

A novel two-ligand system for investigation of signal integration by non-catalytic tyrosine-phosphorylated receptors



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Abstract

Appropriate immune responses require integration of a variety of activating, co-inhibitory, and co-stimulatory signals from cell surface receptors. One important class of immune receptors are the non-catalytic tyrosine-phosphorylated receptors (NTRs), a group of over 100 receptors, such as PD-1, CTLA-4, CD28, and the T and B cell antigen receptors. These receptors have a number of shared features that we propose have a functional significance, such as relatively small receptor-ligand dimensions and binding of surface-associated ligands. The mechanism through which NTR-ligand engagement leads to signal initiation and integration is not well understood and may offer novel targets for emerging immunotherapies, including checkpoint blockade therapies and adoptive cell transfer approaches such as the use of chimeric antigen receptors.

We have developed a generic cellular two-ligand system to investigate signal integration between NTRs by manipulating their extra- and intra-cellular dimensions. We hypothesise that close co-localisation of receptors is important for optimal signal integration and that this requires compatible receptor-ligand dimensions. We also hypothesise that intracellular tail length influences the effectiveness of inhibitory NTRs by determining the “reach” of the tail and any effector molecules recruited to it.

To manipulate the dimensions of receptor-ligand interactions, CHO.K1 cells (“ligand cells”) were transfected with SpyTag constructs of different lengths and either CD48- or HLA-A2 single chain dimer-based constructs. SpyTag can be covalently coupled via SpyCatcher to StrepTactin, which binds to NTRs tagged with the short peptide tag Strep-tag II, such as the co-inhibitory receptor PD-1 studied here. Ligand cells expressing HLA-A2 SCD presenting cognate peptide or expressing CD48 can be used to provide activating signals through binding of the 1G4 TCR and a 2B4-CD64 fusion protein respectively, in THP-1 and Jurkat “receptor cells”.

CD48-based and SCD-based ligand cells were able to activate IL-8 production or upregulation of NF- κ B-eGFP gMFI by THP-1 receptor cells expressing 2B4-CD64 and Jurkat receptor cells expressing the 1G4 TCR, respectively. This activation of THP-1 and Jurkat receptor cells was partially inhibited by engagement of Strep-tag

II-tagged PD-1 with the StrepTactin-based ligand coupled to CHO.K1 ligand cells, demonstrating PD-1-mediated inhibition in the two-ligand system.

Experiments testing the study hypotheses have provided promising preliminary results, including that mismatching of receptor-ligand dimensions partially abrogates PD-1-mediated signalling and that the inhibitory function of PD-1 variants of different lengths is dependent on the presence of an immunoreceptor tyrosine-based switch motif (ITSM). Truncation of the PD-1 intracellular tail abolished PD-1-mediated inhibition, even in the presence of an ITSM. There was no evidence that elongation of the intracellular tail affected PD-1 inhibitory function using preliminary experimental conditions, although this result needs to be confirmed using alternative conditions such as lower ligand density.

These experiments have provided several insights into areas for further development of the two-ligand system. Optimisation of some aspects of the system, such as improving coupling of the soluble StrepTactin-based ligand, increasing the StrepTactin-Strep-tag II affinity, and incorporating co-stimulatory signalling, should provide a suitable platform for studying signal integration between a wide range of NTRs of interest.

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List of Abbreviations

Abbreviation	Full description
°C	degrees celsius
%	percentage
2B4-CD64	extracellular mouse 2B4 fused to transmembrane and intracellular human CD64
ACT	adoptive cell transfer
APC	antigen-presenting cell
B cell	B lymphocyte
BCR	B cell antigen receptor
BiTE	bispecific T cell engagers
BSA	bovine serum albumin
BTLA	B- and T-lymphocyte attenuator
C9V	cancer-testis antigen NY-ESO-1 peptide variant; sequence SLLMWITQV
CAR	chimeric antigen receptor
CD	cluster of differentiation
CHO	Chinese hamster ovary
CLEC	C-type lectin domain family
CO ₂	carbon dioxide
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DAP10	DNAX-activating protein of 10kDa
DAP12	DNAX-activating protein of 12kDa
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eAPC	engineered antigen presenting cell
EAT-2	Ewing sarcoma-associated transcript
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
F	phenylalanine
Fab	fragment, antigen-binding
FACS	fluorescence-activated cell sorting
Fc	fragment, crystallisable
FcR γ	Fc receptor gamma chain
FCS	fetal calf serum
GPI	Glycosylphosphatidylinositol

HLA	human leukocyte antigen
HVEM	herpes virus entry mediator
I27	immunoglobulin-like domain 27
iCAR	inhibitory chimeric antigen receptor
IFN- γ	interferon gamma
Ig	immunoglobulin
IgSF	immunoglobulin super family
IL-8	interleukin-8
irAE	immune-related adverse effect
IRES	internal ribosome entry site
IS	immune synapse or immunological synapse
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
ITTM	immunoglobulin tail tyrosine motif
K_d	equilibrium dissociation constant
KIR	killer-cell immunoglobulin-like receptor
KS model	kinetic-segregation model
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
LILR	leukocyte immunoglobulin-like receptor family
MES	2-(N-morpholino)ethanesulfonic acid
MES buffer	20 mM MES 140 mM NaCl 1% BSA pH 6.0
MHC	major histocompatibility complex
mIg	membrane-bound immunoglobulin
MUC1-ST	tumour-associated MUC1 ST glycoform
n.s.	not statistically significant
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NKT cell	natural killer T cell
NTR	non-catalytic tyrosine-phosphorylated receptor
P/S	penicillin streptomycin
PBA	PBS + 0.1% BSA + 0.05% azide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PE	phosphoethanolamine
PEI	polyethylenimine
PFA	paraformaldehyde

pMHC	peptide-major histocompatibility complex
PTEN	phosphatase and tensin homolog
RPMI	Roswell Park Memorial Institute medium
RPTP	receptor protein tyrosine phosphatase
RT	room temperature
RTK	receptor tyrosine kinase
SAP	SLAM-associated protein
SCD	single chain dimer
scFv	single chain variable fragment
SCT	single chain trimer
SFK	Src family kinase
SH2	Src homology 2
SHIP-1	SH2 domain-containing inositol 5'-phosphatase-1
SHP-1	SH2-containing protein tyrosine phosphatase-1
SHP-2	SH2-containing protein tyrosine phosphatase-2
Siglec	sialic acid-binding immunoglobulin-like lectin
SLE	systemic lupus erythematosus
SRCR	scavenger receptor cysteine-rich
STII	Strep-tag II; sequence WSHPQFEK
T cell	T lymphocyte
TACTILE	T cell activation, increased late expression
TAM	tumour-associated macrophage
TCR	T cell antigen receptor
TfR1	transferrin receptor 1
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TSC	T cell stimulator cell
U/T	untransfected
Y	tyrosine
ZAP70	zeta-chain-associated protein kinase 70

General Introduction

1.1 An introduction to the immune system

The world around us is full of substances and organisms that pose a potential threat to the survival of all living creatures. In order to live in this environment, multicellular organisms have evolved to defend themselves against toxins and pathogens, including a plethora of bacteria, fungi, parasites, and viruses (Murphy & Weaver (2016)). They must also be able to identify and destroy tumourgenic cells, and do so whilst minimising damage to their own healthy tissue. This complex task is carried out by the immune system, which varies substantially between species and can involve a variety of molecules, cells, tissues, and/or organs.

1.1.1 The innate immune system

One aspect of the immune system is the innate immune system, with all multicellular organisms thought to have some form of innate immunity to provide rapid and broad responses. The innate immune system usually targets conserved microbial structures and its components can range from antimicrobial peptides to phagocytic cells to the alternative complement pathway (reviewed by Medzhitov & Janeway (2000)). A strength of the innate immune system is that it has no requirement for previous exposure to an antigen in order to mount a full immune response, resulting in responses on the scale of minutes to hours (Murphy & Weaver (2016)).

The human innate immune system involves rapid defense by cells such as macrophages, granulocytes, and natural killer (NK) cells, as well as the action of inflammatory cytokines, acute phase proteins, and the complement system (reviewed by Parkin &

Cohen (2001); Murphy & Weaver (2016)).

1.1.2 The adaptive immune system

In addition to the broad action of the innate immune system, vertebrates also have an adaptive immune system that provides specific recognition of antigens through antigen receptors on B and T lymphocytes (B and T cells, respectively). Each B or T cell expresses only one antigen receptor specificity, which is generated through somatic gene rearrangements of incomplete receptor gene segments. This results in a population of lymphocytes expressing a hugely diverse repertoire of antigen receptors (Murphy & Weaver (2016)).

Unlike the innate immune system, the adaptive immune response is dependent on the expansion and differentiation of antigen-specific lymphocytes, referred to as clonal expansion, which occurs on the scale of days to weeks (Murphy & Weaver (2016)). An exception to this is the response to antigens that have been encountered previously and that resulted in the generation of memory immune cells. In this case, long-lived memory cells can make a more rapid and effective response to the antigen upon re-exposure (reviewed by Farber *et al.* (2016)). This phenomenon is known as immunological memory and forms the basis for what is arguably one of the most important advances in human health to date: vaccination.

Although the innate and adaptive immune systems have traditionally been considered as separate branches of the immune system, it has become increasingly apparent that there is substantial crossover and important interactions between them (reviewed by Dempsey *et al.* (2003); Borghesi & Milcarek (2007); Paul (2011)).

1.2 Non-catalytic tyrosine-phosphorylated receptors (NTRs)

In order to fulfill their roles in detecting and responding to their environment, cells of both the innate and adaptive immune systems express a wide range of receptors at their surface. These receptors are often transmembrane glycoproteins that can transmit activating or inhibitory signals to the immune cell. Binding of ligand to these receptors can, for example, result in the prevention of immune responses against healthy tissues (“self”) or in the destruction of cancerous or infected cells.

One important group of immune cell surface receptors is known as immunoreceptors or non-catalytic tyrosine-phosphorylated receptors (NTRs) and includes a variety of activating, co-stimulatory, and co-inhibitory receptors (reviewed by Dushek *et al.*

(2012)). There are more than 120 receptors that have been classified by Dushek and colleagues as NTRs according to a number of shared features. We hypothesise that these shared features have a functional importance for NTR signalling.

1.2.1 Shared features of NTRs

NTRs have a modular protein structure, including diverse ectodomains for ligand binding and separate conserved signalling motifs for signal transduction, as detailed in Section 1.2.2. This modular structure makes NTRs amenable to rapid evolution, making them suitably adaptable for their role in pathogen and tumour recognition (Dushek *et al.* (2012)). For example, if an important pathogen targets a host inhibitory NTR to interfere with the host immune response, the host may fairly rapidly generate an activatory version of the same receptor. In line with this, some NTRs have both activating and inhibitory counterparts, termed “paired receptors”, suggesting a functional importance (Yamada & McVicar (2008)).

In addition to this rapid evolvability and modular protein structure, a number of other features are used to classify immune cell-surface receptors as NTRs, as summarised below and represented in Figure 1.1.

1. No intrinsic catalytic domains

Some cell-surface receptors contain intrinsic catalytic domains, such as the receptor tyrosine kinases (RTKs) that bind to growth factors (reviewed by Lemmon & Schlessinger (2010)). This is not the case for NTRs, which contain no intrinsic catalytic domains, leading to their designation as “non-catalytic”.

2. Conserved tyrosine-phosphorylated signalling motifs

With no intrinsic catalytic function, NTRs rely on tyrosine-phosphorylation of conserved motifs within their cytoplasmic tails or associated adaptors to initiate signalling. The most common of these motifs are immunoreceptor tyrosine-based activating motifs (ITAMs), immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and immunoreceptor tyrosine-based switch motifs (ITSMs).

The phosphorylation state of these tyrosine-containing motifs is controlled by the opposing actions of receptor protein tyrosine phosphatases (RPTPs) and membrane-associated tyrosine kinases such as Src family kinases (SFKs; Cooper & Qian (2008)).

Sustained phosphorylation of these motifs results in recruitment of cytoplasmic molecules to initiate downstream signalling.

Like NTRs, RTKs also contain tyrosine-based signalling motifs that become phosphorylated and recruit cytoplasmic adaptor proteins. However, they differ from NTRs in that phosphorylation is the result of dimerisation and/or conformational changes, which result in trans-autophosphorylation by their intrinsic catalytic domains (reviewed by Dushek *et al.* (2012)).

3. Binding of surface-associated ligands

Another difference between RTKs and NTRs is that, unlike RTKs and the many other cell-surface receptors that bind to soluble ligands, NTRs typically bind to surface-presented or surface-associated ligands. This includes a wide range of ligands, from peptide-major histocompatibility complex (pMHC), which is the ligand for the T lymphocyte antigen receptor (TCR; reviewed by Schwartz (1985)), to the surface-like collagen that is recognised by NTRs such as leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1; Lebbink *et al.* (2006)).

4. Small receptor-ligand dimensions

Not only do NTRs usually bind to surface-associated ligands, but they typically do so with relatively small (10 - 15 nm) receptor-ligand dimensions. For the NTRs for which receptor-ligand dimensions have been determined through structural studies, they have been found to have not only relatively small but also strikingly similar dimensions (reviewed by Dushek *et al.* (2012)). These relatively small receptor-ligand dimensions appear to be shared by NTRs despite substantial variation in ectodomain size and binding positions of NTRs and their ligands, suggesting a functional relevance of this feature.

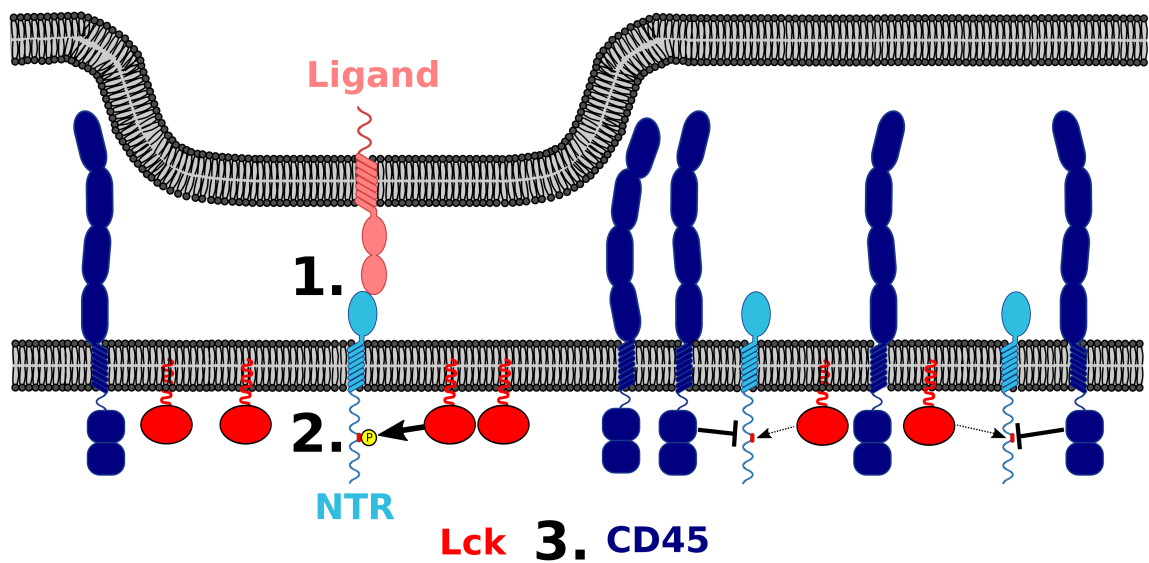


Figure 1.1: Schematic representation of the shared features of non-catalytic tyrosine-phosphorylated receptors (NTRs), including 1. binding of surface-presented or surface-associated ligands with relatively small (10 - 15 nm) receptor-ligand dimensions, 2. no intrinsic catalytic ability; presence of conserved tyrosine-based motifs in their intracellular tail or in associated adaptors, 3. phosphorylation of the tyrosine-based motifs by Src family kinases such as *Lck* and dephosphorylation by receptor protein tyrosine phosphatases such as *CD45*. This figure is based on Dushek et al. (2012).

