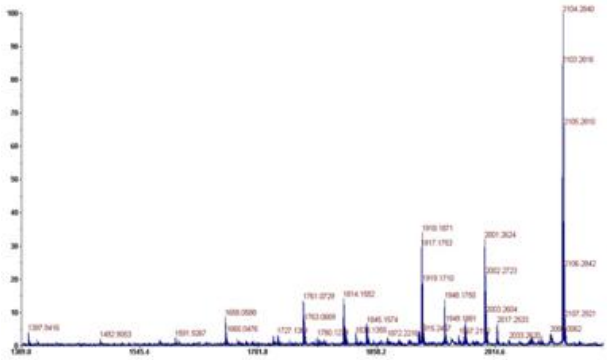

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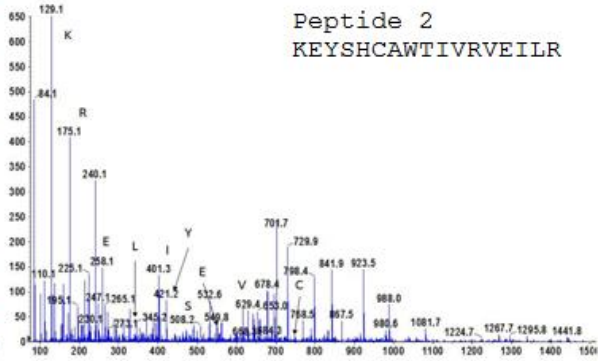

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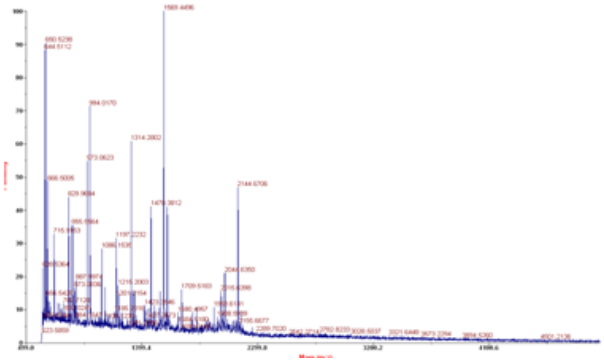

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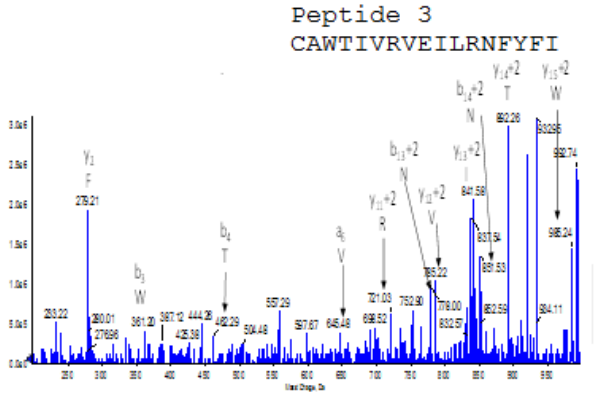

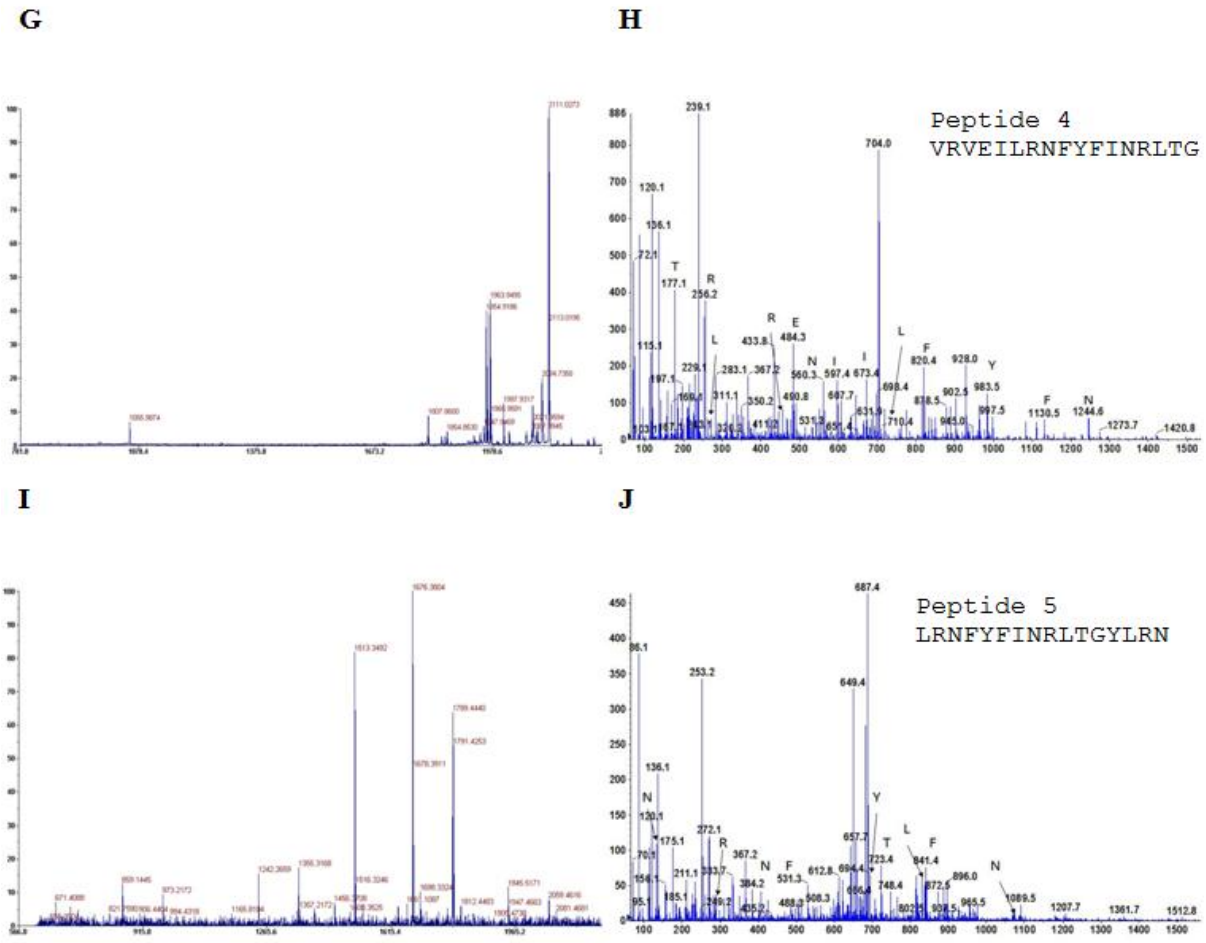

Appendix Figure 1: LC-MS ion current chromatograms for predicted IFN-β-1a Rebif™ peptides. The appendix Figures 1a, b, c, d, e, f, g, h, i and j indicates the molecular mass of each IFN-β-1a Rebif™ peptides 1, 2, 3, 4 and 5 and characteristic fragments are shown in arrows for the individual amino acids.

References

Aalberse, R.C. & Schuurman, J. (2002) 'IgG4 breaking the rules', *Immunology*, vol. 105, no. 1, pp. 9-19.

Aalberse, R.C., van der Gaag, R. & van Leeuwen, J. (1983) 'Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response', *J Immunol*, vol. 130, no. 2, pp. 722-726.

Ahlers, J.D., Belyakov, I.M., Thomas, E.K. & Berzofsky, J.A. (2001) 'High-affinity T helper epitope induces complementary helper and APC polarization, increased CTL, and protection against viral infection', *J Clin Invest*, vol. 108, no. 11, pp. 1677-1685.

Akira, S. & Takeda, K. (2004) 'Toll-like receptor signalling', *Nat Rev Immunol*, vol. 4, no. 7, pp. 499-511.

Alcina, A., Abad-Grau Mdel, M., Fedetz, M., Izquierdo, G., Lucas, M., Fernandez, O., Ndagire, D., Catala-Rabasa, A., Ruiz, A., Gayan, J., Delgado, C., Arnal, C. & Matesanz, F. (2012) 'Multiple sclerosis risk variant HLA-DRB1*1501 associates with high expression of DRB1 gene in different human populations', *PLoS One*, vol. 7, no. 1, p. e29819.

Alfirevic, A., Gonzalez-Galarza, F., Bell, C., Martinsson, K., Platt, V., Bretland, G., Evely, J., Lichtenfels, M., Cederbrant, K., French, N., Naisbitt, D., Park, B.K., Jones, A.R. & Pirmohamed, M. (2012) 'In silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped DNA archive from healthy volunteers', *Genome Med*, vol. 4, no. 6, p. 51.

Alley, S.C., Okeley, N.M. & Senter, P.D. (2010) 'Antibody-drug conjugates: targeted drug delivery for cancer', *Curr Opin Chem Biol*, vol. 14, no. 4, pp. 529-537.

Alonso, A., Jick, S.S., Olek, M.J. & Hernan, M.A. (2007) 'Incidence of multiple sclerosis in the United Kingdom : findings from a population-based cohort', *J Neurol*, vol. 254, no. 12, pp. 1736-1741.

Alzari, P.M., Lascombe, M.B. & Poljak, R.J. (1988) 'Three-dimensional structure of antibodies', *Annu Rev Immunol*, vol. 6, pp. 555-580.

Amit, A.G., Mariuzza, R.A., Phillips, S.E. & Poljak, R.J. (1986) 'Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution', *Science*, vol. 233, no. 4765, pp. 747-753.

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D. & Zychlinsky, A. (2012) 'Neutrophil function: from mechanisms to disease', *Annu Rev Immunol*, vol. 30, pp. 459-489.

Anglister, J., Scherf, T., Zilber, B. & Levy, R. (1995) 'Two-dimensional NMR studies of the interactions between a peptide of cholera toxin and monoclonal antibodies', *Biopolymers*, vol. 37, no. 6, pp. 383-389.

Antonelli, G., Simeoni, E., Bagnato, F., Pozzilli, C., Turriziani, O., Tesoro, R., Di Marco, P., Gasperini, C., Fieschi, C. & Dianzani, F. (1999) 'Further study on the specificity and incidence of neutralizing antibodies to interferon (IFN) in relapsing remitting multiple sclerosis patients treated with IFN beta-1a or IFN beta-1b', *J Neurol Sci*, vol. 168, no. 2, pp. 131-136.

Apetoh, L., Quintana, F.J., Pot, C., Joller, N., Xiao, S., Kumar, D., Burns, E.J., Sherr, D.H., Weiner, H.L. & Kuchroo, V.K. (2010) 'The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27', *Nat Immunol*, vol. 11, no. 9, pp. 854-861.

Askling, J., Baecklund, E., Granath, F., Geborek, P., Fored, M., Backlin, C., Bertilsson, L., Coster, L., Jacobsson, L.T., Lindblad, S., Lysholm, J., Rantapaa-Dahlqvist, S., Saxne, T., van Vollenhoven, R., Klareskog, L. & Feltelius, N. (2009) 'Anti-tumour necrosis factor therapy in rheumatoid arthritis and risk of malignant lymphomas: relative risks and time trends in the Swedish Biologics Register', *Ann Rheum Dis*, vol. 68, no. 5, pp. 648-653.

Askling, J., Fored, C.M., Baecklund, E., Brandt, L., Backlin, C., Ekbom, A., Sundstrom, C., Bertilsson, L., Coster, L., Geborek, P., Jacobsson, L.T., Lindblad, S., Lysholm, J., Rantapaa-Dahlqvist, S., Saxne, T., Klareskog, L. & Feltelius, N. (2005) 'Haematopoietic malignancies in rheumatoid arthritis: lymphoma risk and characteristics after exposure to tumour necrosis factor antagonists', *Ann Rheum Dis*, vol. 64, no. 10, pp. 1414-1420.

Aucouturier, P., Mariault, M., Lacombe, C. & Preud'homme, J.L. (1992) 'Frequency of selective IgG subclass deficiency: a reappraisal', *Clin Immunol Immunopathol*, vol. 63, no. 3, pp. 289-291.

Bachmann, M.F., Kundig, T.M., Kalberer, C.P., Hengartner, H. & Zinkernagel, R.M. (1994) 'How many specific B cells are needed to protect against a virus?', *J Immunol*, vol. 152, no. 9, pp. 4235-4241.

Bachmann, M.F., Rohrer, U.H., Kundig, T.M., Burki, K., Hengartner, H. & Zinkernagel, R.M. (1993) 'The influence of antigen organization on B cell responsiveness', *Science*, vol. 262, no. 5138, pp. 1448-1451.

Bachmann, M.F. & Zinkernagel, R.M. (1997) 'Neutralizing antiviral B cell responses', *Annu Rev Immunol*, vol. 15, pp. 235-270.

Baert, F., Noman, M., Vermeire, S., Van Assche, G., G, D.H., Carbonez, A. & Rutgeerts, P. (2003) 'Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease', *N Engl J Med*, vol. 348, no. 7, pp. 601-608.

Baker, M.P. & Jones, T.D. (2007) 'Identification and removal of immunogenicity in therapeutic proteins', *Curr Opin Drug Discov Devel*, vol. 10, no. 2, pp. 219-227.

Baker, M.P., Reynolds, H.M., Lumicisi, B. & Bryson, C.J. (2010) 'Immunogenicity of protein therapeutics: The key causes, consequences and challenges', *Self Nonself*, vol. 1, no. 4, pp. 314-322.

Baltrukonis, D.J., Finco-Kent, D., Kawabata, T.T., Poirier, M. & Lesauteur, L. (2006) 'Development and Validation of a Quasi-Quantitative Bioassay for Neutralizing Antibodies Against CP-870,893', *J Immunotoxicol*, vol. 3, no. 3, pp. 157-164.

Banchereau, J. & Steinman, R.M. (1998) 'Dendritic cells and the control of immunity', *Nature*, vol. 392, no. 6673, pp. 245-252.

Barbero, P., Bergui, M., Versino, E., Ricci, A., Zhong, J.J., Ferrero, B., Clerico, M., Pipieri, A., Verdun, E., Giordano, L., Durelli, L. & Group, I.T.S. (2006) 'Every-other-day interferon beta-1b versus once-weekly interferon beta-1a for multiple sclerosis (INCOMIN Trial) II: analysis of MRI responses to treatment and correlation with Nab', *Mult Scler*, vol. 12, no. 1, pp. 72-76.

Barbosa, M.D., Vielmetter, J., Chu, S., Smith, D.D. & Jacinto, J. (2006) 'Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity', *Clin Immunol*, vol. 118, no. 1, pp. 42-50.

Barger, T.E., Wrona, D., Goletz, T.J. & Mytych, D.T. (2012) 'A detailed examination of the antibody prevalence and characteristics of anti-ESA antibodies', *Nephrol Dial Transplant*, vol. 27, no. 10, pp. 3892-3899.

Barnard, J.G., Babcock, K. & Carpenter, J.F. (2013) 'Characterization and quantitation of aggregates and particles in interferon-beta products: potential links between product quality attributes and immunogenicity', *J Pharm Sci*, vol. 102, no. 3, pp. 915-928.

Baron, S., Tyring, S.K., Fleischmann, W.R., Jr., Coppenhaver, D.H., Niesel, D.W., Klimpel, G.R., Stanton, G.J. & Hughes, T.K. (1991) 'The interferons. Mechanisms of action and clinical applications', *JAMA*, vol. 266, no. 10, pp. 1375-1383.

Bartelds, G.M., Krieckaert, C.L., Nurmohamed, M.T., van Schouwenburg, P.A., Lems, W.F., Twisk, J.W., Dijkmans, B.A., Aarden, L. & Wolbink, G.J. (2011) 'Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up', *JAMA*, vol. 305, no. 14, pp. 1460-1468.

Basile, E., Gibbs, E., Aziz, T. & Oger, J. (2006) 'During 3 years treatment of primary progressive multiple sclerosis with glatiramer acetate, specific antibodies switch from IgG1 to IgG4', *J Neuroimmunol*, vol. 177, no. 1-2, pp. 161-166.

Batista, F.D. & Harwood, N.E. (2009) 'The who, how and where of antigen presentation to B cells', *Nat Rev Immunol*, vol. 9, no. 1, pp. 15-27.

Battaglia, M., Stabilini, A., Draghici, E., Gregori, S., Mocchetti, C., Bonifacio, E. & Roncarolo, M.G. (2006) 'Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance', *Diabetes*, vol. 55, no. 1, pp. 40-49.

Baudouin, V., Crusiaux, A., Haddad, E., Schandene, L., Goldman, M., Loirat, C. & Abramowicz, D. (2003) 'Anaphylactic shock caused by immunoglobulin E sensitization after retreatment with the chimeric anti-interleukin-2 receptor monoclonal antibody basiliximab', *Transplantation*, vol. 76, no. 3, pp. 459-463.

Bekisz, J., Baron, S., Balinsky, C., Morrow, A. & Zoon, K.C. (2010) 'Antiproliferative Properties of Type I and Type II Interferon', *Pharmaceuticals (Basel)*, vol. 3, no. 4, pp. 994-1015.

Bell, D., Young, J.W. & Banchereau, J. (1999) 'Dendritic cells', *Adv Immunol*, vol. 72, pp. 255-324.