1.2.2 Diversity of NTRs

Although NTRs typically share the features described above, they also have significant diversity. For example, they contain different numbers and combinations of conserved tyrosine-based signalling motifs, associate with different adaptors (or none), and have a variety of ectodomains (Table 1.1). These aspects of NTR diversity will be introduced below.

1.2.2.1 Signalling motifs

Many activating NTRs and adaptors contain ITAMs, which have a conserved amino acid consensus sequence with two tandem tyrosine residues: Yxx(L/I)_{x6-8}Yxx(L/I), where x is any amino acid (Reth (1989); Cambier (1995)). When ITAMs are phosphorylated by SFKs they can recruit Src homology 2 (SH2)-containing signalling molecules including members of the Syk family of tyrosine kinases such as Syk and zeta-chain-associated protein kinase 70 (ZAP70; Chan *et al.* (1992); Wange *et al.* (1993); Kihara & Siraganian (1994); Narula *et al.* (1995)). The number of ITAMs that different NTRs or their adaptors contain varies from one all the way up to a total of ten in the TCR-associated CD3 chains (Table 1.1).

Related to the ITAM is the hemiTAM, which resembles one half of a canonical ITAM, with the consensus sequence YxxL (reviewed by Robinson *et al.* (2006)). HemiTAMs are found in members of the C-type lectin family and the Killer cell lectin-like receptor subfamily (Table 1.1). Two hemiTAMs can be brought together by receptor dimerisation and subsequently recruit Syk family kinases via their SH2 domains (Watson *et al.* (2009)). Another common activating motif is the YxxM or YxNM motif, also known as the immunoglobulin tail tyrosine motif (ITTM) for its presence in some membrane immunoglobulin (mIg) family members (Engels & Wienands (2011)), which is found in NTRs such as members of the CD28 family as well as in the adaptor molecule DAP10, which associates with NKG2D, an activating NK cell receptor (Table 1.1). Phosphorylation of YxxM motifs results in the recruitment of the signaling scaffold proteins PI3K and Grb2 to initiate downstream signalling (reviewed by Lanier (2009)).

In contrast, most inhibitory NTRs contain ITIMs, which have the conserved amino acid sequence (S/I/V/L)xYxx(I/V/L) (Amigorena *et al.* (1992); Muta *et al.* (1994)). Phosphorylation of the conserved tyrosine residue in ITIMs results in recruitment of phosphatases such as SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1) or SH2-containing protein tyrosine phosphatase-1 or -2 (SHP-1 or SHP-2 respectively;

Amigorena *et al.* (1992); Muta *et al.* (1994); D'Ambrosio *et al.* (1995); Vély *et al.* (1996); Ono *et al.* (1997); Kuroiwa *et al.* (1998)). Some receptors also contain ITIM-like motifs, which have sequences similar but not identical to the canonical ITIM. This includes most members of the sialic acid-binding immunoglobulin-like lectin (Siglec) family, which have a conserved ITIM-like sequence of (E/N)YxE(V/I)(R/K) (reviewed by Crocker *et al.* (2007); Table 1.1).

Finally, the signature sequence of the ITSM differs from that of the ITIM due to the presence of a conserved threonine residue at the -2 position relative to the tyrosine: TxYxx(I/V) (Shlapatska *et al.* (2001)). Phosphorylation of ITSMs results in the recruitment of members of the SAP family of cytoplasmic adaptors, which mediate activating signals through recruitment of kinases (reviewed by Cannons *et al.* (2011)). In the absence of SAP family members, ITSMs “switch” to recruitment of inhibitory molecules such as the kinase Csk, as well as the phosphatases SHP-1, SHP-2, and SHIP-1 to initiate inhibitory signalling. Due to this “switch” characteristic, receptors containing ITSMs have been found to have both activating and inhibitory signalling capability in different contexts (reviewed by Veillette *et al.* (2009); Cannons *et al.* (2011)).

1.2.2.2 Adaptors

Most activating NTRs do not themselves contain the conserved tyrosine-based signalling motifs described above but instead associate with adaptors that do (reviewed by Dushek *et al.* (2012)). Most of these adaptors contain one or more ITAMs through which they initiate signalling. For example, DNAX-activating protein of 12kDa (DAP12) contains one ITAM and exists as a homodimer (Call & Wucherpfennig (2007)), an Ig α / β heterodimer contains 2 ITAMs (Siegers *et al.* (2006)), and the CD3 chains associated with the T cell receptor contain a total of 10 ITAMs (Call & Wucherpfennig (2004)). FcR γ contains one ITAM and can exist as a homodimer or as a heterodimer with CD3 ζ , which itself contains 3 ITAMs (Orloff *et al.* (1990)), whereas DNAX-activating protein of 10kDa (DAP10) contains a YxxM motif and forms a homodimer (Call & Wucherpfennig (2007)).

As evident from Table 1.1, different NTRs associate with different adaptors. This preference of NTRs to associate with different adaptors is often dependent on charged or polar residues in the transmembrane domains of the NTRs and adaptors. In some cases the NTRs that adaptors interact with is determined by their preference for specific transmembrane charged residues, such as lysine or arginine (Feng *et al.* (2006)). Positioning of the charged or polar residues within the transmembrane regions (e.g.

central or towards the inner or outer leaflet) also adds to the specificity of adaptors for certain NTRs (reviewed by Call & Wucherpfennig (2007); Lanier (2009)).

1.2.2.3 Ectodomains

Another aspect of variation between NTRs is ectodomain composition. Approximately 80% of NTRs are type I transmembrane receptors (N-terminal ectodomain), with the remaining NTRs being type II receptors (C-terminal ectodomain; Table 1.1). The ectodomains of many NTRs are made up of immunoglobulin (Ig) domains, making them members of the Ig superfamily (IgSF; reviewed by Williams & Barclay (1988)). It is well established that members of the IgSF are abundant in the immune system, often facilitating cell-cell recognition (reviewed by Barclay (2003)). Ig domains do not have a highly conserved amino acid sequence, but instead are defined according to a conserved Ig-fold comprised of a sandwich of two beta sheets, as was originally identified in immunoglobulins (Poljak *et al.* (1973)).

NTRs from the IgSF contain varying numbers of Ig domains, but more than half contain only one or two Ig domains (Table 1.1). It has previously been suggested that the presence of only two Ig domains in many IgSF family members at the leucocyte cell-surface may be functionally important for cell-cell interactions (Wild *et al.* (1999); reviewed by Barclay (2003)), and that relatively small receptor-ligand dimensions are important for NTR signalling (reviewed by Dushek *et al.* (2012)).

In addition to the many NTRs that contain Ig domains in their ectodomains, some NTRs contain scavenger receptor cysteine-rich (SRCR) domains, C-type lectin domains (type II receptors), or mucin domains (Table 1.1; Hohenester *et al.* (1999); Zelensky & Gready (2005); Kane (2010)). This diversity in NTR ectodomains is reflected in the diversity of ligands that they bind to, which includes not only a variety of self- and pathogen-derived cell-surface proteins and oligosaccharides but also immunoglobulins, collagen, and filamentous actin (reviewed by Dushek *et al.* (2012)).

Despite this variation in ectodomain composition and ligand binding, the ectodomains of the vast majority of NTRs are relatively small (<20 nm; Dushek *et al.* (2012)). This is further discussed in relation to NTR signalling mechanisms in Section 1.4.3.2.

Name	UniProtKB Accession	Intrinsic motif/s	Adaptor/s	Ectodomain	Protein type
Antigen receptors					
T cell receptor			2 CD3 ζ , 2 CD3 ϵ , CD3 δ , CD3 γ	2 Ig - TCR α , TCR β 1 Ig - CD3 ϵ , CD3 δ , CD3 γ	type I
B cell receptor		YxxM (mIgG and mIgE)	Ig α , Ig β	Membrane immunoglobulin 1 Ig - Ig α , Ig β	type I
C-type lectin family					
CLEC1A	Q8NC01	1 hemiTAM		1 C-type lectin	type II
CLEC1B	Q9P126	1 hemiTAM		1 C-type lectin	type II
CLEC4A	Q9UMR7	1 ITIM		1 C-type lectin	type II
BDCA2 (CLEC4C)	Q8WTT0		FcR γ	1 C-type lectin	type II
CLEC4D	Q8WXI8		FcR γ	1 C-type lectin	type II
MINCLE (CLEC4E)	Q9ULY5		FcR γ	1 C-type lectin	type II
Dectin-2 (CLEC6A)	Q6EIG7		FcR γ	1 C-type lectin	type II
Dectin-1 (CLEC7A)	Q96PA5	1 hemiTAM		1 C-type lectin	type II
CLEC8A	P78380	1 hemiTAM		1 C-type lectin	type II
DNGR-1 (CLEC9A)	Q6UXN8	1 hemiTAM		1 C-type lectin	type II
CLEC12A	Q5QGZ9	1 ITIM		1 C-type lectin	type II
CLEC12B	Q2HXU8	1 ITIM		1 C-type lectin	type II
NKG2C	P26717		DAP12	1 C-type lectin	type II
NKG2D	P26718		DAP10	1 C-type lectin	type II
NKG2E	Q07444		DAP12	1 C-type lectin	type II
NKG2F	O43908		DAP12	1 C-type lectin	type II

NKG2A	P26715	2 ITIM		1 C-type lectin	type II
NKG2B	P26715	2 ITIM		1 C-type lectin	type II
Killer cell lectin-like receptor subfamily					
KLRB1 (CLEC5B, CD161)	Q12918	1 hemiTAM		1 C-type lectin	type II
KLRF1 (CLEC5C, NKp80)	Q9NZS2	1 hemiTAM		1 C-type lectin	type II
KLRF2 (NKp65)	D3W0D1	1 hemiTAM		1 C-type lectin	type II
KLRG1 (CLEC15a, MAFA)	Q96E93	1 ITIM		1 C-type lectin	type II
CD300 family					
CD300a	Q9UGN4	4 ITIM		1 Ig	type I
CD300b	A8K4G0		DAP12	1 Ig	type I
CD300e	Q496F6		DAP12	1 Ig	type I
CD300f	Q8TDQ1	3 ITIM		1 Ig	type I
Fc receptor-like family					
Fc receptor-like protein 1	Q96LA6	2 ITAM		3 Ig	type I
Fc receptor-like protein 2	Q96LA5	1 ITAM, 2 ITIM		4 Ig	type I
Fc receptor-like protein 3	Q96P31	1 ITAM, 1 ITIM		6 Ig	type I
Fc receptor-like protein 4	Q96PJ5	2 ITIM		4 Ig	type I
Fc receptor-like protein 5	Q96RD9	1 ITAM, 2 ITIM		8 Ig	type I
Fc receptor-like protein 6	Q6DN72	1 ITIM		3 Ig	type I
Classical Fc receptor family					
Fc γ RI (CD64)	P12314		FcR γ	3 Ig	type I

Fc γ RIIa (CD32)	P12318	1 ITAM		2 Ig	type I
Fc γ RIIb (CD32)	P31994	1 ITIM		2 Ig	type I
Fc γ RIIc (CD32)	P31995	1 ITAM		2 Ig	type I
Fc γ RIII (CD16)	P08637		FcR γ	2 Ig	type I
Immunoglobulin alpha Fc receptor	Q53X38		FcR γ	2 Ig	type I
Fc ϵ RI α	P12319		FcR γ	2 Ig	type I
Killer cell immunoglobulin-like receptor family					
KIR2DL1	P43626	2 ITIM		2 Ig	type I
KIR2DL2	P43627	2 ITIM		2 Ig	type I
KIR2DL3	P43628	2 ITIM		2 Ig	type I
KIR2DL4	Q99706	1 ITIM		2 Ig	type I
KIR2DL5	Q8N109	2 ITIM		2 Ig	type I
KIR2DS1	Q14954		DAP12	2 Ig	type I
KIR2DS2	P43631		DAP12	2 Ig	type I
KIR2DS3	Q14952		DAP12	2 Ig	type I
KIR2DS4	P43632		DAP12	2 Ig	type I
KIR2DS5	Q14953		DAP12	2 Ig	type I
KIR3DL1	P43629	2 ITIM		3 Ig	type I
KIR3DL2	P43630	2 ITIM		3 Ig	type I
KIR3DL3	Q8N743	1 ITIM		3 Ig	type I
KIR3DS1	Q14943		DAP12	3 Ig	type I
Leukocyte immunoglobulin-like receptor (LILR) family					
LILRA1	O75019		FcR γ	4 Ig	type I
LILRA2	Q8N149		FcR γ	4 Ig	type I
LILRA4	P59901		FcR γ	4 Ig	type I
LILRA5	A6NI73		FcR γ	2 Ig	type I

LILRA6	Q6PI73		FcR γ	2 Ig	type I
LILRB1	Q8NHL6	4 ITIM		4 Ig	type I
LILRB2	Q8N423	3 ITIM		4 Ig	type I
LILRB3	O75022	3 ITIM		4 Ig	type I
LILRB4	Q8NHJ6	3 ITIM		2 Ig	type I
LILRB5	O75023	2 ITIM		4 Ig	type I
Natural cytotoxicity triggering receptor family					
NKp46	O76036		CD3 ζ , FcR γ	2 Ig	type I
NKp44 (NCR2, CD336)	O95944		DAP12	1 Ig	type I
NKp30	O14931		CD3 ζ , FcR γ	1 Ig	type I
Paired immunoglobulin-like receptor family					
PILR A	Q9UKJ1	2 ITIM		1 Ig	type I
PILR B	Q9UKJ0		DAP12	1 Ig	type I
Sialic acid-binding Ig-like lectin family					
CD22 (Siglec-2)	Q9UQA6	3 ITIM, 1 ITIM-like		7 Ig	type I
CD33 (Siglec-3)	P20138	1 ITIM, 1 ITIM-like		2 Ig	type I
Myelin- associated glycoprotein (Siglec-4)	P20916	Fyn kinase phosphory- lation site		5 Ig	type I
Siglec-5	O15389	1 ITIM, 1 ITIM-like		3 Ig	type I
Siglec-6	O43699	1 ITIM, 1 ITIM-like		3 Ig	type I
Siglec-7	Q9Y286	1 ITIM, 1 ITIM-like		3 Ig	type I
Siglec-8	Q9NYZ4	1 ITIM, 1 ITIM-like		3 Ig	type I