Bellomi, F., Scagnolari, C., Tomassini, V., Gasperini, C., Paolillo, A., Pozzilli, C. & Antonelli, G. (2003) 'Fate of neutralizing and binding antibodies to IFN beta in MS patients treated with IFN beta for 6 years', *J Neurol Sci*, vol. 215, no. 1-2, pp. 3-8.

Belnap, D.M., Watts, N.R., Conway, J.F., Cheng, N., Stahl, S.J., Wingfield, P.T. & Steven, A.C. (2003) 'Diversity of core antigen epitopes of hepatitis B virus', *Proc Natl Acad Sci U S A*, vol. 100, no. 19, pp. 10884-10889.

Bendtzen, K. (2010) 'Critical review: assessment of interferon-beta immunogenicity in multiple sclerosis', *J Interferon Cytokine Res*, vol. 30, no. 10, pp. 759-766.

Bennett, C.L., Luminari, S., Nissenson, A.R., Tallman, M.S., Klinge, S.A., McWilliams, N., McKoy, J.M., Kim, B., Lyons, E.A., Trifilio, S.M., Raisch, D.W., Evens, A.M., Kuzel, T.M., Schumock, G.T., Belknap, S.M., Locatelli, F., Rossert, J. & Casadevall, N. (2004) 'Pure red-cell aplasia and epoetin therapy', *N Engl J Med*, vol. 351, no. 14, pp. 1403-1408.

Benschop, R.J. & Cambier, J.C. (1999) 'B cell development: signal transduction by antigen receptors and their surrogates', *Curr Opin Immunol*, vol. 11, no. 2, pp. 143-151.

Benson, M.J., Pino-Lagos, K., Rosemblatt, M. & Noelle, R.J. (2007) 'All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation', *J Exp Med*, vol. 204, no. 8, pp. 1765-1774.

Berkowitz, S.A. (2006) 'Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals', *AAPS J*, vol. 8, no. 3, pp. E590-605.

Bernton, E.W. (2007) Safety Pharmacology: Similarities and Differences between Small Molecules and Novel Biopharmaceuticals, in, *Preclinical Safety Evaluation of Biopharmaceuticals*, John Wiley & Sons, Inc., pp. 309-335.

Bertolotto, A. (2009) 'Implications of neutralising antibodies on therapeutic efficacy', *J Neurol Sci*, vol. 277 Suppl 1, pp. S29-32.

Bertolotto, A., Gilli, F., Sala, A., Capobianco, M., Malucchi, S., Milano, E., Melis, F., Marnetto, F., Lindberg, R.L., Bottero, R., Di Sapio, A. & Giordana, M.T. (2003) 'Persistent neutralizing antibodies abolish the interferon beta bioavailability in MS patients', *Neurology*, vol. 60, no. 4, pp. 634-639.

Bertolotto, A., Malucchi, S., Milano, E., Castello, A., Capobianco, M. & Mutani, R. (2000) 'Interferon beta neutralizing antibodies in multiple sclerosis: neutralizing activity and cross-reactivity with three different preparations', *Immunopharmacology*, vol. 48, no. 2, pp. 95-100.

Bertolotto, A., Malucchi, S., Sala, A., Orefice, G., Carrieri, P.B., Capobianco, M., Milano, E., Melis, F. & Giordana, M.T. (2002) 'Differential effects of three interferon betas on neutralising antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory', *J Neurol Neurosurg Psychiatry*, vol. 73, no. 2, pp. 148-153.

Bianchini, D., Jayanth, A., Chua, Y.J. & Cunningham, D. (2008) 'Epidermal growth factor receptor inhibitor-related skin toxicity: mechanisms, treatment, and its potential role as a predictive marker', *Clin Colorectal Cancer*, vol. 7, no. 1, pp. 33-43.

Bloomgren, G., Richman, S., Hotermans, C., Subramanyam, M., Goelz, S., Natarajan, A., Lee, S., Plavina, T., Scanlon, J.V., Sandrock, A. & Bozic, C. (2012) 'Risk of natalizumab-associated progressive multifocal leukoencephalopathy', *N Engl J Med*, vol. 366, no. 20, pp. 1870-1880.

Boackle, S.A., Holers, V.M. & Karp, D.R. (1997) 'CD21 augments antigen presentation in immune individuals', *Eur J Immunol*, vol. 27, no. 1, pp. 122-129.

Bonfigli, S., Doro, M.G., Fozza, C., Derudas, D., Dore, F. & Longinotti, M. (2003) 'T-cell receptor repertoire in healthy Sardinian subjects', *Hum Immunol*, vol. 64, no. 7, pp. 689-695.

Boos, L., Campbell, I.L., Ames, R., Wetsel, R.A. & Barnum, S.R. (2004) 'Deletion of the complement anaphylatoxin C3a receptor attenuates, whereas ectopic expression of C3a in the brain exacerbates, experimental autoimmune encephalomyelitis', *J Immunol*, vol. 173, no. 7, pp. 4708-4714.

Borden, E.C. (1992) 'Interferons: pleiotropic cellular modulators', *Clin Immunol Immunopathol*, vol. 62, no. 1 Pt 2, pp. S18-24.

Borden, E.C., Hawkins, M.J., Sielaff, K.M., Storer, B.M., Schiesel, J.D. & Smalley, R.V. (1988) 'Clinical and biological effects of recombinant interferon-beta administered intravenously daily in phase I trial', *J Interferon Res*, vol. 8, no. 3, pp. 357-366.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R. & Stark, G.R. (2007) 'Interferons at age 50: past, current and future impact on biomedicine', *Nat Rev Drug Discov*, vol. 6, no. 12, pp. 975-990.

Bowman, E.P., Chackerian, A.A. & Cua, D.J. (2006) 'Rationale and safety of anti-interleukin-23 and anti-interleukin-17A therapy', *Curr Opin Infect Dis*, vol. 19, no. 3, pp. 245-252.

Bradford, M.M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Anal Biochem*, vol. 72, pp. 248-254.

Brandes, M., Willimann, K., Bioley, G., Levy, N., Eberl, M., Luo, M., Tampe, R., Levy, F., Romero, P. & Moser, B. (2009) 'Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses', *Proc Natl Acad Sci U S A*, vol. 106, no. 7, pp. 2307-2312.

Braun, A. & Alsenz, J. (1997) 'Development and use of enzyme-linked immunosorbent assays (ELISA) for the detection of protein aggregates in interferon-alpha (IFN-alpha) formulations', *Pharm Res*, vol. 14, no. 10, pp. 1394-1400.

Braun, A., Kwee, L., Labow, M.A. & Alsenz, J. (1997) 'Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice', *Pharm Res*, vol. 14, no. 10, pp. 1472-1478.

Bruno, L., Res, P., Dessing, M., Cella, M. & Spits, H. (1997) 'Identification of a committed T cell precursor population in adult human peripheral blood', *J Exp Med*, vol. 185, no. 5, pp. 875-884.

Buchegger, F., Antonescu, C., Delaloye, A.B., Helg, C., Kovacsovics, T., Kosinski, M., Mach, J.P. & Ketterer, N. (2006) 'Long-term complete responses after 131I-tositumomab therapy for relapsed or refractory indolent non-Hodgkin's lymphoma', *Br J Cancer*, vol. 94, no. 12, pp. 1770-1776.

Buck, D., Cepok, S., Hoffmann, S., Grummel, V., Jochim, A., Berthele, A., Hartung, H.P., Wassmuth, R. & Hemmer, B. (2011) 'Influence of the HLA-DRB1 genotype on antibody development to interferon beta in multiple sclerosis', *Arch Neurol*, vol. 68, no. 4, pp. 480-487.

Bui, H.H., Sidney, J., Peters, B., Sathiamurthy, M., Sinichi, A., Purton, K.A., Mothe, B.R., Chisari, F.V., Watkins, D.I. & Sette, A. (2005) 'Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications', *Immunogenetics*, vol. 57, no. 5, pp. 304-314.

Bystry, R.S., Aluvihare, V., Welch, K.A., Kallikourdis, M. & Betz, A.G. (2001) 'B cells and professional APCs recruit regulatory T cells via CCL4', *Nat Immunol*, vol. 2, no. 12, pp. 1126-1132.

Cabaniols, J.P., Fazilleau, N., Casrouge, A., Kourilsky, P. & Kanellopoulos, J.M. (2001) 'Most alpha/beta T cell receptor diversity is due to terminal deoxynucleotidyl transferase', *J Exp Med*, vol. 194, no. 9, pp. 1385-1390.

Cambier, J.C., Gauld, S.B., Merrell, K.T. & Vilen, B.J. (2007) 'B-cell anergy: from transgenic models to naturally occurring anergic B cells?', *Nat Rev Immunol*, vol. 7, no. 8, pp. 633-643.

Cambier, J.C. & Getahun, A. (2010) 'B cell activation versus anergy; the antigen receptor as a molecular switch', *Immunol Lett*, vol. 128, no. 1, pp. 6-7.

Candon, S., Mosca, A., Ruemmele, F., Goulet, O., Chatenoud, L. & Cezard, J.P. (2006) 'Clinical and biological consequences of immunization to infliximab in pediatric Crohn's disease', *Clin Immunol*, vol. 118, no. 1, pp. 11-19.

Carroll, M.C. (2004) 'The complement system in regulation of adaptive immunity', *Nat Immunol*, vol. 5, no. 10, pp. 981-986.

Carson, K.R., Evens, A.M., Richey, E.A., Habermann, T.M., Focosi, D., Seymour, J.F., Laubach, J., Bawn, S.D., Gordon, L.I., Winter, J.N., Furman, R.R., Vose, J.M., Zelenetz, A.D., Mamtani, R., Raisch, D.W., Dorshimer, G.W., Rosen, S.T., Muro, K., Gottardi-Littell, N.R., Talley, R.L., Sartor, O., Green, D., Major, E.O. & Bennett, C.L. (2009) 'Progressive multifocal leukoencephalopathy after rituximab therapy in HIV-negative patients: a report of 57 cases from the Research on Adverse Drug Events and Reports project', *Blood*, vol. 113, no. 20, pp. 4834-4840.

Carter, P. (2001) 'Bispecific human IgG by design', *J Immunol Methods*, vol. 248, no. 1-2, pp. 7-15.
Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B. & Mayeux, P. (2002) 'Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin', *N Engl J Med*, vol. 346, no. 7, pp. 469-475.
Casato, M., Lagana, B., Antonelli, G., Dianzani, F. & Bonomo, L. (1991) 'Long-term results of therapy with interferon-alpha for type II essential mixed cryoglobulinemia', *Blood*, vol. 78, no. 12, pp. 3142-3147.
Castellani, M.L., Anogeianaki, A., Felaco, P., Toniato, E., De Lutiis, M.A., Shaik, B., Fulcheri, M., Vecchiet, J., Tete, S., Salini, V., Theoharides, T.C., Caraffa, A., Antinolfi, P., Frydas, I., Conti, P., Cuccurullo, C., Ciampoli, C., Cerulli, G. & Kempuraj, D. (2010) 'IL-35, an anti-inflammatory cytokine which expands CD4+CD25+ Treg Cells', *J Biol Regul Homeost Agents*, vol. 24, no. 2, pp. 131-135.
Caux, C., Dezutter-Dambuyant, C., Schmitt, D. & Banchereau, J. (1992) 'GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells', *Nature*, vol. 360, no. 6401, pp. 258-261.
Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. & Lanzavecchia, A. (1999) 'Maturation, activation, and protection of dendritic cells induced by double-stranded RNA', *J Exp Med*, vol. 189, no. 5, pp. 821-829.
Chang, K.M., Rehermann, B., McHutchison, J.G., Pasquinelli, C., Southwood, S., Sette, A. & Chisari, F.V. (1997) 'Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus', *J Clin Invest*, vol. 100, no. 9, pp. 2376-2385.
Chatenoud, L. (1993) 'Immunologic monitoring during OKT3 therapy', *Clin Transplant*, vol. 7, no. 4 Pt 2, pp. 422-430.
Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G. & Wahl, S.M. (2003) 'Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3', *J Exp Med*, vol. 198, no. 12, pp. 1875-1886.
Chirino, A.J. & Mire-Sluis, A. (2004) 'Characterizing biological products and assessing comparability following manufacturing changes', *Nat Biotechnol*, vol. 22, no. 11, pp. 1383-1391.
Chirmule, N., Jawa, V. & Meibohm, B. (2012) 'Immunogenicity to therapeutic proteins: impact on PK/PD and efficacy', *AAPS J*, vol. 14, no. 2, pp. 296-302.
Chiu, A.W., Ehrmantraut, M., Richert, N.D., Ikonomidou, V.N., Pellegrini, S., McFarland, H.F., Frank, J.A. & Bagnato, F. (2007) 'A case study on the effect of neutralizing antibodies to interferon beta 1b in multiple sclerosis patients followed for 3 years with monthly imaging', *Clin Exp Immunol*, vol. 150, no. 1, pp. 61-67.
Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J. & Platts-Mills, T.A. (2008) 'Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose', *N Engl J Med*, vol. 358, no. 11, pp. 1109-1117.
Chung, W.H., Hung, S.I., Hong, H.S., Hsih, M.S., Yang, L.C., Ho, H.C., Wu, J.Y. & Chen, Y.T. (2004) 'Medical genetics: a marker for Stevens-Johnson syndrome', *Nature*, vol. 428, no. 6982, p. 486.
Clanet, M., Radue, E.W., Kappos, L., Hartung, H.P., Hohlfeld, R., Sandberg-Wollheim, M., Kooijmans-Coutinho, M.F., Tsao, E.C., Sandrock, A.W. & European, I.-a.D.-C.S.I. (2002) 'A randomized, double-blind, dose-comparison study of weekly interferon beta-1a in relapsing MS', *Neurology*, vol. 59, no. 10, pp. 1507-1517.
Clarke, J.B. (2010) 'Mechanisms of adverse drug reactions to biologics', *Handb Exp Pharmacol*, no. 196, pp. 453-474.
Clegg, A., Bryant, J. & Milne, R. (2000) 'Disease-modifying drugs for multiple sclerosis: a rapid and systematic review', *Health Technol Assess*, vol. 4, no. 9, pp. i-iv, 1-101.
Cohen, B.A., Greenberger, P.A. & Saini, S. (1998) 'Delayed occurrence of a severe cutaneous reaction in a multiple sclerosis patient taking interferon beta-1b', *Allergy Asthma Proc*, vol. 19, no. 2, pp. 85-88.

Cohen, B.A., Oger, J., Gagnon, A. & Giovannoni, G. (2008) 'The implications of immunogenicity for protein-based multiple sclerosis therapies', *J Neurol Sci*, vol. 275, no. 1-2, pp. 7-17.

Coiffier, B. (2002) 'Treatment paradigms in aggressive non-Hodgkin's lymphoma in elderly patients', *Clin Lymphoma*, vol. 3 Suppl 1, pp. S12-18.

Coiffier, B., Lepretre, S., Pedersen, L.M., Gadeberg, O., Fredriksen, H., van Oers, M.H., Wooldridge, J., Kloczko, J., Holowiecki, J., Hellmann, A., Walewski, J., Flensburg, M., Petersen, J. & Robak, T. (2008) 'Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study', *Blood*, vol. 111, no. 3, pp. 1094-1100.

Cole, K.S., Steckbeck, J.D., Rowles, J.L., Desrosiers, R.C. & Montelaro, R.C. (2004) 'Removal of N-linked glycosylation sites in the V1 region of simian immunodeficiency virus gp120 results in redirection of B-cell responses to V3', *J Virol*, vol. 78, no. 3, pp. 1525-1539.

Coles, A. (2009) 'Multiple sclerosis', *Pract Neurol*, vol. 9, no. 2, pp. 118-126.

Coles, A.J., Cox, A., Le Page, E., Jones, J., Trip, S.A., Deans, J., Seaman, S., Miller, D.H., Hale, G., Waldmann, H. & Compston, D.A. (2006) 'The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy', *J Neurol*, vol. 253, no. 1, pp. 98-108.

Collison, L.W., Chaturvedi, V., Henderson, A.L., Giacomin, P.R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C.J., Brown, S.A., Rehg, J.E., Jones, M.L., Ni, H.T., Artis, D., Turk, M.J. & Vignali, D.A. (2010) 'IL-35-mediated induction of a potent regulatory T cell population', *Nat Immunol*, vol. 11, no. 12, pp. 1093-1101.

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S. & Vignali, D.A. (2007) 'The inhibitory cytokine IL-35 contributes to regulatory T-cell function', *Nature*, vol. 450, no. 7169, pp. 566-569.

Colman, P.M. (1988) 'Structure of antibody-antigen complexes: implications for immune recognition', *Adv Immunol*, vol. 43, pp. 99-132.

Compston, A. & Coles, A. (2008) 'Multiple sclerosis', *Lancet*, vol. 372, no. 9648, pp. 1502-1517.

Cook, S.D., Quinless, J.R., Jotkowitz, A., Beaton, P. & Neutralizing Antibody Study, G. (2001) 'Serum IFN neutralizing antibodies and neopterin levels in a cross-section of MS patients', *Neurology*, vol. 57, no. 6, pp. 1080-1084.

Costantino, C.M., Baecher-Allan, C. & Hafler, D.A. (2008) 'Multiple sclerosis and regulatory T cells', *J Clin Immunol*, vol. 28, no. 6, pp. 697-706.

Cousens, L.P., Najafian, N., Mingozzi, F., Elyaman, W., Mazer, B., Moise, L., Messitt, T.J., Su, Y., Sayegh, M., High, K., Khoury, S.J., Scott, D.W. & De Groot, A.S. (2013) 'In vitro and in vivo studies of IgG-derived Treg epitopes (Tregitopes): a promising new tool for tolerance induction and treatment of autoimmunity', *J Clin Immunol*, vol. 33 Suppl 1, pp. S43-49.

Cox, A.L., Thompson, S.A., Jones, J.L., Robertson, V.H., Hale, G., Waldmann, H., Compston, D.A. & Coles, A.J. (2005) 'Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis', *Eur J Immunol*, vol. 35, no. 11, pp. 3332-3342.

Creeke, P.I. & Farrell, R.A. (2013) 'Clinical testing for neutralizing antibodies to interferon-beta in multiple sclerosis', *Ther Adv Neurol Disord*, vol. 6, no. 1, pp. 3-17.

Cruz, A.A., Lima, F., Sarinho, E., Ayre, G., Martin, C., Fox, H. & Cooper, P.J. (2007) 'Safety of anti-immunoglobulin E therapy with omalizumab in allergic patients at risk of geohelminth infection', *Clin Exp Allergy*, vol. 37, no. 2, pp. 197-207.

Dalum, I., Jensen, M.R., Gregorius, K., Thomasen, C.M., Elsner, H.I. & Mouritsen, S. (1997) 'Induction of cross-reactive antibodies against a self protein by immunization with a modified self protein containing a foreign T helper epitope', *Mol Immunol*, vol. 34, no. 16-17, pp. 1113-1120.

Davies, D.R. & Metzger, H. (1983) 'Structural basis of antibody function', *Annu Rev Immunol*, vol. 1, pp. 87-117.

Davies, J.L., Kawaguchi, Y., Bennett, S.T., Copeman, J.B., Cordell, H.J., Pritchard, L.E., Reed, P.W., Gough, S.C., Jenkins, S.C., Palmer, S.M. & et al. (1994) 'A genome-wide search for human type 1 diabetes susceptibility genes', *Nature*, vol. 371, no. 6493, pp. 130-136.

Dayal, A.S., Jensen, M.A., Lledo, A. & Arnason, B.G. (1995) 'Interferon-gamma-secreting cells in multiple sclerosis patients treated with interferon beta-1b', *Neurology*, vol. 45, no. 12, pp. 2173-2177.

De Groot, A.S. & Scott, D.W. (2007) 'Immunogenicity of protein therapeutics', *Trends Immunol*, vol. 28, no. 11, pp. 482-490.

de Groot, D.M., Vogel, G., Dulos, J., Teeuwen, L., Stebbins, K., Hamann, J., Owens, B.M., van Eenennaam, H., Bos, E. & Boots, A.M. (2009) 'Therapeutic antibody targeting of CD97 in experimental arthritis: the role of antigen expression, shedding, and internalization on the pharmacokinetics of anti-CD97 monoclonal antibody 1B2', *J Immunol*, vol. 183, no. 6, pp. 4127-4134.

De Maeyer, E. & De Maeyer-Guignard, J. (1982) 'Immuno-modulating properties of interferons', *Philos Trans R Soc Lond B Biol Sci*, vol. 299, no. 1094, pp. 77-90.

Deem, M.W. & Hejazi, P. (2010) 'Theoretical Aspects of Immunity', *Annual Review of Chemical and Biomolecular Engineering, Vol 1*, vol. 1, pp. 247-276.

Degauque, N., Mariat, C., Kenny, J., Sanchez-Fueyo, A., Alexopoulos, S.P., Kuchroo, V., Zheng, X.X. & Strom, T.B. (2007) 'Regulation of T-cell immunity by T-cell immunoglobulin and mucin domain proteins', *Transplantation*, vol. 84, no. 1 Suppl, pp. S12-16.

Deisenhammer, F., Reindl, M. & Berger, T. (2001) 'Immunoglobulin subclasses in patients with neutralizing and nonneutralizing antibodies against IFN-beta1b', *J Interferon Cytokine Res*, vol. 21, no. 3, pp. 167-171.

Di Marco, P., Pagnotti, P., Bellomi, F., De Vito, G., Sessa, E., Bagnato, F., Scagnolari, C., Bramanti, P. & Antonelli, G. (2006) 'A novel assay to detect low-titred antibodies to interferon beta in multiple sclerosis patients', *New Microbiol*, vol. 29, no. 1, pp. 11-18.

Dintzis, R.Z., Okajima, M., Middleton, M.H., Greene, G. & Dintzis, H.M. (1989) 'The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence', *J Immunol*, vol. 143, no. 4, pp. 1239-1244.

Disis, M.L., Goodell, V., Schiffman, K. & Knutson, K.L. (2004) 'Humoral epitope-spreading following immunization with a HER-2/neu peptide based vaccine in cancer patients', *J Clin Immunol*, vol. 24, no. 5, pp. 571-578.

Dixon, W. & Silman, A. (2006) 'Is there an association between anti-TNF monoclonal antibody therapy in rheumatoid arthritis and risk of malignancy and serious infection? Commentary on the meta-analysis by Bongartz et al', *Arthritis Res Ther*, vol. 8, no. 5, p. 111.

Dore, I., Weiss, E., Altschuh, D. & Van Regenmortel, M.H. (1988) 'Visualization by electron microscopy of the location of tobacco mosaic virus epitopes reacting with monoclonal antibodies in enzyme immunoassay', *Virology*, vol. 162, no. 2, pp. 279-289.

Dorshkind, K. & Swain, S. (2009) 'Age-associated declines in immune system development and function: causes, consequences, and reversal', *Curr Opin Immunol*, vol. 21, no. 4, pp. 404-407.

Durelli, L., Barbero, P., Bergui, M., Versino, E., Bassano, M.A., Verdun, E., Rivoiro, C., Ferrero, C., Picco, E., Ripellino, P., Giuliani, G., Montanari, E., Clerico, M. & G, I.M.S.S. (2008) 'MRI activity and neutralising antibody as predictors of response to interferon beta treatment in multiple sclerosis', *Journal of Neurology Neurosurgery and Psychiatry*, vol. 79, no. 6, pp. 646-651.

Durelli, L., Barbero, P., Cucci, A., Ferrero, B., Ricci, A., Contessa, G., De Mercanti, S., Ripellino, P., Lapuma, D., Viglietta, E., Bergui, M., Versino, E., Clerico, M. & Grp, O.T.N.S.-S. (2009) 'Neutralizing antibodies in multiple sclerosis patients treated with 375 mu g interferon-beta-1b', *Expert Opinion on Biological Therapy*, vol. 9, no. 4, pp. 387-397.

Durelli, L., Bongioanni, M.R., Ferrero, B., Ferri, R., Imperiale, D., Bradac, G.B., Bergui, M., Geuna, M., Bergamini, L. & Bergamasco, B. (1996) 'Interferon alpha-2a treatment of relapsing-remitting multiple sclerosis: disease activity resumes after stopping treatment', *Neurology*, vol. 47, no. 1, pp. 123-129.

Ettinger, R.A., James, E.A., Kwok, W.W., Thompson, A.R. & Pratt, K.P. (2010) 'HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P', *Haemophilia*, vol. 16, no. 102, pp. 44-55.

Farina, C., Vargas, V., Heydari, N., Kumpfel, T., Meinl, E. & Hohlfeld, R. (2002) 'Treatment with glatiramer acetate induces specific IgG4 antibodies in multiple sclerosis patients', *J Neuroimmunol*, vol. 123, no. 1-2, pp. 188-192.

Farrell, R., Kapoor, R., Leary, S., Rudge, P., Thompson, A.J., Millerand, D.H. & Giovannoni, G. (2008) 'Neutralizing anti-interferon beta antibodies are associated with reduced side effects and delayed impact on efficacy of Interferon-beta', *Mult Scler*, vol. 14, no. 2, pp. 212-218.

Feger, U., Luther, C., Poeschel, S., Melms, A., Tolosa, E. & Wiendl, H. (2007) 'Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients', *Clin Exp Immunol*, vol. 147, no. 3, pp. 412-418.

Fehr, T., Naim, H.Y., Bachmann, M.F., Ochsenbein, A.F., Spielhofer, P., Bucher, E., Hengartner, H., Billeter, M.A. & Zinkernagel, R.M. (1998) 'T-cell independent IgM and enduring protective IgG antibodies induced by chimeric measles viruses', *Nat Med*, vol. 4, no. 8, pp. 945-948.

Fernandez, O., Antiguedad, A., Arbizu, T., Burgues, S., Capdevila, A., de Castro, P., Correa de Sa, J.C., Garcia-Merino, J.A., Izquierdo, G., Magalhaes, A., Montalban, X. & Zarranz, J.J. (1999) '[Natural interferon-beta in the treatment of relapsing-remitting multiple sclerosis: a multicenter, randomized, MRI-based, phase II clinical trial]', *Rev Neurol*, vol. 29, no. 12, pp. 1093-1099.

Ferrante, A., Beard, L.J. & Feldman, R.G. (1990) 'IgG subclass distribution of antibodies to bacterial and viral antigens', *Pediatr Infect Dis J*, vol. 9, no. 8 Suppl, pp. S16-24.

Force, T., Krause, D.S. & Van Etten, R.A. (2007) 'Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition', *Nat Rev Cancer*, vol. 7, no. 5, pp. 332-344.

Francis, G.S., Rice, G.P., Alsop, J.C. & Group, P.S. (2005) 'Interferon beta-1a in MS: results following development of neutralizing antibodies in PRISMS', *Neurology*, vol. 65, no. 1, pp. 48-55.

Frank, J.A., Richert, N., Bash, C., Stone, L., Calabresi, P.A., Lewis, B., Stone, R., Howard, T. & Mcfarland, H.F. (2004) 'Interferon-beta-1b slows progression of atrophy in RRMS - Three-year follow-up in NAb- and NAb+ patients', *Neurology*, vol. 62, no. 5, pp. 719-725.

Freedman, M.S., Francis, G.S., Sanders, E.A., Rice, G.P., O'Connor, P., Comi, G., Duquette, P., Metz, L., Murray, T.J., Bouchard, J.P., Abramsky, O., Pelletier, J., O'Brien, F., Once Weekly Interferon beta-1alpha for Multiple Sclerosis Study, G. & University of British Columbia, M.S.M.R.I.R.G. (2005) 'Randomized study of once-weekly interferon beta-1la therapy in relapsing multiple sclerosis: three-year data from the OWIMS study', *Mult Scler*, vol. 11, no. 1, pp. 41-45.

French, M. (1986) 'Serum IgG subclasses in normal adults', *Monogr Allergy*, vol. 19, pp. 100-107.

Frisullo, G., Nociti, V., Iorio, R., Patanella, A.K., Caggiula, M., Marti, A., Sancricca, C., Angelucci, F., Mirabella, M., Tonali, P.A. & Batocchi, A.P. (2009) 'Regulatory T cells fail to suppress CD4T+-bet+ T cells in relapsing multiple sclerosis patients', *Immunology*, vol. 127, no. 3, pp. 418-428.

Fulcher, C.A., de Graaf Mahoney, S., Roberts, J.R., Kasper, C.K. & Zimmerman, T.S. (1985) 'Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments', *Proc Natl Acad Sci U S A*, vol. 82, no. 22, pp. 7728-7732.

Fulkerson, P.C., Zhu, H., Williams, D.A., Zimmermann, N. & Rothenberg, M.E. (2005) 'CXCL9 inhibits eosinophil responses by a CCR3- and Rac2-dependent mechanism', *Blood*, vol. 106, no. 2, pp. 436-443.

Gad, H.H., Dellgren, C., Hamming, O.J., Vends, S., Paludan, S.R. & Hartmann, R. (2009) 'Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family', *J Biol Chem*, vol. 284, no. 31, pp. 20869-20875.

Gamble, C.N. (1966) 'The role of soluble aggregates in the primary immune response of mice to human gamma globulin', *Int Arch Allergy Appl Immunol*, vol. 30, no. 5, pp. 446-455.

Gao, W., Thompson, L., Zhou, Q., Putheti, P., Fahmy, T.M., Strom, T.B. & Metcalfe, S.M. (2009) 'Treg versus Th17 lymphocyte lineages are cross-regulated by LIF versus IL-6', *Cell Cycle*, vol. 8, no. 9, pp. 1444-1450.

Garcia de Vinuesa, C., O'Leary, P., Sze, D.M., Toellner, K.M. & MacLennan, I.C. (1999) 'T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci', *Eur J Immunol*, vol. 29, no. 4, pp. 1314-1323.

Gennery, A.R. & Cant, A.J. (2007) 'Cord blood stem cell transplantation in primary immune deficiencies', *Curr Opin Allergy Clin Immunol*, vol. 7, no. 6, pp. 528-534.

Germain, R.N. (1994) 'MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation', *Cell*, vol. 76, no. 2, pp. 287-299.

Ghaderi, D., Taylor, R.E., Padler-Karavani, V., Diaz, S. & Varki, A. (2010) 'Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins', *Nat Biotechnol*, vol. 28, no. 8, pp. 863-867.

Giannelli, G., Antonelli, G., Fera, G., Del Vecchio, S., Riva, E., Broccia, C., Schiraldi, O. & Dianzani, F. (1994) 'Biological and clinical significance of neutralizing and binding antibodies to interferon-alpha (IFN-alpha) during therapy for chronic hepatitis C', *Clin Exp Immunol*, vol. 97, no. 1, pp. 4-9.

Gibbs, E. & Oger, J. (2007) 'The IgG subclass-specificities of anti-IFNbeta antibodies change with time and differ between the IFNbeta products in relapsing remitting multiple sclerosis patients', *J Neuroimmunol*, vol. 190, no. 1-2, pp. 146-150.

Giezen, T.J., Mantel-Teeuwisse, A.K., Straus, S.M., Schellekens, H., Leufkens, H.G. & Egberts, A.C. (2008) 'Safety-related regulatory actions for biologicals approved in the United States and the European Union', *JAMA*, vol. 300, no. 16, pp. 1887-1896.

Gilli, F., Marnetto, F., Caldano, M., Valentino, P., Granieri, L., Di Sapio, A., Capobianco, M., Sala, A., Malucchi, S., Kappos, L., Lindbergb, R.L.P. & Bertolotto, A. (2007) 'Anti-interferon-beta neutralising activity is not entirely mediated by antibodies', *J Neuroimmunol*, vol. 192, no. 1-2, pp. 198-205.

Giovannoni, G., Barbarash, O., Casset-Semanaz, F., Jaber, A., King, J., Metz, L., Pardo, G., Simsarian, J., Sorensen, P.S., Stubinski, B. & Grp, R.S. (2007) 'Immunogenicity and tolerability of an Investigational formulation of interferon-beta 1a: 24- and 48-week interim analyses of a 2-year, single-arm, historically controlled, phase IIIb study in adults with multiple sclerosis', *Clin Ther*, vol. 29, no. 6, pp. 1128-1145.

Giovannoni, G. & Goodman, A. (2005) 'Neutralizing anti-IFN-beta antibodies: how much more evidence do we need to use them in practice?', *Neurology*, vol. 65, no. 1, pp. 6-8.

Giovannoni, G., Munschauer, F.E., 3rd & Deisenhammer, F. (2002) 'Neutralising antibodies to interferon beta during the treatment of multiple sclerosis', *J Neurol Neurosurg Psychiatry*, vol. 73, no. 5, pp. 465-469.

Gneiss, C., Reindl, M., Berger, T., Lutterotti, A., Ehling, R., Egg, R. & Deisenhammer, F. (2004a) 'Epitope specificity of neutralizing antibodies against IFN-beta', *J Interferon Cytokine Res*, vol. 24, no. 5, pp. 283-290.

Gneiss, C., Reindl, M., Lutterotti, A., Ehling, R., Egg, R., Khalil, M., Berger, T. & Deisenhammer, F. (2004b) 'Interferon-beta: the neutralizing antibody (NAb) titre predicts reversion to NAb negativity', *Mult Scler*, vol. 10, no. 5, pp. 507-510.

Goins, C.L., Chappell, C.P., Shashidharamurthy, R., Selvaraj, P. & Jacob, J. (2010) 'Immune complex-mediated enhancement of secondary antibody responses', *J Immunol*, vol. 184, no. 11, pp. 6293-6298.

Gold, R., Buttgereit, F. & Toyka, K.V. (2001) 'Mechanism of action of glucocorticosteroid hormones: possible implications for therapy of neuroimmunological disorders', *J Neuroimmunol*, vol. 117, no. 1-2, pp. 1-8.

Goodin, D.S., Hartung, H.P., O'Connor, P., Filippi, M., Arnason, B., Comi, G., Cook, S., Jeffery, D., Kappos, L., Bogumil, T., Knappertz, V., Sandbrink, R., Beckmann, K., White, R., Petkau, J., Pohl, C. & Group, B.S. (2012) 'Neutralizing antibodies to interferon beta-1b multiple sclerosis: a clinico-radiographic paradox in the BEYOND trial', *Mult Scler*, vol. 18, no. 2, pp. 181-195.