Siglec-9	Q9Y336	1 ITIM, 1 ITIM-like		3 Ig	type I
Siglec-10	Q96LC7	1 ITIM, 1 ITIM-like		4 Ig	type I
Siglec-11	Q96RL6	1 ITIM, 1 ITIM-like		4 Ig	type I
Siglec-12	Q96PQ1	1 ITIM, 1 ITIM-like		4 Ig	type I
Siglec-14	Q08ET2		DAP12	3 Ig	type I
Siglec-15	Q6ZMC9		DAP12	2 Ig	type I
Siglec-16	A6NMB1		DAP12	4 Ig	type I
Signaling lymphocytic activation molecule family					
SLAMF1	Q13291	2 ITSM		2 Ig	type I
Ly9 (SLAMF3)	Q9HBG7	6 ITIM, 2 ITSM		4 Ig	type I
2B4 (SLAMF4)	Q9BZW8	2 ITIM, 4 ITSM		2 Ig	type I
SLAMF5	Q9UIB8	2 ITSM		2 Ig	type I
SLAMF6	Q96DU3	2 ITSM		2 Ig	type I
SLAMF7 (CRACC)	Q9NQ25	1 ITSM		2 Ig	type I
CD28 family					
CD28	Q9BYV0	1 YxxM		1 Ig	type I
CTLA-4	P16410	1 YxxM		1 Ig	type I
ICOS	Q9Y6W8	1 YxxM		1 Ig	type I
PD-1	Q15116	1 ITIM, 1 ITSM		1 Ig	type I
CD28H	Q96BF3	1 YxxM, 2 non-classi- cal motifs		1 Ig	type I
BTLA	Q7Z6A9	1 ITIM, 1 ITSM		1 Ig	type I
CD200R family					

CD200R1	Q8TD46	non-classical inhibitory motif		2 Ig	type I
CD200R2	Q6Q8B3		DAP12	2 Ig	type I
Signal-regulatory protein family					
SIRP α	P78324	4 ITIM		3 Ig	type I
SIRP β 1	O00241		DAP12	3 Ig	type I
CD5 family					
CD5	P06127	2 ITIM-like		3 SRCR	type I
CD6	P30203	non-classical activation motifs		3 SRCR	type I
Collagen receptors					
LAIR-1	Q6GTX8	2 ITIM		1 Ig	type I
Glycoprotein VI (GPVI)	Q9HCN6		FcR γ	2 Ig	type I
OSCAR	Q8IYS5		FcR γ	2 Ig	type I
T cell immunoglobulin and mucin family					
TIM-1	O46598	non-classical tyrosine motifs		1 Ig, mucin	type I
TIM-3	Q8TDQ0	non-classical inhibitory motifs		1 Ig, mucin	type I
Nectin-binding family					
CD226 (DNAM-1)	Q15762	1 YxxM		2 Ig	type I
TIGIT	Q495A1	1 ITIM		1 Ig	type I
TACTILE (CD96)	P40200	1 ITIM		3 Ig	type I
Triggering receptor expressed on myeloid cells family					
TREM1	Q9NP99		DAP12	1 Ig	type I

TREM2	Q9NZC2		DAP12	1 Ig	type I
TREML1	Q86YW5	2 ITIM		1 Ig	type I
TREML4	Q6UXN2		DAP12	1 Ig	type I
Miscellaneous					
CD7	P09564	1 YxxM		1 Ig	type I
PECAM-1 (CD31)	P16284	2 ITIM		6 Ig	type I
Junctional adhesion molecule-like (JAML)	Q86YT9	1 YxxM		2 Ig	type I
CD19	P15391	7 YxxM		2 Ig	type I

Table 1.1: *Non-catalytic tyrosine-phosphorylated receptors (NTRs)*. *NTR details and characteristics including UniProtKB accession, intrinsic signalling motifs, adaptors, ectodomain composition, and classification as type I (N-terminal ectodomain) or type II (C-terminal ectodomain) transmembrane proteins. NTRs are grouped into families according to shared sequences and/or features. Ig - immunoglobulin; mIgG - membrane-bound immunoglobulin; SCRC - scavenger receptor cysteine-rich domain. This table is adapted from table S1 of Dushek et al. (2012) and from Denham (2016).*

1.3 Regulation of NTR signalling

As detailed in Section 1.2.1, NTR signalling is dependent on being phosphorylated by SFKs and dephosphorylated by RPTPs (reviewed by Dushek *et al.* (2012)). Therefore, in order to understand NTR signalling it is important to understand how these kinases and phosphatases are regulated.

1.3.1 Src family kinases (SFKs)

SFKs are a family of tyrosine kinases found in essentially all metazoan cells. Eleven SFKs have been identified in humans including Blk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, Yes, and Yrk. Of these members, most are preferentially expressed in haematopoietic cells, apart from Fyn, Src, and Yes, which are more ubiquitously expressed (reviewed by Sen & Johnson (2011)).

Sustained phosphorylation of conserved tyrosine-based motifs in the cytoplasmic tails of NTRs by SFKs results in receptor triggering and initiation of signalling through the recruitment of cytoplasmic signalling molecules via their SH2 domains (reviewed by Dushek *et al.* (2012)). For example, the SFK Lck phosphorylates ITAMs within the T cell receptor CD3 chains, resulting in the recruitment the cytoplasmic kinase ZAP70, which Lck then phosphorylates to initiate T cell receptor triggering (reviewed by Weiss & Littman (1994)). Shah and colleagues have recently shown that this sequential phosphorylation of substrates by Lck and then ZAP70 is controlled by the differential preference of these kinases for negatively charged substrates such as the scaffold proteins LAT and SLP-76, which are substrates of ZAP70 but not Lck (Shah *et al.* (2016)).

SFKs such as Lck have a conserved structure, consisting of an SH3 (proline binding), SH2 (phosphotyrosine binding) and SH1 (catalytic) domain (reviewed by Boggon & Eck (2004); Roskoski (2004)). They are not transmembrane proteins, instead being attached to the inner leaflet of the plasma membrane through N-terminal myristoylation and sometimes palmitoylation (Koegl *et al.* (1994); Resh (1999)).

The kinase activity of SFKs is dependent on the phosphorylation state of two conserved tyrosine residues - one towards the C-terminus and one in the kinase domain (reviewed by Roskoski (2004)). Several studies have demonstrated that the C-terminal tyrosine is phosphorylated by the inhibitory kinase Csk and dephosphorylated by CD45/CD148 (Ostergaard *et al.* (1989); Nada *et al.* (1991); Okada *et al.* (1991); Bergman *et al.* (1992); Hurley *et al.* (1993); Cahir-McFarland *et al.* (1993); Klages *et al.* (1994); D'Oro *et al.* (1996); Stepanek *et al.* (2011)). When phosphorylated, the C-terminal tyrosine can form an inactive conformation of the kinase by binding to the SH2 domain, as demonstrated by structural studies (Sicheri *et al.* (1997); Xu *et al.* (1997); Xu *et al.* (1999)).

In contrast, phosphorylation of the conserved tyrosine residue in the kinase domain increases SFK activity (Veillette & Fournel (1990)). This tyrosine residue is trans-autophosphorylated and is dephosphorylated by CD45/CD148 (Ostergaard *et al.* (1989); Veillette & Fournel (1990); D'Oro *et al.* (1996); Yamaguchi & Hendrickson (1996); Baker *et al.* (2000); Stepanek *et al.* (2011); Hui & Vale (2014)).

The role of the CD45 and CD148 phosphatases in regulating SFK activity through dephosphorylation of both an inhibitory and an activating tyrosine highlights the complex nature of the role that these phosphatases play in signalling. It has been proposed that their action may be important for controlling the threshold for activa-

tion and that this is dependent on their localisation relative to SFKs (McNeill *et al.* (2007); Hermiston *et al.* (2009)).

1.3.2 Receptor protein tyrosine phosphatases (RPTPs)

RPTPs such as CD45 and CD148 oppose the action of SFKs by dephosphorylating the conserved tyrosine residues of NTRs (reviewed by Dushek *et al.* (2012)). Both of these phosphatases have relatively large extracellular domains followed by a transmembrane region and a cytoplasmic phosphatase domain. The extracellular domain of CD148 consists of 8 - 10 fibronectin domains and a mucin-like region, estimated to be ≈ 47 - 55 nm in size (Cordoba *et al.* (2013)). CD45 has alternatively spliced isoforms of various lengths (McCall *et al.* (1992); Barclay *et al.* (1997)), with expression of the different isoforms depending on developmental stage, leukocyte population and cell activation state (Trowbridge & Thomas (1994)). The ectodomain of the shortest CD45 isoform, CD45RO, has recently been found to be rigid and reasonably “upright” relative to the plasma membrane, with a length of approximately 22 nm (Chang *et al.* (2016)).

Unlike SFKs, the activity of the phosphatases CD45 and CD148 is not thought to be regulated by the catalytic action of other proteins. In fact, substantial evidence suggests that the major mechanism for regulation of the action of RPTPs is spatial organisation of these molecules relative to their substrates (Irles *et al.* (2002); Lin & Weiss (2003); Varma *et al.* (2006); Cordoba *et al.* (2013); Chang *et al.* (2016)). This forms the foundation of the segregation-based NTR signalling mechanism known as the kinetic-segregation (KS) model (Davis & van der Merwe (1996)), which is described in detail in Section 1.4.3.2.

1.4 Proposed NTR triggering mechanisms

As described in Section 1.2.1, sustained phosphorylation of conserved tyrosine motifs in NTRs results in recruitment of cytoplasmic molecules and downstream signalling. The process of NTR signal initiation is termed NTR “triggering” and there are several mechanisms proposed to explain the process, in particular in the context of the T cell receptor (reviewed by Choudhuri & van der Merwe (2007); van der Merwe & Dushek (2011)). These mechanisms fall into the three broad categories of 1) conformational change, 2) induced proximity or aggregation, and 3) segregation, which are introduced below. It is likely that NTR signalling involves contributions from a number of these

mechanisms, but it has not been definitively determined which are responsible for NTR triggering and to what degree (reviewed by Dushek *et al.* (2012)).

1.4.1 Conformational change

Models for NTR triggering that propose a conformational change in the receptor to initiate signalling can be described by a two-state model. In the basal state the cytoplasmic tail of the NTR is inaccessible to phosphorylation by SFKs (non-signalling state), whereas upon ligand binding a conformational change results in increased accessibility and phosphorylation of the NTR by SFKs (signalling state). Importantly, this model requires that SFK activity dominates RPTP activity so that sustained phosphorylation of NTRs can occur in the signalling state (reviewed by Dushek *et al.* (2012)).

Such a conformational change model has been suggested for the T cell receptor, with the proposal that in the non-signalling state the TCR CD3 chains are sequestered in the plasma membrane, away from the action of SFKs. Ligand binding is then proposed to release the CD3 chains from the membrane, leading to phosphorylation of the ITAMs and subsequent downstream signalling (Aivazian & Stern (2000); Xu *et al.* (2008); reviewed by Kuhns & Davis (2008)). However, alternative studies have found that the release of the CD3 chains from the plasma membrane occurs after phosphorylation, rather than leading to phosphorylation as proposed by the model (Fernandes *et al.* (2010); Zhang *et al.* (2011)). These results suggest that this mechanism is not responsible for initiation of TCR signalling, although it may play a role in signal propagation. Further investigations of conformational change models for TCR triggering are underway, but are yet to definitively demonstrate the importance of such models for initiation of TCR signalling (Lee *et al.* (2015); Swamy *et al.* (2016); Krshnan *et al.* (2016)). Moreover, the plasma membrane-association and subsequent release proposed for the CD3 chains (Aivazian & Stern (2000); Xu *et al.* (2008)) does not appear to be common amongst the large group of NTRs or their adaptors, indicating that such a mechanism is not widely applicable to NTRs.

Another challenge of the plausibility of the applicability of conformational change models for NTR triggering is the substantial diversity in the structure of the extracellular regions of NTRs, as discussed in Section 1.2.2. This variability places considerable constraints on the model for it to be applicable to such a diverse group of receptors.

One type of conformational change mechanism that is compatible with the variability in NTR ectodomains and could conceivably be generally applicable to NTRs

is a mechanical mechanism in which binding of the NTR to ligand results in a pulling force on the receptor (Ma *et al.* (2012); Chen & Zhu (2013)). It is currently unclear how a mechanical force could induce a conformational change across the plasma membrane, or how this could be reconciled with the initiation of NTR phosphorylation upon inhibition of phosphatases in the absence of ligand, such as by pervanadate treatment. As such, further investigation is necessary to determine whether conformational change could be a feasible mechanism for NTR triggering.

1.4.2 Induced proximity or aggregation

The second category of models for NTR triggering is induced proximity or aggregation, which includes models based on two or more NTRs coming together upon ligand binding. These models propose that aggregation or clustering leads to NTR phosphorylation and initiation of signalling such as through trans-autophosphorylation of receptor-associated SFKs and/or phosphorylation of nearby NTRs. Experimental support for these models is provided by the ability to trigger signalling of many NTRs through artificial cross-linking, such as shown by Woodruff *et al.* (1967) and Kaye & Janeway (1984) for the BCR and TCR respectively.

The importance of aggregation for TCR signalling has recently been investigated by Pagon and colleagues, who found that CD3 ζ chains exist in nanoclusters, which increase in density upon TCR engagement (Pagon *et al.* (2016)). TCR nanocluster density was shown to be both independent of and positively correlated with phosphorylation (Pagon *et al.* (2016)), suggesting that clustering occurs prior to phosphorylation. These results are in agreement with aggregation models as well as results from previous studies (Bunnell *et al.* (2002); Campi *et al.* (2005)).

Additional support for aggregation models has been provided by Cooper and Qian, who used a mathematical model to investigate triggering upon NTR dimerisation based on interactions between SFKs, RPTPs, and the NTRs/NTR-like proteins they act upon (Cooper & Qian (2008)). As discussed in Section 1.3.1, SFK activity depends on trans-autophosphorylation of a conserved tyrosine residue within the kinase domain (Veillette & Fournel (1990)). Cooper and Qian propose that by bringing multiple NTRs and their associated SFKs into close proximity, the increased local concentration of kinase and substrate results in enhanced SFK activation through trans-autophosphorylation, leading to increased NTR phosphorylation and subsequent signalling (Cooper & Qian (2008)). This mathematical model is supported experimentally by results from the reconstituted system developed by Hui and Vale,

in which co-clustering of CD3 ζ with Lck resulted in tyrosine phosphorylation (Hui & Vale (2014)).

An aspect of the aggregation model proposed by Cooper and Qian that needs to be addressed to expand its applicability to NTRs is the suggestion that receptor aggregation occurs via binding to dimeric ligands (Cooper & Qian (2008)). As discussed by Dushek *et al.*, most NTR ligands are monovalent and many NTRs are dimers themselves, making aggregation via binding to dimeric ligands unlikely to be widely applicable to NTRs (reviewed by Dushek *et al.* (2012)). However, it is important to note that this does not rule out NTR aggregation through other mechanisms.

An alternative model for NTR signalling via aggregation proposes that a conformational change of certain NTRs upon ligand binding results in receptor clustering (reviewed by van der Merwe & Dushek (2011)). However, as discussed in relation to the models described in the conformational change section above (Section 1.4.1), it is unlikely that a conformational change-based mechanism could be applicable to such a diverse group of receptors. In contrast, a recent study argues that a “kinetic trap” mechanism is responsible for aggregation of integrin at the cell-cell interface and that this could also be applicable to other cell surface receptors (Paszek *et al.* (2014)).