Goodnow, C.C. (2001) 'Pathways for self-tolerance and the treatment of autoimmune diseases', *Lancet*, vol. 357, no. 9274, pp. 2115-2121.

Gopal, A.K., Ramchandren, R., O'Connor, O.A., Berryman, R.B., Advani, R.H., Chen, R., Smith, S.E., Cooper, M., Rothe, A., Matous, J.V., Grove, L.E. & Zain, J. (2012) 'Safety and efficacy of brentuximab vedotin for Hodgkin lymphoma recurring after allogeneic stem cell transplantation', *Blood*, vol. 120, no. 3, pp. 560-568.

Goronzy, J., Weyand, C.M. & Waase, I. (1985) 'T cell subpopulations in inflammatory bowel disease: evidence for a defective induction of T8+ suppressor/cytotoxic T lymphocytes', *Clin Exp Immunol*, vol. 61, no. 3, pp. 593-600.

Gottesman, M.H. & Friedman-Urevich, S. (2006) 'Interferon beta-1b (Betaseron (R)/Betaferon (R)) is well tolerated at a dose of 500 mu g: interferon dose escalation assessment of safety (IDEAS)', *Mult Scler*, vol. 12, no. 3, pp. 271-280.

Gregorek, H., Madalinski, K., Woynarowski, M., Mikolajewicz, J., Syczewska, M. & Socha, J. (2000) 'IgG subclass distribution of hepatitis B surface antigen antibodies induced in children with chronic hepatitis B infection after interferon-alpha therapy', *J Infect Dis*, vol. 181, no. 6, pp. 2059-2062.

Grey, H.M. & Kunkel, H.G. (1964) 'H Chain Subgroups of Myeloma Proteins and Normal 7s Gamma-Globulin', *J Exp Med*, vol. 120, pp. 253-266.

Gross, P.C. & Zeppezauer, M. (2010) 'Infrared spectroscopy for biopharmaceutical protein analysis', *J Pharm Biomed Anal*, vol. 53, no. 1, pp. 29-36.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E. & Roncarolo, M.G. (1997) 'A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis', *Nature*, vol. 389, no. 6652, pp. 737-742.

Guzzo, J.A. & Nichols, T.C. (1999) 'Possible anaphylactic reaction to abciximab', *Catheter Cardiovasc Interv*, vol. 48, no. 1, pp. 71-73.

Han, K., Haley, J.C., Carlson, K., Pinter-Brown, L. & Soriano, T. (2003) 'Regression of cutaneous intravascular lymphoma with rituximab', *Cutis*, vol. 72, no. 2, pp. 137-140.

Harris, J.M., Martin, N.E. & Modi, M. (2001) 'Pegylation: a novel process for modifying pharmacokinetics', *Clin Pharmacokinet*, vol. 40, no. 7, pp. 539-551.

Hart, D.N. (1997) 'Dendritic cells: unique leukocyte populations which control the primary immune response', *Blood*, vol. 90, no. 9, pp. 3245-3287.

Hartmann, G., Weiner, G.J. & Krieg, A.M. (1999) 'CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells', *Proc Natl Acad Sci U S A*, vol. 96, no. 16, pp. 9305-9310.

Hartung, H.P., Bar-Or, A. & Zoukos, Y. (2004) 'What do we know about the mechanism of action of disease-modifying treatments in MS?', *J Neurol*, vol. 251 Suppl 5, pp. v12-v29.

Hartung, H.P., Freedman, M.S., Polman, C.H., Edan, G., Kappos, L., Miller, D.H., Montalban, X., Barkhof, F., Petkau, J., White, R., Sahajpal, V., Knappertz, V., Beckmann, K., Lanius, V., Sandbrink, R., Pohl, C. & Grp, B.S. (2011) 'Interferon beta-1b-neutralizing antibodies 5 years after clinically isolated syndrome', *Neurology*, vol. 77, no. 9, pp. 835-843.

Hashimoto, M., Hirota, K., Yoshitomi, H., Maeda, S., Teradaira, S., Akizuki, S., Prieto-Martin, P., Nomura, T., Sakaguchi, N., Kohl, J., Heyman, B., Takahashi, M., Fujita, T., Mimori, T. & Sakaguchi, S. (2010) 'Complement drives Th17 cell differentiation and triggers autoimmune arthritis', *J Exp Med*, vol. 207, no. 6, pp. 1135-1143.

Hegen, H., Schleiser, M., Gneiss, C., Di Pauli, F., Ehling, R., Kuenz, B., Lutterotti, A., Berger, T. & Deisenhammer, F. (2012a) 'Persistency of neutralizing antibodies depends on titer and interferon-beta preparation', *Multiple Sclerosis Journal*, vol. 18, no. 5, pp. 610-615.

Hegen, H., Schleiser, M., Gneiss, C., Di Pauli, F., Ehling, R., Kuenz, B., Lutterotti, A., Berger, T. & Deisenhammer, F. (2012b) 'Persistency of neutralizing antibodies depends on titer and interferon-beta preparation', *Mult Scler*, vol. 18, no. 5, pp. 610-615.

Henze, T., Rieckmann, P., Toyka, K.V. & Multiple Sclerosis Therapy Consensus Group of the German Multiple Sclerosis, S. (2006) 'Symptomatic treatment of multiple sclerosis. Multiple Sclerosis Therapy Consensus Group (MSTCG) of the German Multiple Sclerosis Society', *Eur Neurol*, vol. 56, no. 2, pp. 78-105.

Heyman, B. (2000) 'Regulation of antibody responses via antibodies, complement, and Fc receptors', *Annu Rev Immunol*, vol. 18, pp. 709-737.

Ho, W.Y., Nguyen, H.N., Wolfl, M., Kuball, J. & Greenberg, P.D. (2006) 'In vitro methods for generating CD8+ T-cell clones for immunotherapy from the naive repertoire', *J Immunol Methods*, vol. 310, no. 1-2, pp. 40-52.

Hochuli, E. (1997) 'Interferon immunogenicity: technical evaluation of interferon-alpha 2a', *J Interferon Cytokine Res*, vol. 17 Suppl 1, pp. S15-21.

Hoffmann, S., Cepok, S., Grummel, V., Lehmann-Horn, K., Hackermuller, J., Stadler, P.F., Hartung, H.P., Berthele, A., Deisenhammer, F., Wassmuth, R. & Hemmer, B. (2008) 'HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of

antibodies against interferon-beta therapy in multiple sclerosis', *Am J Hum Genet*, vol. 83, no. 2, pp. 219-227.
Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., Lassmann, H. & Wekerle, H. (2006) 'The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis', *Neurol Sci*, vol. 27 Suppl 1, pp. S1-7.
Hohlfeld, R. & Wekerle, H. (2004) 'Autoimmune concepts of multiple sclerosis as a basis for selective immunotherapy: from pipe dreams to (therapeutic) pipelines', *Proc Natl Acad Sci U S A*, vol. 101 Suppl 2, pp. 14599-14606.
Hoigne, R., Schlumberger, H.P., Vervloet, D. & Zoppi, M. (1993) 'Epidemiology of allergic drug reactions', *Monogr Allergy*, vol. 31, pp. 147-170.
Hong, J., Li, N., Zhang, X., Zheng, B. & Zhang, J.Z. (2005) 'Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3', *Proc Natl Acad Sci U S A*, vol. 102, no. 18, pp. 6449-6454.
Hori, S., Nomura, T. & Sakaguchi, S. (2003) 'Control of regulatory T cell development by the transcription factor Foxp3', *Science*, vol. 299, no. 5609, pp. 1057-1061.
Hosokawa, T. (1979) 'Studies on B-cell memory. II. T-cell independent antigen can induce B-cell memory', *Immunology*, vol. 38, no. 2, pp. 291-299.
Hosokawa, T., Amagai, T. & Muramatsu, S. (1979) 'Studies on B-cell memory. I. Generation and exhaustion of B-cell memory by thymus-dependent antigen in T-cell depleted mice', *Immunology*, vol. 38, no. 2, pp. 283-289.
Hoyer, L.W. (1993) 'Characterization of dysfunctional factor VIII molecules', *Methods Enzymol*, vol. 222, pp. 169-176.
Hudis, C.A. (2007) 'Trastuzumab--mechanism of action and use in clinical practice', *N Engl J Med*, vol. 357, no. 1, pp. 39-51.
Hughes, R. (1997) 'Wait for further evidence confirming effect of interferon beta in multiple sclerosis', *BMJ*, vol. 314, no. 7096, p. 1763.
Huizinga, T.W., Kerst, M., Nuyens, J.H., Vlug, A., von dem Borne, A.E., Roos, D. & Tetteroo, P.A. (1989) 'Binding characteristics of dimeric IgG subclass complexes to human neutrophils', *J Immunol*, vol. 142, no. 7, pp. 2359-2364.
Hunley, T.E., Corzo, D., Dudek, M., Kishnani, P., Amalfitano, A., Chen, Y.T., Richards, S.M., Phillips, J.A., 3rd, Fogo, A.B. & Tiller, G.E. (2004) 'Nephrotic syndrome complicating alpha-glucosidase replacement therapy for Pompe disease', *Pediatrics*, vol. 114, no. 4, pp. e532-535.
Hussien, Y., Sanna, A., Soderstrom, M., Link, H. & Huang, Y.M. (2001) 'Glatiramer acetate and IFN-beta act on dendritic cells in multiple sclerosis', *J Neuroimmunol*, vol. 121, no. 1-2, pp. 102-110.
'The IFNB Multiple Sclerosis Study Group' (1993), *Neurology*, vol. 43, no. 4, pp. 655-661.
Inada, Y., Furukawa, M., Sasaki, H., Kodera, Y., Hiroto, M., Nishimura, H. & Matsushima, A. (1995) 'Biomedical and biotechnological applications of PEG- and PM-modified proteins', *Trends Biotechnol*, vol. 13, no. 3, pp. 86-91.
Ingram, G., Hakobyan, S., Hirst, C.L., Harris, C.L., Loveless, S., Mitchell, J.P., Pickersgill, T.P., Robertson, N.P. & Morgan, B.P. (2012) 'Systemic complement profiling in multiple sclerosis as a biomarker of disease state', *Mult Scler*, vol. 18, no. 10, pp. 1401-1411.
International, M.H.C., Autoimmunity Genetics, N., Rioux, J.D., Goyette, P., Vyse, T.J., Hammarstrom, L., Fernando, M.M., Green, T., De Jager, P.L., Foisy, S., Wang, J., de Bakker, P.I., Leslie, S., McVean, G., Padyukov, L., Alfredsson, L., Annese, V., Hafler, D.A., Pan-Hammarstrom, Q., Matell, R., Sawcer, S.J., Compston, A.D., Cree, B.A., Mirel, D.B., Daly, M.J., Behrens, T.W., Klareskog, L., Gregersen, P.K., Oksenberg, J.R. & Hauser, S.L. (2009) 'Mapping of multiple susceptibility variants within the MHC region for 7 immune-mediated diseases', *Proc Natl Acad Sci U S A*, vol. 106, no. 44, pp. 18680-18685.
Investigators, C.T., Coles, A.J., Compston, D.A., Selmaj, K.W., Lake, S.L., Moran, S., Margolin, D.H., Norris, K. & Tandon, P.K. (2008) 'Alemtuzumab vs. interferon beta-1a in early multiple sclerosis', *N Engl J Med*, vol. 359, no. 17, pp. 1786-1801.
Isaacs, A. & Lindenmann, J. (1957) 'Virus interference. I. The interferon', *Proc R Soc Lond B Biol Sci*, vol. 147, no. 927, pp. 258-267.

Ito, T., Inaba, M., Inaba, K., Toki, J., Sogo, S., Iguchi, T., Adachi, Y., Yamaguchi, K., Amakawa, R., Valladeau, J., Saeland, S., Fukuhara, S. & Ikehara, S. (1999) 'A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells', *J Immunol*, vol. 163, no. 3, pp. 1409-1419.

Itri, L.M., Campion, M., Dennin, R.A., Palleroni, A.V., Gutterman, J.U., Groopman, J.E. & Trown, P.W. (1987) 'Incidence and clinical significance of neutralizing antibodies in patients receiving recombinant interferon alfa-2a by intramuscular injection', *Cancer*, vol. 59, no. 3 Suppl, pp. 668-674.

Jacobs, L. & Johnson, K.P. (1994) 'A brief history of the use of interferons as treatment of multiple sclerosis', *Arch Neurol*, vol. 51, no. 12, pp. 1245-1252.

Jacobs, L., O'Malley, J., Freeman, A. & Ekes, R. (1982a) 'Intrathecal interferon for multiple sclerosis', *Science*, vol. 217, no. 4556, p. 270.

Jacobs, L., O'Malley, J., Freeman, A., Murawski, J. & Ekes, R. (1982b) 'Intrathecal interferon in multiple sclerosis', *Arch Neurol*, vol. 39, no. 10, pp. 609-615.

Jacquemin, M., Vantomme, V., Buhot, C., Lavend'homme, R., Burny, W., Demotte, N., Chaux, P., Peerlinck, K., Vermylen, J., Maillere, B., van der Bruggen, P. & Saint-Remy, J.M. (2003) 'CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A', *Blood*, vol. 101, no. 4, pp. 1351-1358.

Jalkanen, S. & Salmi, M. (2001) Lymphocytes: Recirculation, in, *eLS*, John Wiley & Sons, Ltd.

Janeway CA Jr, T.P., Walport M, et al. (2001) *Immunobiology: The Immune System in Health and Disease.*, vol. 5th edition, Garland Science, New York.

Janeway, C.A., Jr. (1989) 'Approaching the asymptote? Evolution and revolution in immunology', *Cold Spring Harb Symp Quant Biol*, vol. 54 Pt 1, pp. 1-13.

Janeway, C.A., Jr. & Bottomly, K. (1994) 'Signals and signs for lymphocyte responses', *Cell*, vol. 76, no. 2, pp. 275-285.

Janeway, C.A., Jr. & Medzhitov, R. (2002) 'Innate immune recognition', *Annu Rev Immunol*, vol. 20, pp. 197-216.

Jankowitz, R., Joyce, J. & Jacobs, S.A. (2008) 'Anaphylaxis after administration of ibritumomab tiuxetan for follicular non-hodgkin lymphoma', *Clin Nucl Med*, vol. 33, no. 2, pp. 94-96.

Jean, G.W. & Shah, S.R. (2008) 'Epidermal growth factor receptor monoclonal antibodies for the treatment of metastatic colorectal cancer', *Pharmacotherapy*, vol. 28, no. 6, pp. 742-754.

Jefferis, R. (1986) 'Polyclonal and monoclonal antibody reagents specific for IgG subclasses', *Monogr Allergy*, vol. 19, pp. 71-85.

Jiang, H., Milo, R., Swoveland, P., Johnson, K.P., Panitch, H. & Dhib-Jalbut, S. (1995) 'Interferon beta-1b reduces interferon gamma-induced antigen-presenting capacity of human glial and B cells', *J Neuroimmunol*, vol. 61, no. 1, pp. 17-25.

Johnson, K.P. & Panitch, H.S. (1989) 'Effects of experimental recombinant interferons on multiple sclerosis', *Trans Am Clin Climatol Assoc*, vol. 100, pp. 171-176.

Jones, T.D., Crompton, L.J., Carr, F.J. & Baker, M.P. (2009) 'Deimmunization of monoclonal antibodies', *Methods Mol Biol*, vol. 525, pp. 405-423, xiv.

Joseph, J., Knobler, R.L., D'Imperio, C. & Lublin, F.D. (1988) 'Down-regulation of interferon-gamma-induced class II expression on human glioma cells by recombinant interferon-beta: effects of dosage treatment schedule', *J Neuroimmunol*, vol. 20, no. 1, pp. 39-44.

Kang, S.M., Tang, Q. & Bluestone, J.A. (2007) 'CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects', *Am J Transplant*, vol. 7, no. 6, pp. 1457-1463.

Kappos, L., Clanet, M., Sandberg-Wollheim, M., Radue, E.W., Hartung, H.P., Hohlfeld, R., Xu, J., Bennett, D., Sandrock, A., Goelz, S. & European Interferon Beta-1a, I.M.D.-C.S.I. (2005) 'Neutralizing antibodies and efficacy of interferon beta-1a: a 4-year controlled study', *Neurology*, vol. 65, no. 1, pp. 40-47.

Karpusas, M., Nolte, M., Benton, C.B., Meier, W., Lipscomb, W.N. & Goelz, S. (1997) 'The crystal structure of human interferon beta at 2.2-A resolution', *Proc Natl Acad Sci U S A*, vol. 94, no. 22, pp. 11813-11818.

Kelley, M., Ahene, A.B., Gorovits, B., Kamerud, J., King, L.E., McIntosh, T. & Yang, J. (2013) 'Theoretical Considerations and Practical Approaches to Address the Effect of Anti-drug Antibody (ADA) on Quantification of Biotherapeutics in Circulation', *AAPS J*.