Nonetheless, despite the substantial evidence for the contribution of receptor aggregation to NTR signalling, its role in the initiation of NTR phosphorylation upon ligand binding is called into question by results from multiple studies demonstrating that TCR triggering can occur upon engagement with a single ligand (Sykulev *et al.* (1996); Irvine *et al.* (2002); Purbhoo *et al.* (2004); Ma *et al.* (2008); Huang *et al.* (2013)). Two models attempt to explain these results: the co-receptor heterodimerisation and pseudodimer models.

The co-receptor heterodimerisation model argues that SFKs associated with CD4 or CD8 co-receptors are recruited to the TCR through binding of the co-receptor to the same agonist pMHC as the TCR, resulting in TCR triggering (reviewed by Trautmann & Randriamampita (2003)). However, studies have shown that TCR triggering can occur in the absence of CD4 or CD8 co-receptors (Locksley *et al.* (1993); Schilham *et al.* (1993)), indicating that co-receptor heterodimerisation is not required for TCR triggering.

In contrast to the co-receptor heterodimerisation model, the pseudo-dimer model postulates that self-peptide-MHC molecules and their associated co-receptors facilitate TCR clustering and subsequent signalling (Irvine *et al.* (2002); Krogsgaard *et al.* (2005)). There is evidence for the pseudo-dimer model in CD4⁺ T cells, but conflicting results in the case of CD8⁺ T cells (reviewed by Krogsgaard *et al.* (2007)). As it

stands, the pseudo-dimer model may be feasible for TCR signalling, but it is unlikely that it would be able to explain aggregation and triggering of other NTRs due to the unique nature of the interactions of TCR with self- and agonist-pMHC.

Overall, the aggregation-based models and studies described here have not yet provided definitive evidence of the role of aggregation in initiation of NTR triggering, but there is considerable evidence that it may make an important contribution to NTR signalling. As such, this area of research remains of interest for NTR signalling and warrants further investigation.

1.4.3 Segregation

It is well established that inhibition of tyrosine phosphatases, such as through pervanadate treatment, is generally sufficient for phosphorylation of NTRs, even in the absence of ligand (reviewed by Dushek *et al.* (2012)). This indicates that NTRs are continuously phosphorylated and dephosphorylated, with dephosphorylation dominating at steady state (i.e. without receptor engagement). It therefore follows that an NTR triggering mechanism that somehow shifts the local kinase/phosphatase balance towards the activity of the kinase upon ligand binding could result in sustained NTR phosphorylation and subsequent signalling.

This initiation of signalling by shifting the local kinase-phosphatase balance cannot be accounted for by conformational change models, which require that SFK activity dominates RPTP activity so that sustained phosphorylation of NTRs can occur in the signalling state (reviewed by Dushek *et al.* (2012)). However, it is compatible with the induced proximity or aggregation models described above, and also with the third category of NTR triggering, which is based on redistribution or segregation of SFKs and RPTPs in relation to NTRs upon ligand binding. The two main segregation-based models we will discuss here differ in the mechanism of segregation but share a focus on shifting the local kinase/phosphatase ratio via differences in spatial localisation of SFKs, RPTPs, and their NTR substrates (reviewed by Horejsí (2005); Davis & van der Merwe (2006)).

1.4.3.1 Lipid raft model

The first of these segregation-based models proposes that the distribution of cell-surface molecules is dependent on lipid domains within the plasma membrane known as lipid rafts or membrane rafts (reviewed by Horejsí (2005)). It has been suggested that these lipid rafts are enriched in cholesterol, sphingolipids, and lipid-modified

proteins such as GPI-anchored proteins (Simons & Ikonen (1997); Brown & London (2000)). Most studies of lipid raft structures have focused on TCR signalling, with the lipid raft model hypothesising that preferential distribution and diffusion of engaged TCR within lipid domains that are SFK-enriched/RPTP-depleted favours phosphorylation and results in TCR triggering (Janes *et al.* (2000)).

However, there has been controversy surrounding the techniques used to identify lipid rafts. For example, it has been argued that use of detergents could interfere with lipid structures and create experimental artefacts that may bias the characterisation of lipid rafts (Munro (2003); Simons & Gerl (2010)). There have also been conflicting results in studies investigating the role of lipid rafts in TCR triggering (Pizzo *et al.* (2002); Glebov & Nichols (2004); Hashimoto-Tane & Yokosuka (2010)).

It has previously been suggested that studying the potentially small spatial and temporal scales of lipid rafts may require the use of super-resolution imaging techniques (Eggeling *et al.* (2008); reviewed by van der Merwe & Dushek (2011); Owen *et al.* (2012)). More recently, Bhatia and colleagues have reviewed advances in techniques for analysing lipid domains using giant unilamellar vesicles (Bhatia *et al.* (2016)). Utilisation of methods such as these may help to clarify the presence and characteristics of lipid rafts as well as their possible role in NTR triggering.

1.4.3.2 Kinetic-segregation (KS) model

The second segregation-based model for NTR triggering is known as the KS model and was originally proposed to explain triggering of the TCR (Davis & van der Merwe (1996)). The KS model revolves around the relatively small NTR-ligand dimensions, which require formation of a close contact of approximately 10 - 15 nm between cells in order for receptor-ligand binding to occur (reviewed by Davis & van der Merwe (2006)). It was first proposed by Springer in 1990 that such close contacts would result in the exclusion or segregation of the large components of the glycocalyx such as CD45 and CD43 (Springer (1990)). This proposal was taken further with the development of the KS model for TCR signalling (Davis & van der Merwe (1996)).

The KS model suggests the following sequence of events: in resting T cells, surface proteins diffuse freely in the membrane and hence associate randomly. The action of SFKs, such as Lck, as well as that of abundant RPTPs, such as CD45 and CD148, results in a low net phosphorylation of TCRs (panel A, Figure 1.2). Upon formation of a close contact zone, large, inhibitory RPTPs are excluded, whereas the SFKs are small enough to remain in the area of close contact, resulting in increased net phosphorylation of TCRs. Due to rapid lateral diffusion of proteins on this scale,

phosphorylation of unbound TCR is only transient as the TCR will diffuse out of the close contact area and be exposed to the action of RPTPs within a fraction of a second. However, if the TCR is bound by pMHC it will be trapped within the close contact zone for as long as it remains bound, resulting in sustained phosphorylation and subsequent downstream signalling (panel B, Figure 1.2).

An important feature of the KS model is that it is compatible with triggering of NTRs through the use of phosphatase inhibitors or artificial cross-linking (reviewed by Dushek *et al.* (2012)); since the KS model proposes that simply shifting the local kinase/phosphatase balance in favour of kinases is sufficient for triggering, it follows that steric exclusion of bulky RPTPs through artificial cross-linking or inhibition of phosphatases such as through pervanadate treatment would result in signalling.

The KS model is supported by *in silico* evidence, such as Allard and colleagues' mechanochemistry model of segregation of large surface molecules from small receptor-ligand complexes (Allard *et al.* (2012)). This model is robust to parameter variations, taking into account the diffusion and binding kinetics of receptors and their ligands as well as bending of the plasma membranes, and compression of large surface molecules. In line with the KS model, this mechanochemistry model predicts small spatial scale (approximately 80 nm) and fast temporal scale (1 ms) segregation of large surface molecules from bound receptors (Allard *et al.* (2012)).

Moreover, substantial *in vitro* evidence provides direct support for or is in agreement with the KS model of signalling, in particular for the TCR (reviewed by Davis & van der Merwe (2006)). For example, it has been demonstrated for the TCR that elongation of its ligand, pMHC, results in abrogated signalling (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009)). It has also been shown that CD45 and CD148 are excluded from areas of TCR triggering and that truncating the large ectodomains of CD45 and CD148 results in a lack of segregation of these phosphatases from close contact areas as well as an abrogation of TCR signalling (Irles *et al.* (2002); Lin & Weiss (2003); Varma *et al.* (2006); Cordoba *et al.* (2013)). These results provide further evidence that extracellular dimensions are important for controlling the segregation and function of these RPTPs and their substrates, including NTRs and SFKs such as Lck.

In addition to the substantial evidence for the KS model in relation to TCR triggering, there is also support for the KS model for other NTRs including Dectin-1 (Goodridge *et al.* (2011)), NK cell receptors (McCann *et al.* (2003); Brzostek *et al.* (2010); Köhler *et al.* (2010)), and CD28 family members including cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1),

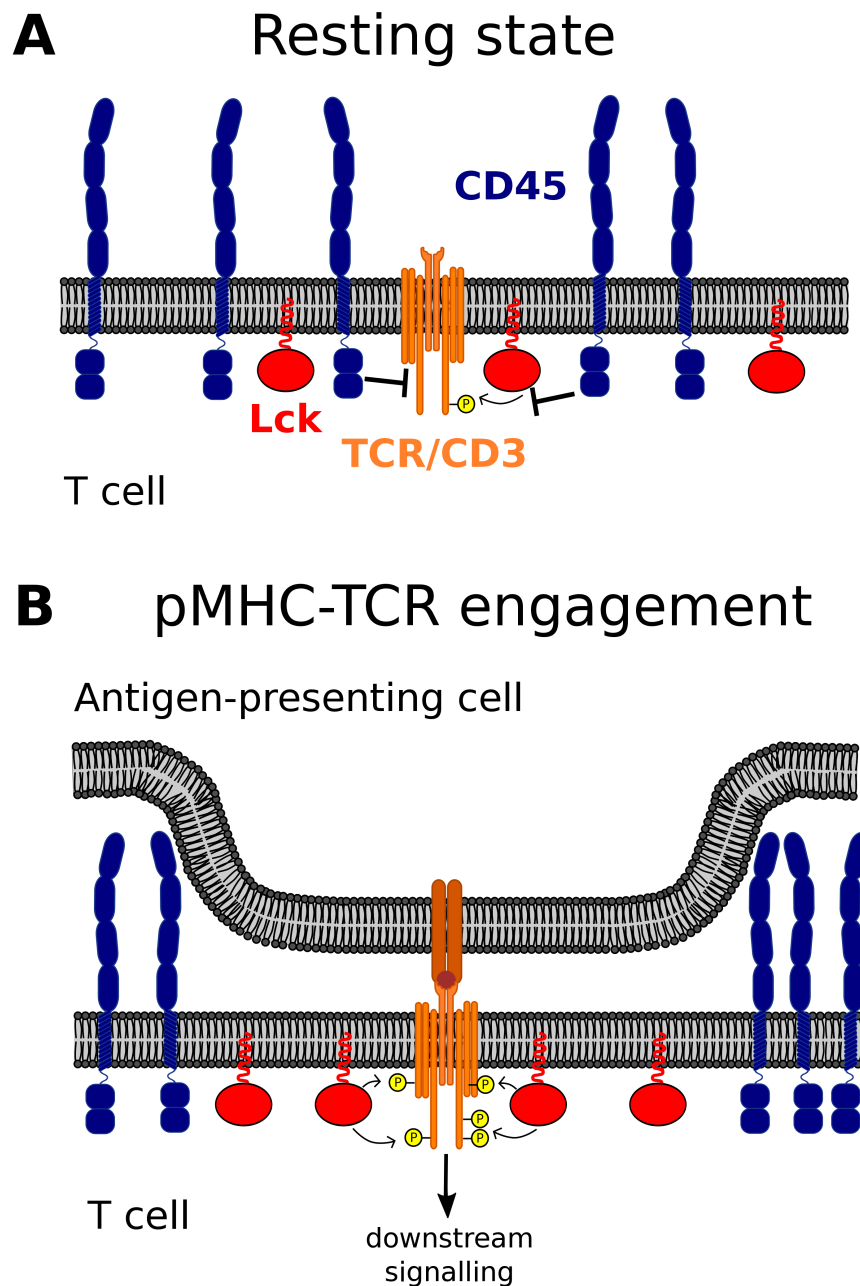


Figure 1.2: Schematic representation of the kinetic-segregation (KS) model for T cell receptor triggering in T lymphocytes. At resting state (A), phosphatase activity (e.g. CD45) dominates over kinase activity (e.g. Lck), resulting in low net phosphorylation of the TCR. Upon binding of the TCR to peptide-MHC (pMHC) an area of close-contact is formed, resulting in segregation of the TCR-CD3 complex from the relatively large phosphatases but not the small kinases. This results in a shift towards kinase activity and sustained phosphorylation of the TCR, leading to downstream signalling. Figure based on van der Merwe & Dushek (2011).

and CD28 (Yokosuka *et al.* (2008); Yokosuka *et al.* (2010); Yokosuka *et al.* (2012); Lim *et al.* (2015)). Overall, these results provide evidence for wider applicability of the KS mechanism for NTR signalling. However, with such a large and diverse group of receptors it is particularly important to demonstrate the applicability of the KS model for a wide range of NTRs.

1.5 Signal integration by NTRs

It is evident from the numerous, and at times conflicting, NTR triggering models described above that much remains to be understood about the signalling mechanism/s of these receptors. As described in Section 1.2, the group of receptors classified as NTRs includes not only activating receptors but also co-stimulatory and co-inhibitory receptors. As such, integration of signals between these different receptors shapes the downstream signalling and subsequent response of the cell to its environment. We refer to the combined action of signalling through different NTRs, such as the combination of signalling through an activating and co-stimulatory pair or an activating and co-inhibitory pair, as signal integration.

Due to the shared characteristics of NTRs described in Section 1.2.1, including the recruitment of cytoplasmic molecules to conserved motifs in their intracellular tails, and subsequent signalling through the action of these recruited molecules, we propose that signal integration between NTRs may require their close co-localisation. This proposal is based on the membrane proximal nature of NTR signal integration, whereby recruited molecules such as cytoplasmic kinases and phosphatases act directly on nearby (membrane proximal) substrates, which would necessarily require close co-localisation of the NTRs or molecules associated with them. This membrane proximal signal integration between NTRs could be important for immune responses that require directionality. For example, cytotoxic T cells and NK cells need a directional response to release cytotoxic granules towards target cells whilst sparing nearby healthy cells (reviewed by Dustin & Long (2010)).

Signal integration is also important for NK cell recognition of “missing self” (Kärre *et al.* (1986); reviewed by Ljunggren & Kärre (1990)), such as through the binding of MHC class I molecules by inhibitory KIR family members, which prevents unwanted immune responses towards “self”. A lack of binding of these inhibitory NK cell receptors, such as by MHC class I-deficient tumour cells, is referred to as the “absence of self” or “missing self” and flags a target cell as infected or tumorigenic. Signals via

activating NK receptors can then lead to NK cell activation and potential killing of the target cell (reviewed by Long *et al.* (2013)).

The process of signal integration has been investigated for several NTRs, including the co-stimulatory receptor CD28 (Lim *et al.* (2015)), as well as co-inhibitory receptors such as PD-1 (Yokosuka *et al.* (2012)), and inhibitory NK cell receptors (Brzostek *et al.* (2010); Köhler *et al.* (2010)). This literature is discussed in detail in Section 4.1.4.

1.6 NTRs for investigation of signal integration

Understanding the process of signal integration between a wider range of activating and co-stimulatory/co-inhibitory NTRs is increasingly important in light of recent advances in immunotherapies such as checkpoint blockade targeting the inhibitory NTRs CTLA-4 and PD-1 (Wolchok *et al.* (2013); Schadendorf *et al.* (2015); Robert *et al.* (2015); Gettinger *et al.* (2015)). These therapies use blocking antibodies against CTLA-4 and/or PD-1 to prevent their inhibitory signalling, which in some cases has been associated with T cell exhaustion (reviewed by Jiang *et al.* (2015)). The rapid progress of these therapies in treating cancers such as melanoma and non-small cell lung cancer has kick-started the field of cancer immunotherapy, with particular attention being given to the great potential of combination therapies (reviewed by Harris *et al.* (2016)). As well as these tumour-related therapies, there has also been a push for targeting co-inhibitory NTRs for the treatment of autoimmune conditions (reviewed by Ceeraz *et al.* (2014); van der Vlist *et al.* (2016); Lee *et al.* (2016)).

In addition to these antibody-based immunotherapies, immune-based treatments known as adoptive cell transfer (ACT) are also under development (reviewed by Rosenberg & Restifo (2015)). Many recent developments in ACT such as chimeric antigen receptor (CAR) therapy utilise NTRs and involve targeting two or more receptors or using a split receptor to improve specificity and functionality (reviewed by Jensen & Riddell (2014)). Signal integration between the receptors then directs the ACT-based immune response against targets such as tumour cells.

Due to the critical role of signal integration in these immune-based therapies, we must develop a better understanding of many of these NTRs and their mechanisms of signal integration. This should accelerate the identification of additional promising targets and maximise the safety and efficacy of potential treatments. A selection of co-inhibitory NTRs that are prime candidates for immunotherapies are introduced below.

1.6.1 Inhibitory KIR family members

In NK cells, inhibitory receptors such as members of the killer cell Ig-like receptor (KIR) family, are responsible for preventing unwanted immune responses by recognising “self” such as through binding to MHC class I molecules. These receptors often contain ITIM signalling motifs to affect inhibitory signalling (reviewed by Long (2008)). This includes the inhibitory NTRs KIR2DL1, KIR2DL2, and KIR2DL3, which bind to the human leukocyte antigen (HLA) C group 2-related alleles (KIR2DL1) and group 1-related alleles (KIR2DL2 and KIR2DL3; reviewed by Rajagopalan & Long (2005)). Engagement of these inhibitory NTRs results in inhibition of the NK-cell triggering mediated by activating NTRs and explains the “missing self” hypothesis, in which MHC class I-deficiency or down-regulation triggers NK cell responses against target cells (reviewed by Long (2008)).

Although many tumours down-regulate MHC class I to avoid immune recognition by T cells (reviewed by Hicklin *et al.* (1999)), it has also been shown that tumour cells can take advantage of these inhibitory receptors to evade NK-mediated immune responses by up-regulating MHC class I (Classen *et al.* (2003)). To prevent this immune escape, a monoclonal antibody against KIR2DL1, -L2, and -L3, known as lirilumab/BMS-986015/IPH2102, is being trialled as a checkpoint inhibitor. Results from murine models (Romagné *et al.* (2009); Kohrt *et al.* (2014)) as well as recent phase I/II trials of lirilumab alone or in combination with lenalidomide (Vey *et al.* (2012); Benson *et al.* (2015)) indicate that inhibitory NK receptors such as KIR2D-L1, -L2, and -L3 are promising targets for immunotherapy.

The rate of response to these treatments is currently modest, with only a subset of patients showing objective responses (Vey *et al.* (2012); Benson *et al.* (2015)). Therefore, understanding more about how these receptors integrate their inhibitory signals in different immune cell subsets and in combination with other receptors may help to improve the applicability of these treatments.

1.6.2 TIGIT

T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is a co-inhibitory receptor in T cells and NK cells that shares the ligands CD112 and CD155 with the activating NTR DNAM-1 (CD226) and also shares the ligand CD155 with the co-inhibitory NTR TACTILE (CD96; Yu *et al.* (2009); Boles *et al.* (2009); Stanitsky *et al.* (2009); Joller *et al.* (2011)). The intracellular tail of TIGIT contains an ITIM and a YxxM motif, and has been shown to recruit the phosphatase SHIP-1, resulting in inhibition

of NK cell cytotoxicity and interferon gamma (IFN- γ) production *in vitro* (Yu *et al.* (2009); Levin *et al.* (2011); Liu *et al.* (2013); Li *et al.* (2014b)).

TIGIT has been implicated in inhibition of DNAM-1-mediated signalling *in vitro* (Lozano *et al.* (2012)), and co-blockade of TIGIT and PD-1 has shown promise both *in vitro* and in a murine colon carcinoma model (Johnston *et al.* (2014); Chauvin *et al.* (2015); Inozume *et al.* (2016)). As such, the inhibitory role of TIGIT and its signalling motifs warrants additional investigation to understand more about this potential target.

1.6.3 TACTILE

Another inhibitory receptor expressed by T and NK cells is T cell activation, increased late expression (TACTILE), also known as CD96 (Wang *et al.* (1992)). TACTILE shares the ligand CD155 with TIGIT (Yu *et al.* (2009); Boles *et al.* (2009)) and DNAM-1 (Shibuya *et al.* (1996)) and is a potential target for immunotherapy due to its inhibitory role in immune regulation.

Despite interest in TACTILE as a therapeutic target, surprisingly little has been elucidated about its signalling mechanism since it was identified in 1992 (Wang *et al.* (1992); reviewed by Bernhardt (2014); Blake *et al.* (2016)). TACTILE contains an ITIM within its intracellular tail (Meyer *et al.* (2009)) and has been shown to inhibit NK cell activation (Chan *et al.* (2014)). TACTILE-deficient mice show increased IFN- γ production with a hyperinflammatory response and lower overall survival upon LPS challenge (Chan *et al.* (2014)). Additionally, TACTILE-deficiency was protective in several tumour models in an NK cell- and IFN- γ -dependent manner.

However, unlike its murine homolog, human TACTILE contains a YxxM domain as well as the ITIM in its intracellular tail (Chan *et al.* (2014)). This may explain the ability of human TACTILE to increase human NK cell-mediated cytotoxicity (Fuchs *et al.* (2004); Stanietsky *et al.* (2009)). Further investigation is required to determine whether human and murine TACTILE function in a similar manner.

One area of human TACTILE signalling that could be investigated using the two-ligand system described in the current work is the positioning of the ITIM and YxxM motif in the intracellular tail. Interestingly, the tyrosine residue of the ITIM is only 26 amino acids from the plasma membrane, which is relatively close compared to ITIMs in other NTRs. It would therefore be interesting to investigate whether the signalling role of TACTILE is impacted by truncation or elongation of its intracellular tail to alter the position of the ITIM.

1.6.4 Siglec-2

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are a family of NTRs that bind to sialic acids such as those found on cell surface glycoproteins. Apart from Siglec-4, which is expressed in the nervous system, Siglecs are expressed by a variety of immune cells with most being preferentially expressed by certain immune cell subtypes (reviewed by Macauley *et al.* (2014)).

Siglec-2, also known as CD22, is expressed by B cells and was originally found to bind to the phosphatase CD45 (Stamenkovic *et al.* (1991); Aruffo *et al.* (1992)). A subsequent study demonstrated binding of Siglec-2 to a number of other glycoproteins in a sialic-acid-dependent manner, leading to the classification of Siglec-2 as a sialic-acid-binding protein (Sgroi *et al.* (1993)). Siglec-2 has been shown to recruit the phosphatase SHP-1 to ITIMs in its intracellular tail and inhibit signalling through the BCR (Doody *et al.* (1995)). Siglec-2 has been explored as a target for B-cell malignancies (reviewed by Sullivan-Chang *et al.* (2013)), with some promising recent results (Satake *et al.* (2014); Weber *et al.* (2015)).

Due to its role in regulating B cell signalling, Siglec-2 has also been investigated as a therapeutic target for systemic lupus erythematosus (SLE; reviewed by van der Vlist *et al.* (2016)). Despite promising early results from targeting Siglec-2 for treatment of SLE (Sieger *et al.* (2013); Wallace *et al.* (2013); Strand *et al.* (2014); Fleischer *et al.* (2015)), two subsequent phase III trials failed to meet their clinical endpoint (reviewed by van der Vlist *et al.* (2016)). Thus, further investigation of Siglec-2 is required to develop more effective treatments targeting this inhibitory NTR.

1.6.5 Siglec-9

Another member of the Siglec family, Siglec-9, has recently been shown to be an exciting potential target for both cancer and autoimmune diseases (Beatson *et al.* (2016); Matsumoto *et al.* (2016)). Siglec-9 is highly expressed by monocytes with lower expression on neutrophils and subpopulations of B, NK and T cells (Zhang *et al.* (2000)). It contains both an ITIM and ITSM in its intracellular tail and has been shown to recruit SHP-1/SHP-2 to inhibit downstream signalling (Ikehara *et al.* (2004); Avril *et al.* (2004); Yamaji *et al.* (2005)). In contrast to PD-1 (Section 1.6.7), it is the ITIM not the ITSM of Siglec-9 that has been found to be critical for its inhibitory function (Avril *et al.* (2004)).

The role of Siglec-9 in tumourogenesis comes from its binding of the transmembrane mucin MUC1 on cancer cells, which has been shown to promote tumour cell

growth (Tanida *et al.* (2013)). Beatson and colleagues have recently demonstrated that binding of Siglec-9 on primary macrophages to a tumor-associated MUC1 glycoform (MUC1-ST) results in induction of a tumour-associated macrophage (TAM) phenotype (Beatson *et al.* (2016)), making Siglec-9 a potential target for checkpoint blockade.

In addition to its role in tumour growth, Siglec-9 has been shown to be a key player in a murine model of collagen-induced arthritis (Matsumoto *et al.* (2016)). Overall these studies highlight Siglec-9 as an exciting target for the development of both tumour- and autoimmune-related immunotherapies. Understanding more about the signalling role of this receptor in different immune cell types should assist in development of such therapies.

1.6.6 BTLA

B- and T-lymphocyte attenuator (BTLA) is a member of the CD28 family of receptors and is expressed by a variety of immune cells including T cells, B cells, NK cells and antigen-presenting cells (APCs; Watanabe *et al.* (2003); Han *et al.* (2004); Hurchla *et al.* (2005); Murphy *et al.* (2006); Vendel *et al.* (2009); reviewed by Baksh & Weber (2015)). BTLA expression on naive T cells is rapidly upregulated upon stimulation and is also high on anergic T cells generated by chronic antigen exposure (Watanabe *et al.* (2003); Hurchla *et al.* (2005)).

It has been shown that BTLA binds to the TNF family member herpes virus entry mediator (HVEM), expressed by some tumor cell types and tumor-associated endothelial cells, as well as the herpes virus protein UL144, which has homology to HVEM (Sedy *et al.* (2005); Gonzalez *et al.* (2005)). The intracellular tail of BTLA is similar in sequence to PD-1 (Section 1.6.7), containing both an ITIM and an ITSM, and with a lack of recruitment of the small adapter SAP to the ITSM of either receptor (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Chemnitz *et al.* (2006)). BTLA has been shown to recruit the cytoplasmic phosphatases SHP-1 and SHP-2 (Watanabe *et al.* (2003); Gavrieli *et al.* (2003); Vendel *et al.* (2009)), although the role of these phosphatases in BTLA signalling has been questioned (Chemnitz *et al.* (2006)).

The potential of BTLA as a target for immunotherapy has been demonstrated by increased anti-tumour immunity from BTLA blockade, which was further enhanced when combined with PD-1 blockade (Fourcade *et al.* (2012)). BTLA has also been implicated in an autoimmune context, with BTLA-deficiency resulting in increased severity of autoimmune disease in several mouse models (Watanabe *et al.* (2003); Oya *et al.* (2008); Steinberg *et al.* (2008); Oya *et al.* (2011)). Additionally, polymorphisms

in the *BTLA* gene have been associated with susceptibility to rheumatoid arthritis (Lin *et al.* (2006); Oki *et al.* (2011)). Overall these results highlight BTLA as a candidate to investigate for both tumour- and autoimmune-related immunotherapies.

1.6.7 PD-1

Like BTLA, PD-1 is a co-inhibitory NTR that belongs to the CD28 family. PD-1 expression is upregulated by activated T and B lymphocytes (Agata *et al.* (1996)) as well as activated monocytes, natural killer T (NKT) cells, NK cells and some dendritic cell subsets (reviewed by Keir *et al.* (2008)).

Studies have shown that PD-1 inhibits T cell signaling by recruiting cytoplasmic phosphatases such as SHP-2 (Latchman *et al.* (2001)) and dephosphorylating membrane proximal substrates such as the TCR CD3 chains, the associated kinase ZAP70, and/or the co-stimulatory receptor CD28 (Sheppard *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012); Zinselmeyer *et al.* (2013)). Which of these substrates is the main target of PD-1-mediated inhibition is currently being investigated, with preliminary results suggesting that CD28 is particularly sensitive to PD-1 inhibitory function (Hui *et al.* (2016)).

PD-1 is of great clinical interest; it is upregulated by activated T cells, is a marker for T cell exhaustion (reviewed by Jiang *et al.* (2015)), was one of the first targets of checkpoint blockade therapy, and has shown promising results in tumour immunotherapy trials both as a monotherapy and in combination therapies (Wolchok *et al.* (2013); Robert *et al.* (2015); Gettinger *et al.* (2015)). Despite being the focus of many clinical studies, several aspects of PD-1 signalling remain poorly understood, such as the importance of the length of its intracellular tail for signal integration. However, enough is known about this inhibitory receptor to use it for validation and optimisation of the system being developed here. As such, PD-1 is the focus of the current study and is described in further detail in Sections 3.1.5, 4.1.5, and 5.1.2.

1.7 Hypotheses and Aims

As evident from the small selection of inhibitory NTRs introduced above, a deeper understanding of the mechanisms of signal integration between these and other NTRs, many of which are much less well characterised than the receptors described here, may assist in the development of effective therapies targeting these receptors. The current study is focused on the development of a system for studying NTR signal integration by exploring the hypotheses outlined below.

1.7.1 Hypothesis 1

Studies of signal integration between NTRs to date have demonstrated a requirement for small and well-matched receptor-ligand dimensions for optimal signal integration (Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2012)). This has been proposed to be due to proximal signal integration by NTRs, which are known to become phosphorylated and then recruit cytoplasmic molecules to their intracellular tail that need to act locally (i.e. nearby at the plasma membrane) on other NTRs and/or proximal signalling components (reviewed by Dushek *et al.* (2012)).

Based on these NTR signal integration studies and the shared features of NTRs described in Section 1.2.1, we hypothesise that optimal NTR signal integration is dependent on close co-localisation, for which well-matched NTR-ligand dimensions are required (“hypothesis 1”, Figure 1.3). According to this hypothesis, a mismatching of receptor-ligand dimensions, such as through elongation of the ligand for one of the NTRs, would be predicted to prevent co-localisation and abrogate subsequent signal integration between NTRs (Figure 1.3). Chapter 4 of the current work details preliminary investigation of this hypothesis using the two-ligand system described in Chapter 3.

1.7.2 Hypothesis 2

Secondly, we hypothesise that NTR signal integration is dependent on intracellular tail length (“hypothesis 2”, Figure 1.4). Specifically, we hypothesise that optimal signal integration by inhibitory NTRs is dependent on the length of their intracellular tail, with signalling motifs further from the plasma membrane enabling inhibition throughout a greater volume.