Kerekes, K., Cooper, P.D., Prechl, J., Jozsi, M., Bajtay, Z. & Erdei, A. (2001) 'Adjuvant effect of gamma-inulin is mediated by C3 fragments deposited on antigen-presenting cells', *J Leukoc Biol*, vol. 69, no. 1, pp. 69-74.

Khan, M.A., Mathieu, A., Sorrentino, R. & Akkoc, N. (2007) 'The pathogenetic role of HLA-B27 and its subtypes', *Autoimmun Rev*, vol. 6, no. 3, pp. 183-189.

Khan, O.A. & Dhib-Jalbut, S.S. (1998) 'Neutralizing antibodies to interferon beta-1a and interferon beta-1b in MS patients are cross-reactive', *Neurology*, vol. 51, no. 6, pp. 1698-1702.

Khattri, R., Cox, T., Yasayko, S.A. & Ramsdell, F. (2003) 'An essential role for Scurfin in CD4+CD25+ T regulatory cells', *Nat Immunol*, vol. 4, no. 4, pp. 337-342.

Killestein, J. & Polman, C.H. (2011) 'Determinants of interferon beta efficacy in patients with multiple sclerosis', *Nat Rev Neurol*, vol. 7, no. 4, pp. 221-228.

Knobler, R.L., Greenstein, J.I., Johnson, K.P., Lublin, F.D., Panitch, H.S., Conway, K., Grantgorsen, S.V., Muldoon, J., Marcus, S.G., Wallenberg, J.C., Williams, G.J. & Yoshizawa, C.N. (1993) 'Systemic Recombinant Human Interferon-Beta Treatment of Relapsing-Remitting Multiple-Sclerosis - Pilot-Study Analysis and 6-Year Follow-Up', *Journal of Interferon Research*, vol. 13, no. 5, pp. 333-340.

Koch-Henriksen, N. (1999) 'The Danish Multiple Sclerosis Registry: a 50-year follow-up', *Mult Scler*, vol. 5, no. 4, pp. 293-296.

Kola, I. & Landis, J. (2004) 'Can the pharmaceutical industry reduce attrition rates?', *Nat Rev Drug Discov*, vol. 3, no. 8, pp. 711-715.

Koren, E. (2002) 'From characterization of antibodies to prediction of immunogenicity', *Dev Biol (Basel)*, vol. 109, pp. 87-95.

Koren, E., De Groot, A.S., Jawa, V., Beck, K.D., Boone, T., Rivera, D., Li, L., Mytych, D., Koscec, M., Weeraratne, D., Swanson, S. & Martin, W. (2007) 'Clinical validation of the "in silico" prediction of immunogenicity of a human recombinant therapeutic protein', *Clin Immunol*, vol. 124, no. 1, pp. 26-32.

Koren, E., Zuckerman, L.A. & Mire-Sluis, A.R. (2002) 'Immune responses to therapeutic proteins in humans--clinical significance, assessment and prediction', *Curr Pharm Biotechnol*, vol. 3, no. 4, pp. 349-360.

Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T.R., Backstrom, B.T., Sobel, R.A., Wucherpfennig, K.W., Strom, T.B., Oukka, M. & Kuchroo, V.K. (2007) 'Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation', *Nat Med*, vol. 13, no. 4, pp. 423-431.

Korporal, M., Haas, J., Balint, B., Fritzsching, B., Schwarz, A., Moeller, S., Fritz, B., Suri-Payer, E. & Wildemann, B. (2008) 'Interferon beta-induced restoration of regulatory T-cell function in multiple sclerosis is prompted by an increase in newly generated naive regulatory T cells', *Arch Neurol*, vol. 65, no. 11, pp. 1434-1439.

Kosmac, M., Avcin, T., Toplak, N., Simonini, G., Cimaz, R. & Curin Serbec, V. (2011) 'Exploring the binding sites of anti-infliximab antibodies in pediatric patients with rheumatic diseases treated with infliximab', *Pediatr Res*, vol. 69, no. 3, pp. 243-248.

Krieckaert, C., Rispens, T. & Wolbink, G. (2012) 'Immunogenicity of biological therapeutics: from assay to patient', *Curr Opin Rheumatol*, vol. 24, no. 3, pp. 306-311.

Kumar, D., Bouldin, T.W. & Berger, R.G. (2010) 'A case of progressive multifocal leukoencephalopathy in a patient treated with infliximab', *Arthritis Rheum*, vol. 62, no. 11, pp. 3191-3195.

Kuramochi, Y., Guo, X., Sawyer, D.B. & Lim, C.C. (2006) 'Rapid electrical stimulation induces early activation of kinase signal transduction pathways and apoptosis in adult rat ventricular myocytes', *Exp Physiol*, vol. 91, no. 4, pp. 773-780.

Kwon, H.J., Cote, T.R., Cuffe, M.S., Kramer, J.M. & Braun, M.M. (2003) 'Case reports of heart failure after therapy with a tumor necrosis factor antagonist', *Ann Intern Med*, vol. 138, no. 10, pp. 807-811.

Lal, G. & Bromberg, J.S. (2009) 'Epigenetic mechanisms of regulation of Foxp3 expression', *Blood*, vol. 114, no. 18, pp. 3727-3735.
Lanzavecchia, A. (1990) 'Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes', *Annu Rev Immunol*, vol. 8, pp. 773-793.
Lasser, K.E., Allen, P.D., Woolhandler, S.J., Himmelstein, D.U., Wolfe, S.M. & Bor, D.H. (2002) 'Timing of new black box warnings and withdrawals for prescription medications', *JAMA*, vol. 287, no. 17, pp. 2215-2220.
Lazarski, C.A., Chaves, F.A., Jenks, S.A., Wu, S., Richards, K.A., Weaver, J.M. & Sant, A.J. (2005) 'The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance', *Immunity*, vol. 23, no. 1, pp. 29-40.
Leach, M.W. (2013) 'Regulatory forum opinion piece: differences between protein-based biologic products (biotherapeutics) and chemical entities (small molecules) of relevance to the toxicologic pathologist', *Toxicol Pathol*, vol. 41, no. 1, pp. 128-136.
Leader, B., Baca, Q.J. & Golan, D.E. (2008) 'Protein therapeutics: a summary and pharmacological classification', *Nat Rev Drug Discov*, vol. 7, no. 1, pp. 21-39.
Leary, S.M., Porter, B. & Thompson, A.J. (2005) 'Multiple sclerosis: diagnosis and the management of acute relapses', *Postgrad Med J*, vol. 81, no. 955, pp. 302-308.
Leckband, S.G., Kelsoe, J.R., Dunnenberger, H.M., George, A.L., Jr., Tran, E., Berger, R., Muller, D.J., Whirl-Carrillo, M., Caudle, K.E. & Pirmohamed, M. (2013) 'Clinical pharmacogenetics implementation consortium guidelines for hla-B genotype and carbamazepine dosing', *Clin Pharmacol Ther*, vol. 94, no. 3, pp. 324-328.
Leppert, D., Waubant, E., Burk, M.R., Oksenberg, J.R. & Hauser, S.L. (1996) 'Interferon beta-1b inhibits gelatinase secretion and in vitro migration of human T cells: a possible mechanism for treatment efficacy in multiple sclerosis', *Ann Neurol*, vol. 40, no. 6, pp. 846-852.
Levin, L.I., Munger, K.L., Rubertone, M.V., Peck, C.A., Lennette, E.T., Spiegelman, D. & Ascherio, A. (2003) 'Multiple sclerosis and Epstein-Barr virus', *JAMA*, vol. 289, no. 12, pp. 1533-1536.
Levings, M.K., Sangregorio, R. & Roncarolo, M.G. (2001) 'Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function', *J Exp Med*, vol. 193, no. 11, pp. 1295-1302.
Li, D.K., Zhao, G.J., Paty, D.W. & University of British Columbia, M.S.M.R.I.A.R.G.T.S.S.G. (2001a) 'Randomized controlled trial of interferon-beta-1a in secondary progressive MS: MRI results', *Neurology*, vol. 56, no. 11, pp. 1505-1513.
Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M. & Kuter, D.J. (2001b) 'Thrombocytopenia caused by the development of antibodies to thrombopoietin', *Blood*, vol. 98, no. 12, pp. 3241-3248.
Liebler, D.C. & Guengerich, F.P. (2005) 'Elucidating mechanisms of drug-induced toxicity', *Nat Rev Drug Discov*, vol. 4, no. 5, pp. 410-420.
Lin, L. (1998) 'Betaseron', *Dev Biol Stand*, vol. 96, pp. 97-104.
Lindsay, J.O., Ciesielski, C.J., Scheinin, T., Hodgson, H.J. & Brennan, F.M. (2001) 'The prevention and treatment of murine colitis using gene therapy with adenoviral vectors encoding IL-10', *J Immunol*, vol. 166, no. 12, pp. 7625-7633.
Ling, P.D., Warren, M.K. & Vogel, S.N. (1985) 'Antagonistic effect of interferon-beta on the interferon-gamma-induced expression of Ia antigen in murine macrophages', *J Immunol*, vol. 135, no. 3, pp. 1857-1863.
Liu, Y., Reidler, H., Pan, J., Milunic, D., Qin, D., Chen, D., Vallejo, Y.R. & Yin, R. (2011) 'A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers', *J Pharmacol Toxicol Methods*, vol. 64, no. 3, pp. 238-245.
Loertscher, R. & Lavery, P. (2002) 'The role of glycosyl phosphatidyl inositol (GPI)-anchored cell surface proteins in T-cell activation', *Transpl Immunol*, vol. 9, no. 2-4, pp. 93-96.
Lok, A.S., Lai, C.L. & Leung, E.K. (1990) 'Interferon antibodies may negate the antiviral effects of recombinant alpha-interferon treatment in patients with chronic hepatitis B virus infection', *Hepatology*, vol. 12, no. 6, pp. 1266-1270.
Lorenzi, A.R., Clarke, A.M., Wooldridge, T., Waldmann, H., Hale, G., Symmons, D., Hazleman, B.L. & Isaacs, J.D. (2008) 'Morbidity and mortality in rheumatoid arthritis patients with prolonged

therapy-induced lymphopenia: twelve-year outcomes', *Arthritis Rheum*, vol. 58, no. 2, pp. 370-375.

Lu, L.F., Thai, T.H., Calado, D.P., Chaudhry, A., Kubo, M., Tanaka, K., Loeb, G.B., Lee, H., Yoshimura, A., Rajewsky, K. & Rudensky, A.Y. (2009) 'Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein', *Immunity*, vol. 30, no. 1, pp. 80-91.

Lublin, F.D., Whitaker, J.N., Eidelman, B.H., Miller, A.E., Arnason, B.G. & Burks, J.S. (1996) 'Management of patients receiving interferon beta-1b for multiple sclerosis: report of a consensus conference', *Neurology*, vol. 46, no. 1, pp. 12-18.

Ludwig-Portugall, I., Hamilton-Williams, E.E., Gotot, J. & Kurts, C. (2009) 'CD25+ T(reg) specifically suppress auto-Ab generation against pancreatic tissue autoantigens', *Eur J Immunol*, vol. 39, no. 1, pp. 225-233.

Ludwig-Portugall, I., Hamilton-Williams, E.E., Gottschalk, C. & Kurts, C. (2008) 'Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens', *J Immunol*, vol. 181, no. 7, pp. 4447-4451.

Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., Antoni, C., Leeb, B., Elliott, M.J., Woody, J.N., Schaible, T.F. & Feldmann, M. (1998) 'Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis', *Arthritis Rheum*, vol. 41, no. 9, pp. 1552-1563.

Major, E.O. (2010) 'Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies', *Annu Rev Med*, vol. 61, pp. 35-47.

Mallal, S., Nolan, D., Witt, C., Masel, G., Martin, A.M., Moore, C., Sayer, D., Castley, A., Mamotte, C., Maxwell, D., James, I. & Christiansen, F.T. (2002) 'Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir', *Lancet*, vol. 359, no. 9308, pp. 727-732.

Malucchi, S., Capobianco, M., Gilli, F., Marnetto, F., Caldano, M., Sala, A. & Bertolotto, A. (2005) 'Fate of multiple sclerosis patients positive for neutralising antibodies towards interferon beta shifted to alternative treatments', *Neurol Sci*, vol. 26 Suppl 4, pp. S213-214.

Malucchi, S., Sala, A., Gilli, F., Bottero, R., Di Sapio, A., Capobianco, M. & Bertolotto, A. (2004) 'Neutralizing antibodies reduce the efficacy of beta IFN during treatment of multiple sclerosis', *Neurology*, vol. 62, no. 11, pp. 2031-2037.

Marrosu, M.G., Muntoni, F., Murru, M.R., Costa, G., Pischedda, M.P., Pirastu, M., Sotgiu, S., Rosati, G. & Cianchetti, C. (1992) 'HLA-DQB1 genotype in Sardinian multiple sclerosis: evidence for a key role of DQB1 *0201 and *0302 alleles', *Neurology*, vol. 42, no. 4, pp. 883-886.

Martyn, C.N., Cruddas, M. & Compston, D.A. (1993) 'Symptomatic Epstein-Barr virus infection and multiple sclerosis', *J Neurol Neurosurg Psychiatry*, vol. 56, no. 2, pp. 167-168.

McCormack, M., Alfirevic, A., Bourgeois, S., Farrell, J.J., Kasperaviciute, D., Carrington, M., Sills, G.J., Marson, T., Jia, X., de Bakker, P.I., Chinthapalli, K., Molokhia, M., Johnson, M.R., O'Connor, G.D., Chaila, E., Alhusaini, S., Shianna, K.V., Radtke, R.A., Heinzen, E.L., Walley, N., Pandolfo, M., Pichler, W., Park, B.K., Depondt, C., Sisodiya, S.M., Goldstein, D.B., Deloukas, P., Delanty, N., Cavalleri, G.L. & Pirmohamed, M. (2011) 'HLA-A*3101 and carbamazepine-induced hypersensitivity reactions in Europeans', *N Engl J Med*, vol. 364, no. 12, pp. 1134-1143.

McWilliam, A.S., Napoli, S., Marsh, A.M., Pemper, F.L., Nelson, D.J., Pimm, C.L., Stumbles, P.A., Wells, T.N. & Holt, P.G. (1996) 'Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli', *J Exp Med*, vol. 184, no. 6, pp. 2429-2432.

McWilliam, A.S., Nelson, D., Thomas, J.A. & Holt, P.G. (1994) 'Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces', *J Exp Med*, vol. 179, no. 4, pp. 1331-1336.

Medzhitov, R. & Janeway, C.A., Jr. (2002) 'Decoding the patterns of self and nonself by the innate immune system', *Science*, vol. 296, no. 5566, pp. 298-300.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A., Jr. (1997) 'A human homologue of the Drosophila Toll protein signals activation of adaptive immunity', *Nature*, vol. 388, no. 6640, pp. 394-397.

Meritet, J.F., Maury, C. & Tovey, M.G. (2001) 'Induction of tolerance to recombinant therapeutic proteins', *J Interferon Cytokine Res*, vol. 21, no. 12, pp. 1031-1038.

Milella, M., Antonelli, G., Santantonio, T., Currenti, M., Monno, L., Mariano, N., Angarano, G., Dianzani, F. & Pastore, G. (1993) 'Neutralizing antibodies to recombinant alpha-interferon and response to therapy in chronic hepatitis C virus infection', *Liver*, vol. 13, no. 3, pp. 146-150.

Miller, J.F. & Basten, A. (1996) 'Mechanisms of tolerance to self', *Curr Opin Immunol*, vol. 8, no. 6, pp. 815-821.

Minagar, A., Murray, T.J. & Investigators, P.S. (2008) 'Efficacy and tolerability of intramuscular interferon beta-la compared with subcutaneous interferon beta-1a in relapsing MS: results from PROOF', *Current Medical Research and Opinion*, vol. 24, no. 4, pp. 1049-1055.

Mocciaro, F., Russo, G., Di Mitri, R. & Marino, A. (2013) 'Infliximab-induced thrombocytopenia in an elderly patient with ileocolonic Crohn's disease', *Inflamm Bowel Dis*, vol. 19, no. 4, pp. E52-53.

Mommaas, A.M., Mulder, A.A., Jordens, R., Out, C., Tan, M.C., Cresswell, P., Kluin, P.M. & Koning, F. (1999) 'Human epidermal Langerhans cells lack functional mannose receptors and a fully developed endosomal/lysosomal compartment for loading of HLA class II molecules', *Eur J Immunol*, vol. 29, no. 2, pp. 571-580.

Mongey, A.B. & Hess, E.V. (2008) 'Drug insight: autoimmune effects of medications-what's new?', *Nat Clin Pract Rheumatol*, vol. 4, no. 3, pp. 136-144.

Monshi, M.M., Faulkner, L., Gibson, A., Jenkins, R.E., Farrell, J., Earnshaw, C.J., Alfirevic, A., Cederbrant, K., Daly, A.K., French, N., Pirmohamed, M., Park, B.K. & Naisbitt, D.J. (2013) 'Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury', *Hepatology*, vol. 57, no. 2, pp. 727-739.

Moody, D.B., Besra, G.S., Wilson, I.A. & Porcelli, S.A. (1999) 'The molecular basis of CD1-mediated presentation of lipid antigens', *Immunol Rev*, vol. 172, pp. 285-296.