As for hypothesis 1, this hypothesis is based on the shared features of NTRs described in Section 1.2.1 as well as evidence for the importance of proximal signal

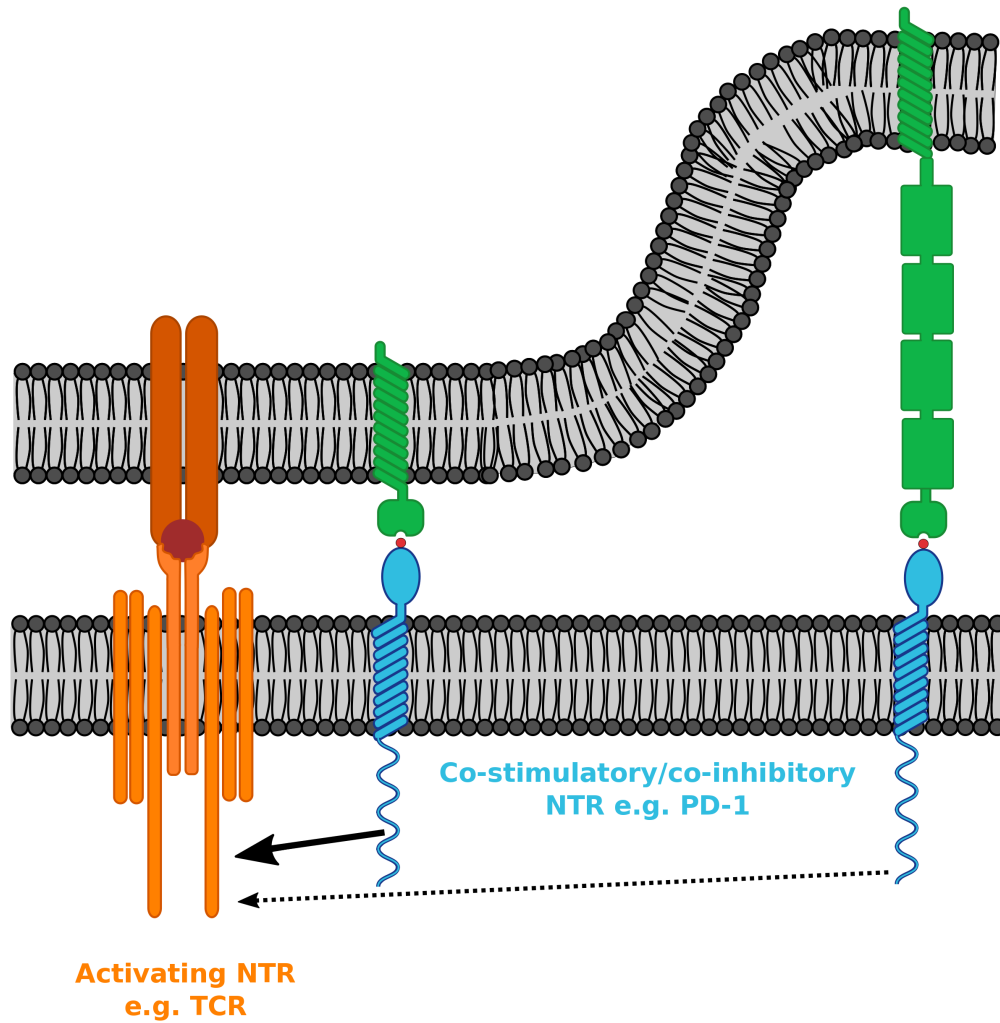
integration by NTRs (Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2012)). It has been suggested that the intracellular tails of NTRs are mostly unstructured (Dushek *et al.* (2012)) and we expect the position of the motifs within the tails of NTRs to affect the reach of the intracellular tail and any recruited molecules bound to it (Figure 1.4).

As discussed by Riley, many related inhibitory NTRs possess not only amino acid sequence similarity but also strikingly similar positioning of signalling motifs within the intracellular tail (Riley (2009)). It is also interesting to note that the conserved tyrosine residues found in ITIM- and ITSM-containing NTRs are on average more than 20 amino acids further from the plasma membrane than those in ITAMs (Dr Jesse Goyette, unpublished). These observations raise the question - is there a functional relevance of long intracellular tails for signal integration by inhibitory NTRs? This question is explored in relation to the inhibitory receptor PD-1 in Chapter 5 of the current work.

1.7.3 Aims

Due to the significant number of receptors that have been classified as NTRs and the need for a systematic approach for analysing them, the first aim of this study is to develop a generic cellular two-ligand system for analysis of signal integration by NTRs (Chapter 3). The subsequent aims are to utilise the two-ligand system to investigate the importance of NTR-ligand dimensions (Chapter 4) and intracellular tail length (Chapter 5) for NTR signal integration.

Ligand-presenting cell



Receptor cell

Figure 1.3: Schematic representation of “hypothesis 1”, that optimal signal integration by co-stimulatory or co-inhibitory NTRs (blue) acting on an activating NTR (orange) requires close co-localisation of the NTRs, which is dependent on well-matched NTR-ligand dimensions. The requirement for close co-localisation follows from the fact that NTRs are known to recruit effector molecules such as cytoplasmic phosphatases that have to be able to “reach” their targets while remaining attached to the receptor. Optimal signal integration is represented by a bold arrow; sub-optimal signal integration is represented by a dashed arrow.

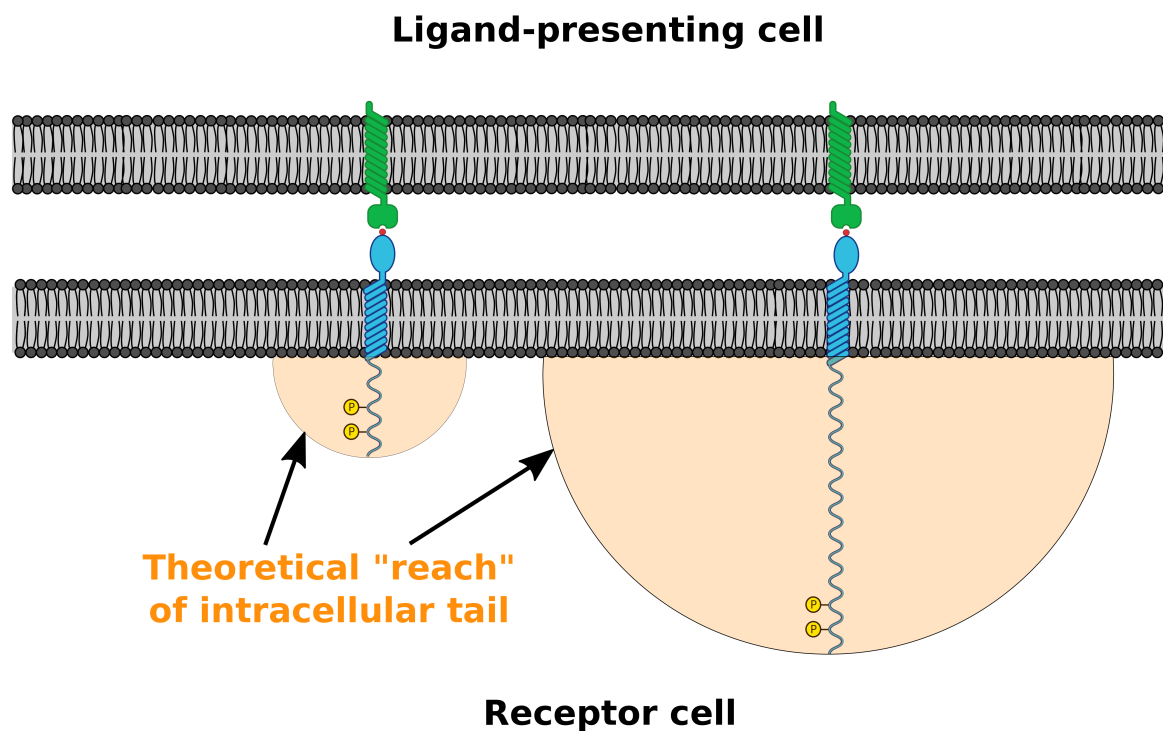


Figure 1.4: Schematic representation of “hypothesis 2”, showing the theoretical reach (orange semi-circles) of ligand-bound NTRs with unstructured intracellular tails of different lengths, to which they recruit cytoplasmic molecules to carry out membrane proximal signalling. We hypothesise that optimal signal integration by inhibitory NTRs is dependent on the length of their intracellular tail, with signalling motifs further from the plasma membrane enabling inhibition throughout a greater volume.

Materials and Methods

2.1 Materials

The experiments performed during the current study were carried out using the materials detailed in Table 2.1 and the antibodies listed in Table 2.2.

2.2 Cloning of ligand constructs

2.2.1 Avi tag approach

For the initial generic ligand approach, a pcDNA3.1(+)-CD80-Avi vector was used. This consisted of an Ig κ signal sequence, an Avi tag, and an HA tag followed by the stalk, transmembrane and intracellular regions of mouse CD80. pcDNA3.1(+)-CD80-Avi was used along with a pDisplay vector encoding the BirA biotin-protein ligase with endoplasmic reticulum (ER) retention signal sequence (pDisplay-BirA-ER) for biotinylation of the Avi tag. These vectors were both supplied by Dr Eleanor Denham and were cloned as described in Denham (2016).

Item	Supplier	Catalogue number
0.22 μm filter	Millex [®]	SLGP033RS
0.45 μm filter	Sartorius Stedim Biotech	16555-K
1.5 mL eppendorf	TreffLab	96.07246.9.01
15 mL falcon tube	Greiner Bio-one	188271
16% formaldehyde solution (w/v), methanol-free	Thermo Scientific	28906
2-(N-morpholino)ethanesulfonic acid (MES)	Sigma Aldrich	M8250-1KG
5 mL polypropylene round-bottom falcon tube (with cap)	Falcon/Corning	352063
5 mL polystyrene round-bottom tube	Falcon/Corning	352052
50 mg/mL ampicillin	Sigma Aldrich	A9393-25G
50 mg/mL gentamicin	Sigma	G1272-10ml
50 mL falcon tube	Falcon/Corning	352070
96-well plate, flat-bottomed	CoStar	3596
96-well plate, Nunc-Immuno Maxisorp X60 ELISA plate	Thermo Scientific	442404
96-well plate, Nunc-Immuno MaxiSorp X60 sterile	Thermo Scientific	456537
96-well plate, round-bottomed	CoStar	3799
96-well plate, v-bottomed	CoStar	3894
Amaza Cell line Nucleofector [™] kit	Lonza	VACA-1003
Avidin	Sigma Aldrich	A9275
Biotin-ATTO 647	ATTO-TEC GmbH	AD 647-71
Bovine serum albumin (BSA)	Sigma Aldrich	A7906-500G
Centrifuge	Beckman Coulter	Allegra X-12R
D-biotin	Avidity	BIO-200
Dialysed fetal calf serum (FCS)	Gibco (LT)	26400-044
Dulbecco's Modified Eagle Medium (DMEM)	Sigma	D6429-500ML
Dimethyl sulfoxide (DMSO) for polymerase chain reaction (PCR)	Agilent	600260-53

DMSO for tissue culture	Sigma	D2650
Dynabeads M-280 sheep anti-mouse IgG	Invitrogen Dynal AS	M-280 112.01D
Dynabeads M-280 streptavidin	Invitrogen Dynal AS	M-280 112.05D
FCS	Gibco (LT)	10500-064
Geneticin [®] G418	Gibco (LT)	10131-027
Glutamine-free DMEM	Sigma	D6546-500ML
GSEM supplement	Sigma	G9785
Human IL-8 ELISA Ready-SET-Go! 2nd Generation kit	eBioscience	88-8086-77
Hygromycin B	Invitrogen	10687-010
L-Methionine sulfoximine (MSX)	Sigma	M5379
Magnetic rack	Dynal	MPC-L
Nucleocounter	Chemometec	NC-3000
Nunc [™] Cryotube [™] vials	Thermo Scientific	375418
Phosphate-buffered saline, 1X (PBS)	Lonza BioWhittaker [®]	BE17-516F
Penicillin streptomycin (P/S)	Gibco (LT)	15140-122
Petri dish	Corning	430167
Phosphoric acid	Sigma Aldrich	79622-500G
Plate shaker	Dynatech	AM69
Plate washer	BioTeK Instruments	ELx405
Quick Ligation [™] kit	NEB	M2200S
Roswell Park Memorial Institute medium (RPMI) 1640	Sigma	R8758-500ML
Solution 13 AO/DAPI dye	Chemometec	910-3013
Streptavidin:RPE	SeroTec	STAR4B
TrypLE [™] Express (trypsin)	Gibco (LT)	12604-013
Tween [®] 20	Sigma Aldrich	P2287-500ML
Vitamin D (1-alpha,25-Dihydroxyvitamin D3)	Santa Cruz Biotechnology Inc	sc-202877

Table 2.1: Details of the materials and equipment used for the current study (continued from previous page).

Antibody	Clone	Species and isotype	Supplier	Catalogue number
anti-human CD3	UCHT1	Mouse IgG1	TONBO biosciences	70-0038
anti-human CD45-Alexa FLUOR 647 [®]	F10-89-4	Mouse IgG2a	BIO-RAD	MCA87A647
anti-human CD64 (anti-FCGR1A)	10.1	Mouse IgG1	Thermo Scientific	MA1-10270
anti-HA-Alexa FLUOR [®] 647	6E2	Mouse IgG1	Cell Signaling Technology [®]	3444S
anti-human HLA-A,-B,C	W6/32	Mouse IgG2a	Provided by Dr Marion H Brown	N/A
anti-human HLA-A2-FITC	BB7.2	Mouse IgG2b	Santa Cruz Biotechnology	sc-32236
anti-human PD-1 (anti-CD279)-Alexa FLUOR 647 [®]	EH12.1	Mouse IgG1	BD Pharmingen [™]	560838
anti-human transferrin receptor (anti-CD71)	MG2a-53	Mouse IgG2a	BioLegend	334102
anti-mouse 2B4 (anti-CD244.2) with or without FITC	m2B4 (B6)458.1	Mouse IgG1	BioLegend	133501/133503 (FITC)
anti-mouse CD48-FITC	OX78	Rat IgG2a	Santa Cruz Biotechnology	sc-53053
anti-mouse IgG1 isotype control (LEAF [™] Purified)	MG1-45	Mouse IgG1	BioLegend	401405
anti-mouse IgG1 isotype control-Alexa FLUOR [®] 647	N/A	Goat polyclonal	Thermo Fisher Scientific	A-21240
anti-Strep-tag II (StrepMAB Classic)		Mouse IgG1	iba	2-1507-001

Table 2.2: *Details of the antibodies used for the current study.*

2.2.2 SpyTag approach

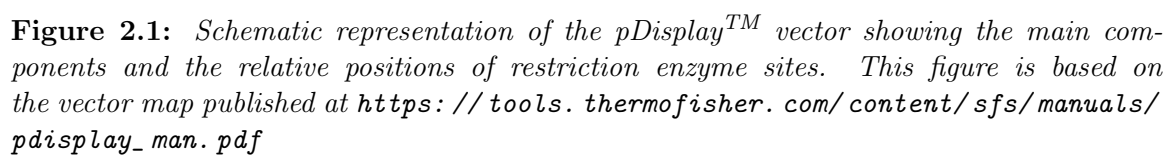
For the SpyTag approach, a construct encoding the the SpyTag peptide (sequence AHIVMVDAYKPTK) followed by the short extracellular stalk, transmembrane and cytoplasmic regions of mouse CD80 in the pEE14 vector (Figure 2.2) was provided by Dr Jesse Goyette (Denham (2016)).

Elongated ligand constructs were generated by inserting Ig domains from CD2 or CD4. To prevent binding to CD58 and MHC class II, mutations were made to the CD2 and CD4 Ig domains, respectively, as detailed in Piatier-Tonneau *et al.* (1991) and van der Merwe *et al.* (1995). These mutations, namely K43E in CD2 and R54D in CD4, were made using primers 1 - 4 (Table 2.3). The mutated CD2 and CD4 Ig domains were then inserted into the ligand constructs using primers 5 - 8 (Table 2.3).

Glycosylphosphatidylinositol (GPI)-anchored mouse CD48 and CD48-CD4 constructs in the pEE14 vector were provided by Dr Jesse Goyette and were cloned into the pDisplayTM vector (Figure 2.1) using primers 9 and 10 (Table 2.3). Mutations to the CD4 were once again made using primers 3 and 4. SCD constructs in the pDisplayTM vector were provided by Dr Jesse Goyette and Dr Ben de Wet.

Primer number	Primer name	Sequence (5' to 3')
1	hCD2_K43E_F	gggaaaaaacttcagacaagaa g agattgcacaattcagaaaagag
2	hCD2_K43E_R	ctcttttctgaattgtgcaatct ct tttctgtctgaagtttttccc
3	hCD4_R54D_F	ggtccatccaagctgaatgat gac gctgactcaagaagaagcc
4	hCD4_R54D_R	ggcttcttcttgagtcagc gtc atcattcagcttggatggacc
5	hCD2_BamHI_F	ttattaGGATCCgagattacgaatgccttggaaacc
6	hCD2_BamHI_R	attattGGATCCtttctctggacagctgacagg
7	hCD4_BamHI_F	ttactcGGATCCaaagtggactgggcaaaaaag
8	hCD4_BglII_R	ttattaAGATCTtgtgggcagaaccttgatgtttgattccagca
9	mCD48_HindIII_F	ttattaAAGCTTctaagtattatgtgcttcat
10	mCD48_XhoI_R	ttattaCTCGAGtcagggttaacaagatcctgtgaatg

Table 2.3: Primers used for cloning ligand constructs for expression in CHO.K1 ligand cells. Restriction enzyme sites are shown in capital letters and introduced mutations are shown in bold text.



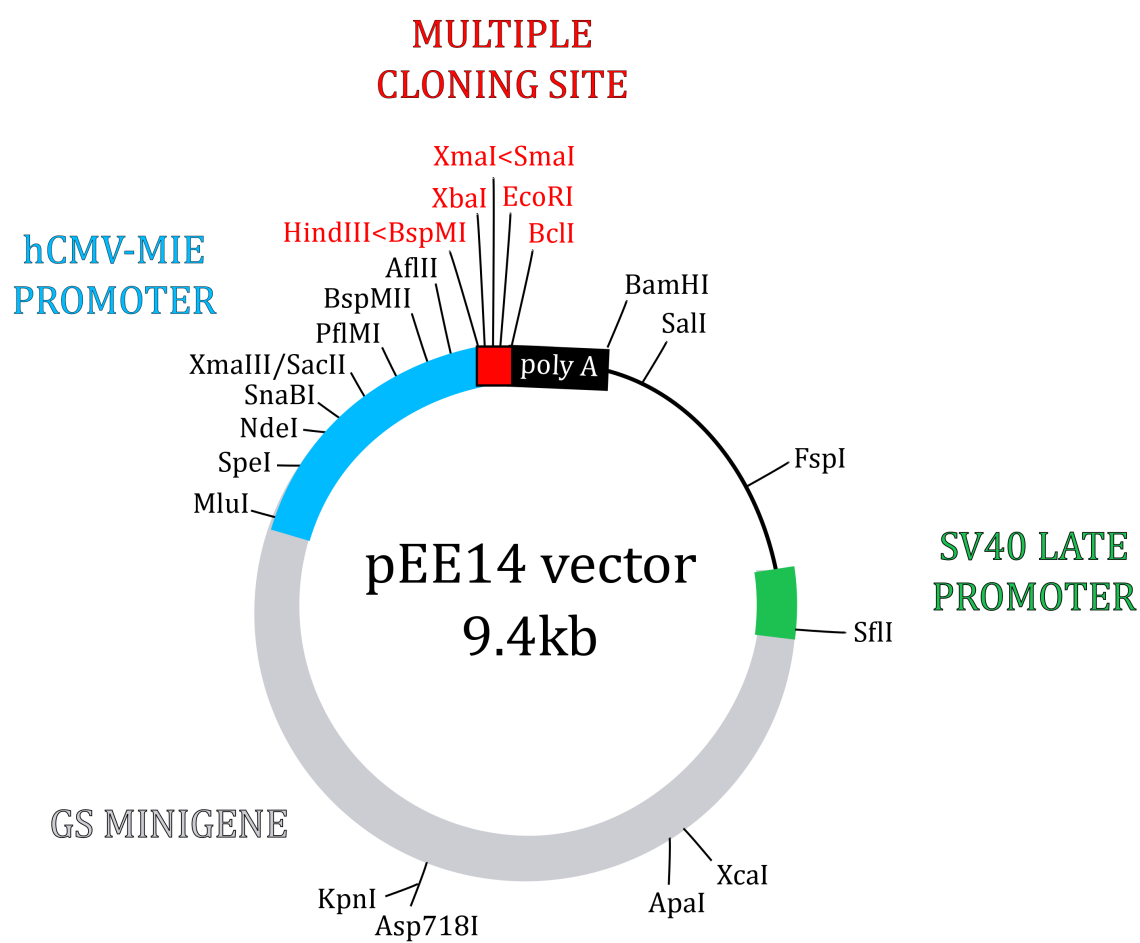


Figure 2.2: Schematic representation of the pEE14 vector showing the main components and relative positions of restriction enzyme sites. This figure is based on a vector map provided by Dr Marion H Brown.

2.3 CHO.K1 cell culture

CHO.K1 cells were cultured in DMEM + 10% FCS + 1X P/S at 37 °C with 10% CO₂. Cells were passaged every 2 - 3 days by removing media, washing with sterile PBS and then using TrypLE™ Express to harvest the cells. Cell stocks were frozen down at -80 °C in 1 mL FCS + 10% DMSO in cryotubes using a NALGENE cryo freezing container. To revive frozen stocks, media was pre-warmed in the incubator before thawing the cryotube and transferring the thawed cells to the flask of pre-warmed media.

CHO.K1 cells expressing constructs in pcDNA3.1(+) and pDisplay vectors were selected using 100 µg/mL hygromycin or 1 mg/mL G418 respectively. For cells expressing constructs in the pEE14 vector, selection was performed using MSX media made up of glutamine-free DMEM, 1X GS supplement, 10% dialysed FCS and 25 µM MSX (referred to as “MSX media”). CHO.K1 cells expressing constructs in both pEE14 and pDisplay vectors were selected using a combination of 25 µM MSX plus 1 mg/mL G418 in MSX media.

2.4 Transfection of CHO.K1 cells with ligand constructs

The different methods used for transfection of the CHO.K1 cells with ligand constructs are described below.

2.4.1 FuGENE X-tremeGENE™ 9

Cells were passaged 1 - 2 days prior to transfection to be 50 - 80% confluent at the time of transfection. For transfecting cells in 6-well plates, 97 µL serum-free DMEM was pipetted into a 1.5 mL eppendorf, followed by 3 µL of FuGENE X-tremeGENE™ 9 reagent directly into the media without touching the walls of the eppendorf. The eppendorf was flicked to mix and incubated at room temperature (RT) for 5'. The relevant DNA (1 µg) was then added and the eppendorf was once again flicked to mix before incubating for at least 15' at RT. The FuGENE-DNA-DMEM mixture was added to the cells dropwise before swirling to mix and returning the cells to the incubator at 37 °C and 10% CO₂. Approximately 1 day post-transfection the cells were passaged to 30 - 50% confluency in larger flasks. Approximately 2 days post-transfection relevant selection agents were added.

In the case of co-transfection with two DNA constructs the amount of all reagents was doubled with 1 μg of each DNA construct added. For transfection of cells in T25 flasks, 291 μL of serum-free DMEM plus 9 μL of FuGENE reagent and 3 μg of DNA was used (or double each of these for co-transfections with 3 μg of each DNA construct in T25 flasks). For T75 flasks, 1 mL of serum-free DMEM plus 30 μL of FuGENE reagent and 10 μg of DNA was used.

2.4.2 Polyethylenimine (PEI)

Cells were passaged 1 - 2 days prior to transfection to be 50 - 80% confluent at the time of transfection. Serum-free DMEM was warmed to 37 °C. The serum-containing DMEM was removed from the flasks of cells to be transfected and any remaining media was gently washed off with PBS. The pre-warmed serum-free DMEM was then added to the cells which were returned to the incubator at 37 °C and 10% CO₂.

Serum-free DMEM (50 μL) was pipetted into two eppendorfs per sample (one eppendorf for DNA, one eppendorf for PEI). DNA was added to one eppendorf to obtain a final concentration of 2 $\mu\text{g}/\text{mL}$ in culture (e.g. 14 μg DNA for a T25 flask with 7 mL of media) and mixed thoroughly. PEI was added to the other eppendorf to obtain a final concentration of 4 $\mu\text{g}/\text{mL}$ in culture (e.g. 28 μg PEI for a T25 flask with 7 mL of media). The DNA and PEI mixtures were then combined, pipetting up and down thoroughly to mix. After incubating for 20' at RT, the mixture was pipetted onto the cells and swirled to mix.

The cells were incubated for 4h at 37 °C and 10% CO₂ before replacing the media with serum-containing media and incubating overnight at 37 °C and 10% CO₂. Approximately 1 day post-transfection the cells were passaged to 30 - 50% confluency in larger flasks. Approximately 2 days post-transfection relevant selection agents were added.

2.4.3 Amaxa

Cells were passaged 1 - 2 days prior to transfection to be 80 - 90% confluent at the time of transfection. Plates were prepared by filling the appropriate number of wells in 6-well plates with 1 mL of DMEM + 10% FCS + 1X P/S and pre-incubating at 37 °C and 10% CO₂. Cells were harvested by trypsination and counted using Solution 13 AO/DAPI dye with a Nucleocounter cell counter. Enough cells to give 1×10^6 cells per sample were centrifuged at $200 \times g$ for 10' at RT and the supernatant was discarded.

The following steps were performed promptly to avoid extended suspension of the cells in the Nucleofector Solution as this may reduce cell viability and gene transfer efficiency: 82 μL Nucleofector Solution T plus 18 μL supplement was mixed per sample and used to carefully resuspend the cell pellet. DNA (2 μg) was added to the cell suspension and the DNA/cell suspension was transferred into an Amaxa cuvette, closing the cuvette with the cap. Nucleofector program H-014 for high viability or U-023 for high expression level was selected on the Nucleofector I Device. The cuvette containing the cell/DNA suspension was inserted into the Nucleofector Cuvette Holder and the selected program was applied.

Once the program was finished the cuvette was removed and approximately 500 μL of the pre-incubated DMEM was immediately added to the cuvette using one of the supplied pipettes. The sample was gently transferred into the prepared 6-well plate avoiding repeated aspiration of the sample. The cells were then returned to the incubator at 37 °C and 10% CO_2 . Approximately 1 day post-transfection the cells were passaged to 30 - 50% confluency in larger flasks. Approximately 2 days post-transfection relevant selection agents were added.

2.5 Flow cytometry of ligand cell lines to assess expression levels

All flow cytometry washing and resuspension steps were performed using PBS + 0.1% BSA + 0.05% azide (PBA) unless otherwise specified.

Cells were harvested using TrypLE™ Express and resuspended in serum-containing media to neutralise the trypsin. Cells were pipetted into flow tubes, centrifuged for 5' at $524 \times g$ and 4 °C and then the supernatant was discarded. The cells were resuspended in 1 mL PBA to wash before repeating the centrifugation. After discarding the supernatant, cells were stained for approximately 15' at RT in 25 μL of the relevant antibody diluted to 10 $\mu\text{g}/\text{mL}$ in PBA unless otherwise stated.

Washing and centrifugation steps were repeated 3 times and, if required, staining was repeated with secondary antibody followed by another 3 rounds of washing and centrifugation. Cells were fixed in 0.3 - 0.5 mL of PBS + 1% PFA before analysis on a FACScan™ Cytex 8 flow cytometer.

2.6 Ligand cell sorting

To obtain CHO.K1 ligand cells with high and well-matched expression of the ligand constructs, sorting was performed using antibody-coated dynabeads and/or fluorescence-activated cell sorting (FACS). Cells were harvested using TrypLE™ Express and resuspended in serum-containing media to neutralise the trypsin. All steps were performed in sterile conditions.

2.6.1 Dynabeads

Sheep anti-mouse IgG dynabeads (100 μ g per sample) were added to 1 mL PBS in a 5 mL polypropylene round-bottom falcon tube (with cap). A Dynal magnetic rack was used to hold the beads at the back of the tube while pipetting off the solution. The beads were resuspended in PBS plus an excess of antibody (e.g. anti-CD48 (OX78) or anti-HLA-A,-B-C (W6/32); Table 2.2) and incubated at RT for approximately 15'. The magnetic rack was used to hold the beads at the back of the tube while pipetting off the solution. The tube was removed from the magnetic rack and the beads were washed by resuspending with 1 mL PBS. This wash step was repeated 3 times before resuspending in a total volume of PBS to give 500 μ L per sample. The antibody-coated dynabeads were aliquoted out into separate 5 mL polypropylene round-bottom falcon tubes (with caps; 500 μ L per sample) and then the relevant cells were added. The cells were incubated with the antibody-coated dynabeads for approximately 30' at RT before using the magnetic rack to wash and resuspend the cells in PBS 3 - 4 times as done previously. The remaining cells were resuspended in 1 mL of DMEM + 10% FCS + 1X P/S and added to a pre-warmed 6-well plate at 37 °C with 10% CO₂.

2.6.2 FACS

Cells were harvested and then pipetted into 5 mL polypropylene round-bottom falcon tubes (with caps), centrifuged for 5' at $524 \times g$ and 4 °C and then the supernatant was discarded. The cells were resuspended in 1 mL PBS + 10% FCS to wash before repeating the centrifugation. The desired antibody was diluted to 10 μ g/mL in PBS and filtered using a 0.22 μ m filter. After discarding the supernatant, cells were stained for approximately 15' at RT in approximately 100 μ L of the relevant antibody e.g. anti-HA-Alexa FLUOR® 647 against the SpyTag construct, anti-CD48-FITC, or anti-HLA-A2-FITC antibodies as appropriate (Table 2.2).

Washing and centrifugation steps were repeated 3 times and, if necessary, staining was repeated with filtered secondary antibody followed by another 3 rounds of washing and centrifugation. Cells were then resuspended in a volume of PBS to provide a suitable density of cells for FACS (usually around 0.5 - 1 mL). Cells were kept on ice and sorted by Nigel Rust at the Sir William Dunn School of Pathology flow cytometry facility using a Beckman Coulter MoFlo[®] Astrios[™].