Morgan, B.P. (2000) 'The complement system: an overview', *Methods Mol Biol*, vol. 150, pp. 1-13.

Mosmann, T.R., Schumacher, J.H., Fiorentino, D.F., Leverah, J., Moore, K.W. & Bond, M.W. (1990) 'Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay', *J Immunol*, vol. 145, no. 9, pp. 2938-2945.

Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C. & et al. (1993) 'The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction', *Nature*, vol. 366, no. 6451, pp. 129-135.

Myhr, K.M., Riise, T., Green Lilleas, F.E., Beiske, T.G., Celius, E.G., Edland, A., Jensen, D., Larsen, J.P., Nilsen, R., Nortvedt, M.W., Smievoll, A.I., Vedeler, C. & Nyland, H.I. (1999) 'Interferon-alpha2a reduces MRI disease activity in relapsing-remitting multiple sclerosis. Norwegian Study Group on Interferon-alpha in Multiple Sclerosis', *Neurology*, vol. 52, no. 5, pp. 1049-1056.

Nagler-Anderson, C. & Shi, H.N. (2001) 'Peripheral nonresponsiveness to orally administered soluble protein antigens', *Crit Rev Immunol*, vol. 21, no. 1-3, pp. 121-131.

Najafian, N., Chitnis, T., Salama, A.D., Zhu, B., Benou, C., Yuan, X., Clarkson, M.R., Sayegh, M.H. & Khoury, S.J. (2003) 'Regulatory functions of CD8+CD28- T cells in an autoimmune disease model', *J Clin Invest*, vol. 112, no. 7, pp. 1037-1048.

Nakatsui, T., Wong, J. & Groot, D. (2008) 'Survival of densely packed follicular unit grafts using the lateral slit technique', *Dermatol Surg*, vol. 34, no. 8, pp. 1016-1022; discussion 1022-1015.

Namdar, A., Nikbin, B., Ghabaee, M., Bayati, A. & Izad, M. (2010) 'Effect of IFN-beta therapy on the frequency and function of CD4(+)CD25(+) regulatory T cells and Foxp3 gene expression in relapsing-remitting multiple sclerosis (RRMS): a preliminary study', *J Neuroimmunol*, vol. 218, no. 1-2, pp. 120-124.

Napier, J.C. (1997) 'Interferon beta in multiple sclerosis. Interferon beta reduces overall relapse rate by one third', *BMJ*, vol. 314, no. 7080, p. 601.

Nechansky, A. (2010) 'HAHA--nothing to laugh about. Measuring the immunogenicity (human anti-human antibody response) induced by humanized monoclonal antibodies applying ELISA and SPR technology', *J Pharm Biomed Anal*, vol. 51, no. 1, pp. 252-254.

Nepom, G.T. & Erlich, H. (1991) 'MHC class-II molecules and autoimmunity', *Annu Rev Immunol*, vol. 9, pp. 493-525.

Nesburn, A.B., Bettahi, I., Dasgupta, G., Chentoufi, A.A., Zhang, X., You, S., Morishige, N., Wahlert, A.J., Brown, D.J., Jester, J.V., Wechsler, S.L. & BenMohamed, L. (2007) 'Functional Foxp3+ CD4+ CD25(Bright+) "natural" regulatory T cells are abundant in rabbit conjunctiva and suppress virus-specific CD4+ and CD8+ effector T cells during ocular herpes infection', *J Virol*, vol. 81, no. 14, pp. 7647-7661.

Nielsen, M. & Lund, O. (2009) 'NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction', *BMC Bioinformatics*, vol. 10, p. 296.

Nielsen, M., Lundegaard, C. & Lund, O. (2007) 'Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method', *BMC Bioinformatics*, vol. 8, p. 238.

Nirula, A., Glaser, S.M., Kalled, S.L. & Taylor, F.R. (2011) 'What is IgG4? A review of the biology of a unique immunoglobulin subtype', *Curr Opin Rheumatol*, vol. 23, no. 1, pp. 119-124.

Noonan, C.W., Kathman, S.J. & White, M.C. (2002) 'Prevalence estimates for MS in the United States and evidence of an increasing trend for women', *Neurology*, vol. 58, no. 1, pp. 136-138.

Noronha, A., Toscas, A. & Jensen, M.A. (1992) 'Contrasting effects of alpha, beta, and gamma interferons on nonspecific suppressor function in multiple sclerosis', *Ann Neurol*, vol. 31, no. 1, pp. 103-106.

Noronha, A., Toscas, A. & Jensen, M.A. (1993) 'Interferon beta decreases T cell activation and interferon gamma production in multiple sclerosis', *J Neuroimmunol*, vol. 46, no. 1-2, pp. 145-153.

'North American Study Group' (2004), *Neurology*, vol. 63, no. 10, pp. 1788-1795.

Novick, D., Cohen, B. & Rubinstein, M. (1994) 'The human interferon alpha/beta receptor: characterization and molecular cloning', *Cell*, vol. 77, no. 3, pp. 391-400.

Novotny, J. (1987) 'A static accessibility model of protein antigenicity', *Int Rev Immunol*, vol. 2, no. 4, pp. 379-389.

Ohta, H., Takahashi, I., Kojima, T., Takamatsu, J., Shima, M., Yoshioka, A., Saito, H. & Kamiya, T. (1999) 'Histocompatibility antigens and alleles in Japanese haemophilia A patients with or without factor VIII antibodies', *Tissue Antigens*, vol. 54, no. 1, pp. 91-97.

Olerup, O. & Hillert, J. (1991) 'HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation', *Tissue Antigens*, vol. 38, no. 1, pp. 1-15.

Orstavik, K.H. (1990) 'Inhibitors to factor IX contain all IgG subclasses except IgG3', *Folia Haematol Int Mag Klin Morphol Blutforsch*, vol. 117, no. 4, pp. 545-548.

Ozeki, T., Mushiroda, T., Yowang, A., Takahashi, A., Kubo, M., Shirakata, Y., Ikezawa, Z., Iijima, M., Shiohara, T., Hashimoto, K., Kamatani, N. & Nakamura, Y. (2011) 'Genome-wide association study identifies HLA-A*3101 allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population', *Hum Mol Genet*, vol. 20, no. 5, pp. 1034-1041.

Pachner, A.R. (2003) 'An improved ELISA for screening for neutralizing anti-IFN-beta antibodies in MS patients', *Neurology*, vol. 61, no. 10, pp. 1444-1446.

Pachner, A.R., Bertolotto, A. & Deisenhammer, F. (2003) 'Measurement of MxA mRNA or protein as a biomarker of IFNbeta bioactivity: detection of antibody-mediated decreased bioactivity (ADB)', *Neurology*, vol. 61, no. 9 Suppl 5, pp. S24-26.

Pachner, A.R., Cadavid, D., Wolansky, L. & Skurnick, J. (2009a) 'Effect of anti-IFN beta antibodies on MRI lesions of MS patients in the BECOME study', *Neurology*, vol. 73, no. 18, pp. 1485-1492.

Pachner, A.R., Cadavid, D., Wolansky, L. & Skurnick, J. (2009b) 'Effect of anti-IFN{beta} antibodies on MRI lesions of MS patients in the BECOME study', *Neurology*, vol. 73, no. 18, pp. 1485-1492.

Pachner, A.R., Dail, D., Pak, E. & Narayan, K. (2005) 'The importance of measuring IFNbeta bioactivity: monitoring in MS patients and the effect of anti-IFNbeta antibodies', *J Neuroimmunol*, vol. 166, no. 1-2, pp. 180-188.

Pachner, A.R., Oger, J. & Palace, J. (2003) 'The measurement of antibodies binding to IFNbeta in MS patients treated with IFNbeta', *Neurology*, vol. 61, no. 9 Suppl 5, pp. S18-20.

Pachner, A.R., Warth, J.D., Pace, A. & Goelz, S. (2009c) 'Effect of neutralizing antibodies on biomarker responses to interferon beta: the INSIGHT study', *Neurology*, vol. 73, no. 18, pp. 1493-1500.

Panitch, H., Goodin, D.S., Francis, G., Chang, P., Coyle, P.K., O'Connor, P., Monaghan, E., Li, D., Weinshenker, B., Efficacy, E.S.G.E.o.I.D.-r.E.N.A.C. & University of British Columbia, M.S.M.R.I.R.G. (2002) 'Randomized, comparative study of interferon beta-1a treatment regimens in MS: The EVIDENCE Trial', *Neurology*, vol. 59, no. 10, pp. 1496-1506.

Park, B.K., Pirmohamed, M. & Kitteringham, N.R. (1998) 'Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective', *Chem Res Toxicol*, vol. 11, no. 9, pp. 969-988.

Park, K. (2009) 'Molecular aspects of adverse drug reactions – from molecule to man', *Toxicology Letters*, vol. 189, Supplement, no. 0, p. S14.

Parker, D.C. (1993) 'T cell-dependent B cell activation', *Annu Rev Immunol*, vol. 11, pp. 331-360.

Parker, E.T., Craddock, H.N., Barrow, R.T. & Lollar, P. (2004) 'Comparative immunogenicity of recombinant B domain-deleted porcine factor VIII and Hyate:C in hemophilia A mice presensitized to human factor VIII', *J Thromb Haemost*, vol. 2, no. 4, pp. 605-611.

Parkin, J. & Cohen, B. (2001) 'An overview of the immune system', *Lancet*, vol. 357, no. 9270, pp. 1777-1789.

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P.S., Bruck, W., Lucchinetti, C. & Lassmann, H. (2006) 'Remyelination is extensive in a subset of multiple sclerosis patients', *Brain*, vol. 129, no. Pt 12, pp. 3165-3172.

Patton, A., Mullenix, M.C., Swanson, S.J. & Koren, E. (2005) 'An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen', *J Immunol Methods*, vol. 304, no. 1-2, pp. 189-195.

Paty, D.W. & Li, D.K. (1993) 'Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group', *Neurology*, vol. 43, no. 4, pp. 662-667.

Perez-Alvarez, R., Perez-de-Lis, M., Ramos-Casals, M. & group, B.s. (2013) 'Biologics-induced autoimmune diseases', *Curr Opin Rheumatol*, vol. 25, no. 1, pp. 56-64.

Perez-Soler, R., Delord, J.P., Halpern, A., Kelly, K., Krueger, J., Sureda, B.M., von Pawel, J., Temel, J., Siena, S., Soulieres, D., Saltz, L. & Leyden, J. (2005) 'HER1/EGFR inhibitor-associated rash: future directions for management and investigation outcomes from the HER1/EGFR inhibitor rash management forum', *Oncologist*, vol. 10, no. 5, pp. 345-356.

Perez-Soler, R. & Saltz, L. (2005) 'Cutaneous adverse effects with HER1/EGFR-targeted agents: is there a silver lining?', *J Clin Oncol*, vol. 23, no. 22, pp. 5235-5246.

Peters, B., Sidney, J., Bourne, P., Bui, H.H., Buus, S., Doh, G., Fleri, W., Kronenberg, M., Kubo, R., Lund, O., Nemazee, D., Ponomarenko, J.V., Sathiamurthy, M., Schoenberger, S.P., Stewart, S., Surko, P., Way, S., Wilson, S. & Sette, A. (2005) 'The design and implementation of the immune epitope database and analysis resource', *Immunogenetics*, vol. 57, no. 5, pp. 326-336.

Petersen, B., Bendtzen, K., Koch-Henriksen, N., Ravnborg, M., Ross, C., Sorensen, P.S. & Danish Multiple Sclerosis, G. (2006) 'Persistence of neutralizing antibodies after discontinuation of IFNbeta therapy in patients with relapsing-remitting multiple sclerosis', *Mult Scler*, vol. 12, no. 3, pp. 247-252.

Petkau, A.J., White, R.A., Ebers, G.C., Reder, A.T., Sibley, W.A., Lublin, F.D., Paty, D.W. & Grp, I.M.S.S. (2004) 'Longitudinal analyses of the effects of neutralizing antibodies on interferon beta-1b in relapsing-remitting multiple sclerosis', *Mult Scler*, vol. 10, no. 2, pp. 126-138.

Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J. & Harding, C.V. (1993) 'Phagocytic processing of bacterial antigens for class I MHC presentation to T cells', *Nature*, vol. 361, no. 6410, pp. 359-362.

Phillips, J.T., Rice, G., Frohman, E., Gaer, L.V., Scott, T., Haas, J., Eggenberger, E., Freedman, M.S., Stuart, W., Cunha, L., Jacobs, L., Oger, J., Arnold, D., Murry, T.J., DiBiase, M., Jethwa, V. & Goelz, S. (2004) 'A multicenter, open-label, phase II study of the immunogenicity and safety of a new prefilled syringe (liquid) formulation of Avonex in patients with multiple sclerosis', *Clin Ther*, vol. 26, no. 4, pp. 511-521.

Pichler, W.J. (2006) 'Adverse side-effects to biological agents', *Allergy*, vol. 61, no. 8, pp. 912-920.

Pierce, S.K. (2002) 'Lipid rafts and B-cell activation', *Nat Rev Immunol*, vol. 2, no. 2, pp. 96-105.

Pirmohamed, M., Breckenridge, A.M., Kitteringham, N.R. & Park, B.K. (1998) 'Adverse drug reactions', *BMJ*, vol. 316, no. 7140, pp. 1295-1298.

Platanias, L.C. (2005) 'Mechanisms of type-I- and type-II-interferon-mediated signalling', *Nat Rev Immunol*, vol. 5, no. 5, pp. 375-386.

Polman, C., Kappos, L., White, R., Dahlke, F., Beckmann, K., Pozzilli, C., Thompson, A., Petkau, J., Miller, D. & European Study Group in Interferon Beta-1b in Secondary Progressive, M.S. (2003) 'Neutralizing antibodies during treatment of secondary progressive MS with interferon beta-1b', *Neurology*, vol. 60, no. 1, pp. 37-43.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. & Beutler, B. (1998) 'Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene', *Science*, vol. 282, no. 5396, pp. 2085-2088.

Ponce, R., Abad, L., Amaravadi, L., Gelzleichter, T., Gore, E., Green, J., Gupta, S., Herzyk, D., Hurst, C., Ivens, I.A., Kawabata, T., Maier, C., Mounho, B., Rup, B., Shankar, G., Smith, H., Thomas, P. & Wierda, D. (2009) 'Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies', *Regul Toxicol Pharmacol*, vol. 54, no. 2, pp. 164-182.

Porres, J.C., Carreno, V., Ruiz, M., Marron, J.A. & Bartolome, J. (1989) 'Interferon antibodies in patients with chronic HBV infection treated with recombinant interferon', *J Hepatol*, vol. 8, no. 3, pp. 351-357.

Porter, S. (2001) 'Human immune response to recombinant human proteins', *J Pharm Sci*, vol. 90, no. 1, pp. 1-11.

Pozzilli, C., Romano, S. & Cannoni, S. (2002) 'Epidemiology and current treatment of multiple sclerosis in Europe today', *J Rehabil Res Dev*, vol. 39, no. 2, pp. 175-185.

Praditpornsilpa, K., Kupatawintu, P., Mongkonsritagoon, W., Supasyndh, O., Jootar, S., Intarakumthornchai, T., Pongskul, C., Prasithsirikul, W., Achavanuntakul, B., Ruangkarnchanasetr, P., Laohavinij, S. & Eiam-Ong, S. (2009) 'The association of anti-r-HuEpo-associated pure red cell aplasia with HLA-DRB1*09-DQB1*0309', *Nephrol Dial Transplant*, vol. 24, no. 5, pp. 1545-1549.

Presta, L.G. (2006) 'Engineering of therapeutic antibodies to minimize immunogenicity and optimize function', *Adv Drug Deliv Rev*, vol. 58, no. 5-6, pp. 640-656.

'PRISMS-4: Long-term efficacy of interferon-beta-1a in relapsing MS' (2001), *Neurology*, vol. 56, no. 12, pp. 1628-1636.

Pungor, E., Jr., Files, J.G., Gabe, J.D., Do, L.T., Foley, W.P., Gray, J.L., Nelson, J.W., Nestaas, E., Taylor, J.L. & Grossberg, S.E. (1998) 'A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b', *J Interferon Cytokine Res*, vol. 18, no. 12, pp. 1025-1030.

Radhakrishnan, R., Walter, L.J., Hruza, A., Reichert, P., Trotta, P.P., Nagabhushan, T.L. & Walter, M.R. (1996) 'Zinc mediated dimer of human interferon-alpha 2b revealed by X-ray crystallography', *Structure*, vol. 4, no. 12, pp. 1453-1463.

Ragnhammar, P., Friesen, H.J., Frodin, J.E., Lefvert, A.K., Hassan, M., Osterborg, A. & Mellstedt, H. (1994) 'Induction of anti-recombinant human granulocyte-macrophage colony-stimulating factor (Escherichia coli-derived) antibodies and clinical effects in nonimmunocompromised patients', *Blood*, vol. 84, no. 12, pp. 4078-4087.

Ragonese, P., Aridon, P., Salemi, G., D'Amelio, M. & Savettieri, G. (2008) 'Mortality in multiple sclerosis: a review', *Eur J Neurol*, vol. 15, no. 2, pp. 123-127.

Ramos-Casals, M., Brito-Zeron, P., Munoz, S., Soria, N., Galiana, D., Bertolaccini, L., Cuadrado, M.J. & Khamashta, M.A. (2007) 'Autoimmune diseases induced by TNF-targeted therapies: analysis of 233 cases', *Medicine (Baltimore)*, vol. 86, no. 4, pp. 242-251.

'Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group' (1998), *Lancet*, vol. 352, no. 9139, pp. 1498-1504.

Ransohoff, R.M., Devajyothi, C., Estes, M.L., Babcock, G., Rudick, R.A., Frohman, E.M. & Barna, B.P. (1991) 'Interferon-beta specifically inhibits interferon-gamma-induced class II major histocompatibility complex gene transcription in a human astrocytoma cell line', *J Neuroimmunol*, vol. 33, no. 2, pp. 103-112.

Ransohoff, R.M. & Zamvil, S.S. (2007) 'Neuroimmunotherapeutics comes of age', *Neurotherapeutics*, vol. 4, no. 4, pp. 569-570.

Rao, K.V.S. (2001) Antigens, in, *eLS*, John Wiley & Sons, Ltd.

Reding, M.T. (2006) 'Immunological aspects of inhibitor development', *Haemophilia*, vol. 12 Suppl 6, pp. 30-35; discussion 35-36.

Reding, M.T., Lei, S., Lei, H., Green, D., Gill, J. & Conti-Fine, B.M. (2002) 'Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients', *Thromb Haemost*, vol. 88, no. 4, pp. 568-575.

Reek, J.N., de Groot, D., Oosterom, G.E., Kamer, P.C. & van Leeuwen, P.W. (2002) 'Core and periphery functionalized dendrimers for transition metal catalysis; a covalent and a non-covalent approach', *J Biotechnol*, vol. 90, no. 3-4, pp. 159-181.

Rescigno, M., Granucci, F., Citterio, S., Foti, M. & Ricciardi-Castagnoli, P. (1999) 'Coordinated events during bacteria-induced DC maturation', *Immunol Today*, vol. 20, no. 5, pp. 200-203.

Reske, D., Walser, A., Haupt, W.F. & Petereit, H.F. (2004) 'Long-term persisting interferon beta-1b neutralizing antibodies after discontinuation of treatment', *Acta Neurol Scand*, vol. 109, no. 1, pp. 66-70.

Reth, M. & Wienands, J. (1997) 'Initiation and processing of signals from the B cell antigen receptor', *Annu Rev Immunol*, vol. 15, pp. 453-479.

Revel, M. (2003) 'Interferon-beta in the treatment of relapsing-remitting multiple sclerosis', *Pharmacol Ther*, vol. 100, no. 1, pp. 49-62.

Rice, G.P., Paszner, B., Oger, J., Lesaux, J., Paty, D. & Ebers, G. (1999) 'The evolution of neutralizing antibodies in multiple sclerosis patients treated with interferon beta-1b', *Neurology*, vol. 52, no. 6, pp. 1277-1279.

Rifkin, I.R., Leadbetter, E.A., Busconi, L., Viglianti, G. & Marshak-Rothstein, A. (2005) 'Toll-like receptors, endogenous ligands, and systemic autoimmune disease', *Immunol Rev*, vol. 204, pp. 27-42.

Rock, E.P., Sibbald, P.R., Davis, M.M. & Chien, Y.H. (1994) 'CDR3 length in antigen-specific immune receptors', *J Exp Med*, vol. 179, no. 1, pp. 323-328.

Roda, B., Zattoni, A., Reschiglian, P., Moon, M.H., Mirasoli, M., Michelini, E. & Roda, A. (2009) 'Field-flow fractionation in bioanalysis: A review of recent trends', *Anal Chim Acta*, vol. 635, no. 2, pp. 132-143.

Rojas, J.R., Taylor, R.P., Cunningham, M.R., Rutkoski, T.J., Vennarini, J., Jang, H., Graham, M.A., Geboes, K., Rousselle, S.D. & Wagner, C.L. (2005) 'Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomolgus monkeys', *J Pharmacol Exp Ther*, vol. 313, no. 2, pp. 578-585.

Romani, N. & Schuler, G. (1989) 'Structural and functional relationships between epidermal Langerhans cells and dendritic cells', *Res Immunol*, vol. 140, no. 9, pp. 895-898; discussion 918-826.

Rosati, G. (2001) 'The prevalence of multiple sclerosis in the world: an update', *Neurol Sci*, vol. 22, no. 2, pp. 117-139.

Ross, C., Clemmesen, K.M., Sorensen, P.S., Koch-Henriksen, N. & Bendtzen, K. (2006) 'Measuring and evaluating interferon beta-induced antibodies in patients with multiple sclerosis', *Mult Scler*, vol. 12, no. 1, pp. 39-46.

Ross, C., Clemmesen, K.M., Svenson, M., Sorensen, P.S., Koch-Henriksen, N., Skovgaard, G.L. & Bendtzen, K. (2000a) 'Immunogenicity of interferon-beta in multiple sclerosis patients: influence of preparation, dosage, dose frequency, and route of administration. Danish Multiple Sclerosis Study Group', *Ann Neurol*, vol. 48, no. 5, pp. 706-712.

Ross, C., Clemmesen, K.M., Svenson, M., Sorensen, P.S., Koch-Henriksen, N., Skovgaard, G.L., Bendtzen, K. & Study, D.M.S. (2000b) 'Immunogenicity of interferon-beta in multiple sclerosis patients: Influence of preparation, dosage, dose frequency, and route of administration', *Ann Neurol*, vol. 48, no. 5, pp. 706-712.

Rossert, J., Casadevall, N. & Eckardt, K.U. (2004) 'Anti-erythropoietin antibodies and pure red cell aplasia', *J Am Soc Nephrol*, vol. 15, no. 2, pp. 398-406.

Rossman, H.S. (2004) 'Neutralizing antibodies to multiple sclerosis treatments', *J Manag Care Pharm*, vol. 10, no. 3 Suppl B, pp. S12-19.

Roy, S., Barnes, P.F., Garg, A., Wu, S., Cosman, D. & Vankayalapati, R. (2008) 'NK cells lyse T regulatory cells that expand in response to an intracellular pathogen', *J Immunol*, vol. 180, no. 3, pp. 1729-1736.

Rudick, R.A., Ransohoff, R.M., Peppler, R., VanderBrug Medendorp, S., Lehmann, P. & Alam, J. (1996) 'Interferon beta induces interleukin-10 expression: relevance to multiple sclerosis', *Ann Neurol*, vol. 40, no. 4, pp. 618-627.

Rudick, R.A., Simonian, N.A., Alam, J.A., Campion, M., Scaramucci, J.O., Jones, W., Coats, M.E., Goodkin, D.E., Weinstock-Guttman, B., Herndon, R.M., Mass, M.K., Richert, J.R., Salazar, A.M., Munschauer, F.E., 3rd, Cookfair, D.L., Simon, J.H. & Jacobs, L.D. (1998) 'Incidence and significance of neutralizing antibodies to interferon beta-1a in multiple sclerosis. Multiple Sclerosis Collaborative Research Group (MSCRG)', *Neurology*, vol. 50, no. 5, pp. 1266-1272.

Rudolph, M.G., Stanfield, R.L. & Wilson, I.A. (2006) 'How TCRs bind MHCs, peptides, and coreceptors', *Annu Rev Immunol*, vol. 24, pp. 419-466.

Ruf, P., Kluge, M., Jager, M., Burges, A., Volovat, C., Heiss, M.M., Hess, J., Wimberger, P., Brandt, B. & Lindhofer, H. (2010) 'Pharmacokinetics, immunogenicity and bioactivity of the therapeutic antibody catumaxomab intraperitoneally administered to cancer patients', *Br J Clin Pharmacol*, vol. 69, no. 6, pp. 617-625.

Sacks, S.H. & Zhou, W. (2012) 'The role of complement in the early immune response to transplantation', *Nat Rev Immunol*, vol. 12, no. 6, pp. 431-442.

Sakaguchi, S. (2005) 'Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self', *Nat Immunol*, vol. 6, no. 4, pp. 345-352.

Sakaguchi, S., Miyara, M., Costantino, C.M. & Hafler, D.A. (2010) 'FOXP3+ regulatory T cells in the human immune system', *Nat Rev Immunol*, vol. 10, no. 7, pp. 490-500.

Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. (2008) 'Regulatory T cells and immune tolerance', *Cell*, vol. 133, no. 5, pp. 775-787.

Sathish, J.G., Sethu, S., Bielsky, M.C., de Haan, L., French, N.S., Govindappa, K., Green, J., Griffiths, C.E., Holgate, S., Jones, D., Kimber, I., Moggs, J., Naisbitt, D.J., Pirmohamed, M., Reichmann, G., Sims, J., Subramanyam, M., Todd, M.D., Van Der Laan, J.W., Weaver, R.J. & Park, B.K. (2013) 'Challenges and approaches for the development of safer immunomodulatory biologics', *Nat Rev Drug Discov*, vol. 12, no. 4, pp. 306-324.

Scagnolari, C., Bellomi, F., Turriziani, O., Bagnato, F., Tomassini, V., Lavolpe, V., Ruggieri, M., Bruschi, F., Meucci, G., Dicuonzo, G. & Antonelli, G. (2002a) 'Neutralizing and binding antibodies to IFN-beta: Relative frequency in relapsing-remitting multiple sclerosis patients treated with different IFN-beta preparations', *Journal of Interferon and Cytokine Research*, vol. 22, no. 2, pp. 207-213.

Scagnolari, C., Bellomi, F., Turriziani, O., Bagnato, F., Tomassini, V., Lavolpe, V., Ruggieri, M., Bruschi, F., Meucci, G., Dicuonzo, G. & Antonelli, G. (2002b) 'Neutralizing and binding antibodies to IFN-beta: relative frequency in relapsing-remitting multiple sclerosis patients treated with different IFN-beta preparations', *J Interferon Cytokine Res*, vol. 22, no. 2, pp. 207-213.

Scandella, D., Gilbert, G.E., Shima, M., Nakai, H., Eagleson, C., Felch, M., Prescott, R., Rajalakshmi, K.J., Hoyer, L.W. & Saenko, E. (1995) 'Some factor VIII inhibitor antibodies recognize a common epitope corresponding to C2 domain amino acids 2248 through 2312, which overlap a phospholipid-binding site', *Blood*, vol. 86, no. 5, pp. 1811-1819.

Schellekens, H. (2003) 'Immunogenicity of therapeutic proteins', *Nephrol Dial Transplant*, vol. 18, no. 7, pp. 1257-1259.

Schellekens, H. (2005) 'Factors influencing the immunogenicity of therapeutic proteins', *Nephrol Dial Transplant*, vol. 20 Suppl 6, pp. vi3-9.
Schellekens, H. (2008) 'Recombinant human erythropoietins, biosimilars and immunogenicity', *J Nephrol*, vol. 21, no. 4, pp. 497-502.
Schernthaner, G. (1993) 'Immunogenicity and allergenic potential of animal and human insulins', *Diabetes Care*, vol. 16 Suppl 3, pp. 155-165.
Schur, P.H. (1987) 'IgG subclasses--a review', *Ann Allergy*, vol. 58, no. 2, pp. 89-96, 99.
Schwid, S.R. & Panitch, H.S. (2007) 'Full results of the Evidence of Interferon Dose-Response-European North American Comparative Efficacy (EVIDENCE) study: a multicenter, randomized, assessor-blinded comparison of low-dose weekly versus high-dose, high-frequency interferon beta-1a for relapsing multiple sclerosis', *Clin Ther*, vol. 29, no. 9, pp. 2031-2048.
Scott, D.L. (2006) 'Rheumatoid arthritis: acute presentations and urgent complications', *Br J Hosp Med (Lond)*, vol. 67, no. 5, pp. 235-239.
Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K.T. & Mitsui, Y. (1992) 'Three-dimensional crystal structure of recombinant murine interferon-beta', *EMBO J*, vol. 11, no. 9, pp. 3193-3201.
Seong, S.Y. & Matzinger, P. (2004) 'Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses', *Nat Rev Immunol*, vol. 4, no. 6, pp. 469-478.
Sethu, S., Govindappa, K., Alhaidari, M., Pirmohamed, M., Park, K. & Sathish, J. (2012) 'Immunogenicity to biologics: mechanisms, prediction and reduction', *Arch Immunol Ther Exp (Warsz)*, vol. 60, no. 5, pp. 331-344.
Shahkarami, M.A., Vaziri, B., Salami, S., Harandi, A.A. & Oger, J. (2013) 'Neutralizing antibodies in multiple sclerosis patients on weekly intramuscular Avonex and biosimilar interferon beta-1a (CinnoVex): comparing results of measurements in two different laboratories', *J Immunol Methods*, vol. 388, no. 1-2, pp. 46-48.
Shapiro, A.M., Jack, C.S., Lapierre, Y., Arbour, N., Bar-Or, A. & Antel, J.P. (2006) 'Potential for interferon beta-induced serum antibodies in multiple sclerosis to inhibit endogenous interferon-regulated chemokine/cytokine responses within the central nervous system', *Arch Neurol*, vol. 63, no. 9, pp. 1296-1299.
Sharief, M.K., Semra, Y.K., Seidi, O.A. & Zoukos, Y. (2001) 'Interferon-beta therapy downregulates the anti-apoptosis protein FLIP in T cells from patients with multiple sclerosis', *J Neuroimmunol*, vol. 120, no. 1-2, pp. 199-207.
Sharma, M., Hegde, P., Aimanianda, V., Beau, R., Senechal, H., Poncet, P., Latge, J.P., Kaveri, S.V. & Bayry, J. (2013) 'Circulating human basophils lack the features of professional antigen presenting cells', *Sci Rep*, vol. 3, p. 1188.
Shearer, M. & Taylor-Papadimitriou, J. (1987) 'Regulation of cell growth by interferon', *Cancer Metastasis Rev*, vol. 6, no. 3, pp. 199-221.
Shiina, T., Inoko, H. & Kulski, J.K. (2004) 'An update of the HLA genomic region, locus information and disease associations: 2004', *Tissue Antigens*, vol. 64, no. 6, pp. 631-649.
Siber, G.R., Schur, P.H., Aisenberg, A.C., Weitzman, S.A. & Schiffman, G. (1980) 'Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens', *N Engl J Med*, vol. 303, no. 4, pp. 178-182.
Sidney, J., Assarsson, E., Moore, C., Ngo, S., Pinilla, C., Sette, A. & Peters, B. (2008) 'Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries', *Immunome Res*, vol. 4, p. 2.
Simonney, N., De Bosch, N., Argueyo, A., Garcia, E. & Layrisse, Z. (1985) 'HLA antigens in hemophiliacs A with or without factor VIII antibodies in a Venezuelan Mestizo population', *Tissue Antigens*, vol. 25, no. 4, pp. 216-219.
Singh, B., Lee, K.C. & Fraga, E. (1989) 'Hydrophobic amino acid residues in peptide antigens determine the genetic control of immune responses', *Pept Res*, vol. 2, no. 1, pp. 120-127.
Singh, H. & Raghava, G.P. (2001) 'ProPred: prediction of HLA-DR binding sites', *Bioinformatics*, vol. 17, no. 12, pp. 1236-1237.
Sjoberg, A.P., Trouw, L.A. & Blom, A.M. (2009) 'Complement activation and inhibition: a delicate balance', *Trends Immunol*, vol. 30, no. 2, pp. 83-90.

Smith, K.J., Pyrdol, J., Gauthier, L., Wiley, D.C. & Wucherpfennig, K.W. (1998) 'Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein', *J Exp Med*, vol. 188, no. 8, pp. 1511-1520.

Somerfield, J., Hill-Cawthorne, G.A., Lin, A., Zandi, M.S., McCarthy, C., Jones, J.L., Willcox, M., Shaw, D., Thompson, S.A., Compston, A.S., Hale, G., Waldmann, H. & Coles, A.J. (2010) 'A novel strategy to reduce the immunogenicity of biological therapies', *J Immunol*, vol. 185, no. 1, pp. 763-768.

Sominanda, A., Lundkvist, M., Fogdell-Hahn, A., Hemmer, B., Hartung, H.P., Hillert, J., Menge, T. & Kieseier, B.C. (2010) 'Inhibition of endogenous interferon beta by neutralizing antibodies against recombinant interferon beta', *Arch Neurol*, vol. 67, no. 9, pp. 1095-1101.

Sorensen, P.S. (2008) 'Neutralizing antibodies against interferon-Beta', *Ther Adv Neurol Disord*, vol. 1, no. 2, pp. 125-141.

Sorensen, P.S., Deisenhammer, F., Duda, P., Hohlfeld, R., Myhr, K.M., Palace, J., Polman, C., Pozzilli, C., Ross, C. & Sclerosis, E.T.F.o.A.-I.-b.A.i.M. (2005a) 'Guidelines on use of anti-IFN-beta antibody measurements in multiple sclerosis: report of an EFNS Task Force on IFN-beta antibodies in multiple sclerosis', *Eur J Neurol*, vol. 12, no. 11, pp. 817-827.

Sorensen, P.S., Jensen, P.E., Haghikia, A., Lundkvist, M., Vedeler, C., Sellebjerg, F., Koch-Henriksen, N., Fogdell-Hahn, A., Myhr, K.M., Hillert, J. & Gold, R. (2011) 'Occurrence of antibodies against natalizumab in relapsing multiple sclerosis patients treated with natalizumab', *Mult Scler*, vol. 17, no. 9, pp. 1074-1078.

Sorensen, P.S., Koch-Henriksen, N., Ross, C., Clemmesen, K.M. & Bendtzen, K. (2005b) 'Appearance and disappearance of neutralizing antibodies during interferon-beta therapy', *Neurology*, vol. 65, no. 1, pp. 33-39.

Sorensen, P.S., Koch-Henriksen, N., Ross, C., Clemmesen, K.M., Bendtzen, K. & Danish Multiple Sclerosis Study, G. (2005c) 'Appearance and disappearance of neutralizing antibodies during interferon-beta therapy', *Neurology*, vol. 65, no. 1, pp. 33-39.

Sorensen, P.S., Koch-Henriksen, N., Ross, C., Clemmesen, K.M., Bendtzen, K. & Grp, D.M.S. (2005d) 'Appearance and disappearance of neutralizing antibodies during interferon-beta therapy', *Neurology*, vol. 65, no. 1, pp. 33-39.

Sorensen, P.S., Ross, C., Clemmesen, K.M., Bendtzen, K., Frederiksen, J.L., Jensen, K., Kristensen, O., Petersen, T., Rasmussen, S., Ravnborg, M., Stenager, E., Koch-Henriksen, N. & Danish Multiple Sclerosis Study, G. (2003) 'Clinical importance of neutralising antibodies against interferon beta in patients with relapsing-remitting multiple sclerosis', *Lancet*, vol. 362, no. 9391, pp. 1184-1191.

Sospedra, M. & Martin, R. (2005) 'Immunology of multiple sclerosis', *Annu Rev Immunol*, vol. 23, pp. 683-747.

Spear, G.T., Paulnock, D.M., Jordan, R.L., Meltzer, D.M., Merritt, J.A. & Borden, E.C. (1987) 'Enhancement of monocyte class I and II histocompatibility antigen expression in man by in vivo beta-interferon', *Clin Exp Immunol*, vol. 69, no. 1, pp. 107-115.

Starr, T.K., Jameson, S.C. & Hogquist, K.A. (2003) 'Positive and negative selection of T cells', *Annu Rev Immunol*, vol. 21, pp. 139-176.

Steinitz, K.N., van Helden, P.M., Binder, B., Wraith, D.C., Unterthurner, S., Hermann, C., Schuster, M., Ahmad, R.U., Weiller, M., Lubich, C., de la Rosa, M., Schwarz, H.P. & Reipert, B.M. (2012) 'CD4+ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*1501 mice', *Blood*, vol. 119, no. 17, pp. 4073-4082.

Stevens, C. (2010) *Nature of immune system*, Davis Company, Philadelphia.

Stickler, M., Valdes, A.M., Gebel, W., Razo, O.J., Faravashi, N., Chin, R., Rochanayon, N. & Harding, F.A. (2004) 'The HLA-DR2 haplotype is associated with an increased proliferative response to the immunodominant CD4(+) T-cell epitope in human interferon-beta', *Genes Immun*, vol. 5, no. 1, pp. 1-7.

Strainic, M.G., Liu, J., Huang, D., An, F., Lalli, P.N., Muqim, N., Shapiro, V.S., Dubyak, G.R., Heeger, P.S. & Medof, M.E. (2008) 'Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells', *Immunity*, vol. 28, no. 3, pp. 425-435.

Strayer, D.R. & Carter, W.A. (2012) 'Recombinant and natural human interferons: analysis of the incidence and clinical impact of neutralizing antibodies', *J Interferon Cytokine Res*, vol. 32, no. 3, pp. 95-102.

Sturniolo, T., Bono, E., Ding, J., Raddrizzani, L., Tuereci, O., Sahin, U., Braxenthaler, M., Gallazzi, F., Protti, M.P., Sinigaglia, F. & Hammer, J. (1999) 'Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices', *Nat Biotechnol*, vol. 17, no. 6, pp. 555-561.

Swanson, S.J., Ferbas, J., Mayeux, P. & Casadevall, N. (2004) 'Evaluation of methods to detect and characterize antibodies against recombinant human erythropoietin', *Nephron Clin Pract*, vol. 96, no. 3, pp. c88-95.

Tani, K., Su, S.B., Utsunomiya, I., Oppenheim, J.J. & Wang, J.M. (1998) 'Interferon-gamma maintains the binding and functional capacity of receptors for IL-8 on cultured human T cells', *Eur J Immunol*, vol. 28, no. 2, pp. 502-507.

Tatarewicz, S.M., Wei, X., Gupta, S., Masterman, D., Swanson, S.J. & Moxness, M.S. (2007) 'Development of a maturing T-cell-mediated immune response in patients with idiopathic Parkinson's disease receiving r-metHuGDNF via continuous intraputaminal infusion', *J Clin Immunol*, vol. 27, no. 6, pp. 620-627.

Taylor, A., Verhagen, J., Akdis, C.A. & Akdis, M. (2004) 'T regulatory cells in allergy and health: a question of allergen specificity and balance', *Int Arch Allergy Immunol*, vol. 135, no. 1, pp. 73-82.

Theis, V.S. & Rhodes, J.M. (2008) 'Review article: minimizing tuberculosis during anti-tumour necrosis factor-alpha treatment of inflammatory bowel disease', *Aliment Pharmacol Ther*, vol. 27, no. 1, pp. 19-30.

Todd, J.A., Acha-Orbea, H., Bell, J.I., Chao, N., Fronek, Z., Jacob, C.O., McDermott, M., Sinha, A.A., Timmerman, L., Steinman, L. & et al. (1988) 'A molecular basis for MHC class II--associated autoimmunity', *Science*, vol. 240, no. 4855, pp. 1003-1009.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. & Tada, K. (1980) 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *Int J Cancer*, vol. 26, no. 2, pp. 171-176.

UBC (1996) 'The IFNB Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group', *Neurology*, vol. 47, no. 4, pp. 889-894.

Uetrecht, J. (2008) 'Idiosyncratic drug reactions: past, present, and future', *Chem Res Toxicol*, vol. 21, no. 1, pp. 84-92.

Uze, G., Lutfalla, G. & Gresser, I. (1990) 'Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA', *Cell*, vol. 60, no. 2, pp. 225-234.

Valentin, J.P., Bialecki, R., Ewart, L., Hammond, T., Leishmann, D., Lindgren, S., Martinez, V., Pollard, C., Redfern, W. & Wallis, R. (2009) 'A framework to assess the translation of safety pharmacology data to humans', *J Pharmacol Toxicol Methods*, vol. 60, no. 2, pp. 152-158.

van de Stadt, L.A., van Schouwenburg, P.A., Bryde, S., Kruithof, S., van Schaardenburg, D., Hamann, D., Wolbink, G. & Rispens, T. (2013) 'Monoclonal anti-citrullinated protein antibodies selected on citrullinated fibrinogen have distinct targets with different cross-reactivity patterns', *Rheumatology (Oxford)*, vol. 52, no. 4, pp. 631-635.

van den Beemd, R., Boor, P.P., van Lochem, E.G., Hop, W.C., Langerak, A.W., Wolvers-Tettero, I.L., Hooijkaas, H. & van Dongen, J.J. (2000) 'Flow cytometric analysis of the Vbeta repertoire in healthy controls', *Cytometry*, vol. 40, no. 4, pp. 336-345.

van der Voort, L.F., Visser, A., Knol, D.L., Oudejans, C.B., Polman, C.H. & Killestein, J. (2009) 'Lack of interferon-beta bioactivity is associated with the occurrence of relapses in multiple sclerosis', *Eur J Neurol*, vol. 16, no. 9, pp. 1049-1052.

Vanderlugt, C.J. & Miller, S.D. (1996) 'Epitope spreading', *Curr Opin Immunol*, vol. 8, no. 6, pp. 831-836.

Vasquez, E.M., Fabrega, A.J. & Pollak, R. (1995) 'OKT3-induced cytokine-release syndrome: occurrence beyond the second dose and association with rejection severity', *Transplant Proc*, vol. 27, no. 1, pp. 873-874.

Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J.L. & Stinissen, P. (2008) 'Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression', *J Immunol*, vol. 180, no. 9, pp. 6411-6420.

Verthelyi, D. & Wang, V. (2010) 'Trace levels of innate immune response modulating impurities (IIRMIs) synergize to break tolerance to therapeutic proteins', *PLoS One*, vol. 5, no. 12, p. e15252.

Vlug, A., Nieuwenhuys, E.J., van Eijk, R.V., Geertzen, H.G. & van Houte, A.J. (1994) 'Nephelometric measurements of human IgG subclasses and their reference ranges', *Ann Biol Clin (Paris)*, vol. 52, no. 7-8, pp. 561-567.

von Wussow, P., Hartmann, F., Freund, M., Poliwoda, H. & Deicher, H. (1988) 'Leucocyte-derived interferon-alpha in patients with antibodies to recombinant IFN-alpha 2b', *Lancet*, vol. 1, no. 8590, pp. 882-883.

Vos, Q., Lees, A., Wu, Z.Q., Snapper, C.M. & Mond, J.J. (2000) 'B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms', *Immunol Rev*, vol. 176, pp. 154-170.

Vu, M.D., Xiao, X., Gao, W., Degauque, N., Chen, M., Kroemer, A., Killeen, N., Ishii, N. & Li, X.C. (2007) 'OX40 costimulation turns off Foxp3+ Tregs', *Blood*, vol. 110, no. 7, pp. 2501-2510.

Vugmeyster, Y., Xu, X., Theil, F.P., Khawli, L.A. & Leach, M.W. (2012) 'Pharmacokinetics and toxicology of therapeutic proteins: Advances and challenges', *World J Biol Chem*, vol. 3, no. 4, pp. 73-92.

Vultaggio, A., Maggi, E. & Matucci, A. (2011) 'Immediate adverse reactions to biologicals: from pathogenic mechanisms to prophylactic management', *Curr Opin Allergy Clin Immunol*, vol. 11, no. 3, pp. 262-268.

Vultaggio, A., Matucci, A., Nencini, F., Pratesi, S., Parronchi, P., Rossi, O., Romagnani, S. & Maggi, E. (2010) 'Anti-infliximab IgE and non-IgE antibodies and induction of infusion-related severe anaphylactic reactions', *Allergy*, vol. 65, no. 5, pp. 657-661.

Wadhwa, M. & Thorpe, R. (2010) 'Unwanted immunogenicity: lessons learned and future challenges', *Bioanalysis*, vol. 2, no. 6, pp. 1073-1084.

Walther, E.U. & Hohlfeld, R. (1999) 'Multiple sclerosis: side effects of interferon beta therapy and their management', *Neurology*, vol. 53, no. 8, pp. 1622-1627.

Wang, J., Lozier, J., Johnson, G., Kirshner, S., Verthelyi, D., Pariser, A., Shores, E. & Rosenberg, A. (2008) 'Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment', *Nat Biotechnol*, vol. 26, no. 8, pp. 901-908.

Wang, W. (2005) 'Protein aggregation and its inhibition in biopharmaceutics', *Int J Pharm*, vol. 289, no. 1-2, pp. 1-30.

Waxman, S.G. (2006a) 'Axonal conduction and injury in multiple sclerosis: the role of sodium channels', *Nat Rev Neurosci*, vol. 7, no. 12, pp. 932-941.

Waxman, S.G. (2006b) 'Ions, energy and axonal injury: towards a molecular neurology of multiple sclerosis', *Trends Mol Med*, vol. 12, no. 5, pp. 192-195.

Weaver, D.J., Jr., Reis, E.S., Pandey, M.K., Kohl, G., Harris, N., Gerard, C. & Kohl, J. (2010) 'C5a receptor-deficient dendritic cells promote induction of Treg and Th17 cells', *Eur J Immunol*, vol. 40, no. 3, pp. 710-721.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D. & Shaw, G.M. (2003) 'Antibody neutralization and escape by HIV-1', *Nature*, vol. 422, no. 6929, pp. 307-312.

Weintraub, A. (2003) 'Immunology of bacterial polysaccharide antigens', *Carbohydr Res*, vol. 338, no. 23, pp. 2539-2547.

Whelan, S.F., Hofbauer, C.J., Horling, F.M., Allacher, P., Wolfsegger, M.J., Oldenburg, J., Male, C., Windyga, J., Tiede, A., Schwarz, H.P., Scheiflinger, F. & Reipert, B.M. (2013) 'Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients', *Blood*, vol. 121, no. 6, pp. 1039-1048.

Wing, K. & Sakaguchi, S. (2010) 'Regulatory T cells exert checks and balances on self tolerance and autoimmunity', *Nat Immunol*, vol. 11, no. 1, pp. 7-13.

Wussow, P.V., Jakschies, D., Freund, M., Hehlmann, R., Brockhaus, F., Hochkeppel, H., Horisberger, M. & Deicher, H. (1991) 'Treatment of anti-recombinant interferon-alpha 2 antibody positive CML patients with natural interferon-alpha', *Br J Haematol*, vol. 78, no. 2, pp. 210-216.

Xinqiang, S., Fei, L., Nan, L., Yuan, L., Fang, Y., Hong, X., Lixin, T., Juan, L., Xiao, Z., Yuying, S. & Yongzhi, X. (2010) 'Therapeutic efficacy of experimental rheumatoid arthritis with low-dose methotrexate by increasing partially CD4+CD25+ Treg cells and inducing Th1 to Th2 shift in both cells and cytokines', *Biomed Pharmacother*, vol. 64, no. 7, pp. 463-471.

Yang, J., Brook, M.O., Carvalho-Gaspar, M., Zhang, J., Ramon, H.E., Sayegh, M.H., Wood, K.J., Turka, L.A. & Jones, N.D. (2007) 'Allograft rejection mediated by memory T cells is resistant to regulation', *Proc Natl Acad Sci U S A*, vol. 104, no. 50, pp. 19954-19959.

Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H.S., Chung, Y., Pappu, B.P., Shah, B., Chang, S.H., Schluns, K.S., Watowich, S.S., Feng, X.H., Jetten, A.M. & Dong, C. (2008) 'Molecular antagonism and plasticity of regulatory and inflammatory T cell programs', *Immunity*, vol. 29, no. 1, pp. 44-56.

Yong, V.W. (2002) 'Differential mechanisms of action of interferon-beta and glatiramer aetate in MS', *Neurology*, vol. 59, no. 6, pp. 802-808.

Yong, V.W., Chabot, S., Stuve, O. & Williams, G. (1998) 'Interferon beta in the treatment of multiple sclerosis - Mechanisms of action', *Neurology*, vol. 51, no. 3, pp. 682-689.

You, S., Leforban, B., Garcia, C., Bach, J.F., Bluestone, J.A. & Chatenoud, L. (2007) 'Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment', *Proc Natl Acad Sci U S A*, vol. 104, no. 15, pp. 6335-6340.

Young, K.J. & Zhang, L. (2002) 'The nature and mechanisms of DN regulatory T-cell mediated suppression', *Hum Immunol*, vol. 63, no. 10, pp. 926-934.

Zang, Y.C., Yang, D., Hong, J., Tejada-Simon, M.V., Rivera, V.M. & Zhang, J.Z. (2000) 'Immunoregulation and blocking antibodies induced by interferon beta treatment in MS', *Neurology*, vol. 55, no. 3, pp. 397-404.

Zhang, Q., Wang, P., Kim, Y., Haste-Andersen, P., Beaver, J., Bourne, P.E., Bui, H.H., Buus, S., Frankild, S., Greenbaum, J., Lund, O., Lundegaard, C., Nielsen, M., Ponomarenko, J., Sette, A., Zhu, Z. & Peters, B. (2008) 'Immune epitope database analysis resource (IEDB-AR)', *Nucleic Acids Res*, vol. 36, no. Web Server issue, pp. W513-518.

Zhang, Y., Wang, H.B., Chi, L.J. & Wang, W.Z. (2009) 'The role of FoxP3+CD4+CD25hi Tregs in the pathogenesis of myasthenia gravis', *Immunol Lett*, vol. 122, no. 1, pp. 52-57.

Zhou, L., Chong, M.M. & Littman, D.R. (2009) 'Plasticity of CD4+ T cell lineage differentiation', *Immunity*, vol. 30, no. 5, pp. 646-655.

Zhou, W., Patel, H., Li, K., Peng, Q., Villiers, M.B. & Sacks, S.H. (2006) 'Macrophages from C3-deficient mice have impaired potency to stimulate alloreactive T cells', *Blood*, vol. 107, no. 6, pp. 2461-2469.

Zhou, X., Bailey-Bucktrout, S., Jeker, L.T. & Bluestone, J.A. (2009) 'Plasticity of CD4(+) FoxP3(+) T cells', *Curr Opin Immunol*, vol. 21, no. 3, pp. 281-285.

Zhu, J., Yamane, H. & Paul, W.E. (2010) 'Differentiation of effector CD4 T cell populations (*)', *Annu Rev Immunol*, vol. 28, pp. 445-489.