The sorted cells were collected in 1 mL filtered FCS in 5 mL polypropylene round-bottom falcon tubes (with caps). Once the sorting was complete the cells were resuspended in pre-warmed DMEM + 10% FCS in 6-well plates at 37 °C with 10% CO₂.

2.7 Cloning of receptor constructs

Lentiviral vectors (pHR-SIN-BX) encoding activating NTR constructs were gifts from Dr Andre Cohnen and Professor Oreste Acuto (1G4 TCR) and Dr Jesse Goyette (mouse 2B4-human CD64, abbreviated as 2B4-CD64). The 1G4 TCR was cloned with the alpha and beta chains separated by a 2A peptide sequence in the order β -2A- α (Dr Wolfgang Paster, personal communication). Further details of these constructs are shown in Table 3.1.

Human PD-1 constructs were generated from an I.M.A.G.E. clone (Clone ID 30915198; GE Healthcare). A control PD-1 construct without the Strep-tag II tag was cloned into the pHR-SIN-BX lentiviral vector, originally a gift from Professor Oreste Acuto, using primers 11 and 12 with 5' BamHI and 3' NotI restriction sites (Table 2.4). This construct is referred to as PD-1 control.

Primers 12 and 13 with 5' BamHI and 3' NotI sites were used to clone PD-1 into the same vector but with an N-terminal Strep-tag II cloned in by Dr Eleanor Denham (Denham (2016)), generating a PD-1 construct with N-terminal Strep-tag II (WSHPQFEK). This construct, referred to as PD-1 Strep-tag II, was then used for generation of all other Strep-tag II-tagged PD-1 constructs (Table 5.1). The vector contains an Ig κ signal peptide, with the Strep-tag II tag preceded by serine and alanine residues (SA) and followed by a (GS)₃ linker (Denham (2016)). Primers 14 and 15 were used to introduce a silent AgeI mutation at PD-1 amino acid position 207, on the membrane proximal side of the ITIM, to facilitate truncation and elongation of the intracellular tail.

This PD-1 construct was cloned into the pPCR-Script vector using 5' BamHI and 3' NotI restriction sites. Generation of all of the other PD-1 constructs was

then performed using the pPCR-Script vector. Firstly, Y223F and Y248F mutations were made to the ITIM and/or ITSM respectively using primers 16 - 19 (Table 2.4). This resulted in the PD-1 ITIM mutant, PD-1 ITSM mutant and PD-1 ITIM/ITSM mutant constructs. These mutants were then elongated by inserting one or two copies of the PD-1 ITIM/ITSM mutant intracellular tail generated from PCR of the PD-1 ITIM/ITSM mutant using primers 14 and 20, and inserting the PCR product into the AgeI site of the various PD-1 mutant constructs (PD-1 ITIM mutant, PD-1 ITSM mutant and PD-1 ITIM/ITSM mutant constructs). This approach was used to generate all of the elongated (elongated x1 and elongated x2) PD-1 constructs.

Truncated PD-1 constructs were generated by performing a PCR of the intracellular tail from two amino acids 5' of the ITSM onwards using primers 12 and 20 (Table 2.4), and then inserting the PCR product into the PD-1 Strep-tag II vector digested with AgeI and NotI restriction enzymes. This approach was used to generate both the PD-1 truncated and PD-1 truncated ITSM mutant constructs by using either the PD-1 WT or PD-1 ITSM mutant constructs respectively as the template DNA for the PCR.

Once all of these constructs were generated in pPCR-Script they were cloned into the pHR-SIN-BX lentiviral vector using 5' BamHI and 3' NotI restriction sites and the DNA sequences were confirmed by bi-directional Sanger sequencing. Further details of the mutated PD-1 constructs are set out in Table 5.1 and the full set of PD-1 variants, including PD-1 control and PD-1 Strep-tag II, are represented in Figure 5.1.

Primer number	Primer Name	Sequence (5' to 3')
11	hPD-1_Kozak_BamHI_F	ttattaGGATCCctgctccaggcatgcagatcc
12	hPD-1_NotI_R	attattGCGGCCGCtcagagggccaagagcagtg
13	hPD-1_BamHI_F	ttattaGGATCCccaggatggttcttagactcc
14	hPD-1_AgeI_F	ccaggcgcACCGGTcagcccctgaag
15	hPD-1_AgeI_R	cttcaggggctg ACCGGT gcgcctgg
16	hPD-1_Y223F_F	gtggact tt ggggagctg
17	hPD-1_Y223F_R	cagctcccca a agtccac
18	hPD-1_Y248F_F	cagacggagt tt gccaccattg
19	hPD-1_Y248F_R	caatggtggca a actccgtctg
20	hPD-1_XmaI_No STOP_R	attattCCCGGGgagggccaagagcagtgtc
21	hPD-1_AgeI_ITSM_F	ttataaCCCGGTgagcagacggagtatgccacca

Table 2.4: *Primers used for cloning PD-1 receptor constructs for expression in THP-1 and Jurkat receptor cells. Restriction enzyme sites are shown in capitals and introduced mutations are shown in bold text.*

2.8 THP-1 and Jurkat cell culture

A Jurkat JE6.1 reporter cell line expressing a nuclear factor κ -light-chain-enhancer of activated B cells-enhanced green fluorescent protein (NF- κ B-eGFP) reporter (Ratzinger et al. Scientific Reports 2014) and subsequently lentivirally transduced with the 1G4 TCR described above plus CD8 co-receptor (pHR-SIN-BX-CD8- β -IRES- α ; Dr Wolfgang Paster, personal communication) was a gift from Sabrina Jutz, Dr Wolfgang Paster, and Professor Peter Steinberger. THP-1 cells were provided by Dr Eleanor Denham.

All Jurkat and THP-1 cells were cultured in RPMI + 10% FCS + 1X P/S at 37 °C with 5% CO₂. Cells were passaged every 2 - 3 days to maintain cell density of approximately 0.2 - 1 × 10⁶ cells/mL. Cell stocks were frozen down at -80 °C in 1 mL FCS + 10% DMSO in cryotubes using a NALGENE cryo freezing container. To revive frozen stocks, media was pre-warmed in the incubator before thawing the cryotube and transferring the thawed cells to the flask of pre-warmed media.

For differentiation of THP-1 cells, the cells were passaged and resuspended at 0.2 × 10⁶ cells/mL in RPMI + 100 nM vitamin D. The cells were cultured in this media for 3 days prior to stimulation. Petri dishes were used for culturing the THP-1 cells during differentiation to minimise the adhesion that occurs when differentiating the cells in standard culture flasks.

2.9 Lentiviral transduction of cell lines with receptor constructs

2.9.1 FuGENE X-tremeGENE™ 9

Jurkat cells were lentivirally transduced using virus particles packaged in HEK 293T cells transfected with FuGENE X-tremeGENE™ 9. To package the lentiviral particles, HEK 293T cells were transfected with packaging and pHR-SIN-BX constructs using FuGENE X-tremeGENE™ 9. Briefly, HEK 293T cells were cultured for 1 week in RPMI and the day before the transfection they were passaged into RPMI plus 10% FCS, 1X P/S and 10 mM HEPES to be 50 - 80% confluent the next day. Serum-free and antibiotic-free RPMI + 10 mM HEPES was warmed to 37 °C and the FuGENE transfection reagent was brought to RT.

For transfecting HEK 293T cells in a T25 flask, 0.5 mL RPMI plus 10 mM HEPES was added to a 1.5 mL eppendorf tube and 29 μ L of FuGENE transfection reagent

was slowly added. The tube was inverted four times to mix and then incubated at RT for 20'. During this time the DNA was prepared as per Table 2.5. The pRSV.REV, pGAG and pVSV-G were mixed first followed by the pHR-SIN-BX vector of interest.

Component	Amount required (μg)
pRSV.REV	3
pMDLg/pRRE (pGAG)	3
pVSV-G	1.17
pHR-SIN-BX vector of interest	2.5

Table 2.5: DNA required for transfection of a T25 flask of HEK 293T cells for packaging of lentiviral particles.

The RPMI/transfection reagent mixture (0.4 mL) was slowly added into the DNA mixture and pipetted to mix before transferring it all back into the remaining RPMI/transfection mix. The eppendorf was inverted four times and incubated at RT for 30'. The media was carefully removed from the flask of HEK 293T cells and the RPMI/FuGENE/DNA mixture was added to the side. The flask was tilted to cover the cells with the transfection mixture.

After 1' at RT a further 9.5 mL of RPMI plus 10% FCS, 1X P/S and 10 mM HEPES was added to the side of the flask and tilted to cover the cells. The cells were incubated overnight at 37 °C and 5% CO₂ before transferring the culture supernatant to a 15 mL falcon tube and centrifuging at $456 \times g$ for 6' at 4 °C to pellet the cell debris. The supernatant was transferred to a new 15 mL falcon tube and the cell debris was discarded. The virus-containing supernatant was kept on ice for the rest of the procedure.

Polybrene (10 μL of 5 mg/mL stock) was added to the 10 mL of virus-containing supernatant, which was then filtered through a 0.22 μm filter. Enough Jurkat cells for 3×10^6 cells per sample were centrifuged in a 50 mL falcon at $524 \times g$ for 5'. The cells were resuspended in 3 mL of the virus-containing supernatant and transferred to a 6-well plate for lentiviral transduction. The cells were centrifuged for 2h at $2095 \times g$ at 32 °C before incubating at 32 °C and 8% CO₂ for 6h then returning to 37 °C and 5% CO₂. Expression of the relevant proteins was analysed by flow cytometry approximately 3 days post-transduction.

2.9.2 PEI

THP-1 cells were lentivirally transduced using virus particles packaged in HEK 293T cells transfected using a PEI-based approach. To package the lentiviral particles, HEK 293T cells were transfected with packaging and pHR-SIN-BX constructs using PEI. Briefly, HEK 293T cells were passaged 1 - 2 days prior to transfection to be 50 - 80% confluent at the time of transfection. Serum-free RPMI was warmed to 37 °C. The serum-containing RPMI was removed from the cells to be transfected and replaced with pre-warmed serum-free RPMI.

DNA was diluted in 400 μ L of serum-free RPMI in an eppendorf according to Table 2.6, with pRSV, pGAG and pVSV-G mixed together first before adding pHR-SIN-BX DNA. PEI was diluted in another 400 μ L of serum-free RPMI according to Table 2.6 before mixing the DNA and PEI solutions together and pipetting up and down vigorously for 30 - 60".

Flask size	6-well	T25	T75	T175
Volume of media (mL)	3	7	20	40
pRSV (μ g)	0.95	2.5	7.5	17.5
pMDLg/pRRE (pGAG; μ g)	0.95	2.5	7.5	17.5
pVSV-G (μ g)	0.37	0.97	2.9	6.8
pHR-SIN-BX (μ g)	0.8	2.1	6.3	14.7
Total DNA (μ g)	3	8	24	56
PEI (μ g)	6	16	48	112

Table 2.6: *DNA and PEI required for lentiviral transduction in different volumes.*

The DNA-PEI mixture was incubated at RT for approximately 20' before adding it to the HEK 293T cells and incubating at 37 °C and 5% CO₂. After 4 - 5h the media was replaced with complete RPMI. The supernatant was harvested 24 - 48h later, 5 μ g/mL polybrene was added and it was filtered using a 0.45 μ m filter. Virus-containing supernatant (3 - 5 mL per well) was added to a 6-well plate and 2×10^6 THP-1 cells/well were added for lentiviral transduction.

The plate was centrifuged at $2095 \times g$ for 90' then incubated at 32 °C and 8% CO₂ for 4h before returning to 37 °C and 5% CO₂. Expression of the relevant proteins was analysed by flow cytometry approximately 3 days post-transduction.

2.10 Tetramer production

Fluorescent streptavidin tetramers of biotinylated HLA-A2 refolded with the cancer-testis antigen NY-ESO-1 C9V peptide (peptide sequence SLLMWITQV) were used for staining and subsequent flow cytometry analysis of the 1G4 TCR throughout the current study. These tetramers were produced by transferring 66.6 μg of biotinylated HLA-A2-C9V monomers to a 1.5 mL eppendorf and adding 10 μL of PE-streptavidin every 10', ten times (100 μL total), shaking at RT for the duration of the process. The eppendorf was covered in aluminium foil and stored at 4 °C.

2.11 Flow cytometry of receptor cell lines to assess expression levels

All flow cytometry washing and resuspension steps were performed using PBA unless otherwise specified. For analysis of THP-1 cells, the cells were incubated with PBA + 10% human serum for 30' prior to staining to block Fc receptor binding sites. Subsequent washing steps were also carried out with PBA + 10% human serum.

For flow cytometry of THP-1 and Jurkat cells, the cells of interest were transferred into flow tubes, centrifuged for 5' at $524 \times g$ and 4 °C and then the supernatant was discarded. The cells were resuspended in 1 mL PBA to wash before repeating the centrifugation. After discarding the supernatant, cells were stained for approximately 15' at RT in 25 μL of the relevant antibody diluted to 10 $\mu\text{g}/\text{mL}$ in PBA unless otherwise stated. For staining of the 1G4 TCR, cells were stained with undiluted PE-HLA-A2-C9V tetramer, which binds to the 1G4 TCR and was produced as per Section 2.10.

Washing and centrifugation steps were repeated 3 times and, if necessary, staining was repeated with secondary antibody followed by another 3 rounds of washing and centrifugation. Cells were then fixed in 0.3 - 0.5 mL of PBS + 1% PFA before analysis on a FACScanTM Cytex 8 flow cytometer.

2.12 Receptor cell sorting

To obtain THP-1 receptor cells with high and well-matched expression levels of the receptors, sorting was performed using antibody-coated dynabeads and/or FACS. To obtain Jurkat receptor cells with high and well-matched expression levels of the

receptors, sorting was performed using FACS. All steps were performed in sterile conditions.

2.12.1 Dynabeads

Sheep anti-mouse IgG dynabeads (100 μ g per sample) were added to 1 mL PBS in a 5 mL polypropylene round-bottom falcon tube (with cap). A magnetic rack was used to hold the beads at the back of the tube while pipetting off the solution. The beads were resuspended in PBS plus an excess of antibody (e.g. anti-Strep-tag II or anti-2B4; Table 2.2) and incubated at RT for approximately 15'. The magnetic rack was used to hold the beads at the back of the tube while pipetting off the solution. The tube was removed from the magnetic rack and the beads were washed by resuspending with 1 mL PBS. This wash step was repeated 3 times before resuspending in a total volume of PBS to give 500 μ L per sample. The bead-antibody solution was aliquoted out into separate tubes (500 μ L per sample) and then the relevant cells were added. The cells were incubated with the beads for approximately 30' at RT before using the magnetic rack to wash and resuspend the cells in PBS 3 - 4 times as done previously. The remaining cells were resuspended in 1 mL of DMEM + 10% FCS 1X P/S and added to a pre-warmed 6-well plate at 37 °C with 10% CO₂.

2.12.2 FACS

Cells were pipetted into 5 mL polypropylene round-bottom falcon tubes (with caps), centrifuged for 5' at $524 \times g$ and 4 °C and then the supernatant was discarded. The cells were resuspended in 1 mL PBS + 10% FCS to wash before repeating the centrifugation. The desired antibody (e.g. anti-2B4-FITC or anti-PD-1-Alexa 647; Table 2.2) was diluted to 10 μ g/mL in PBS and filtered using a 0.22 μ m filter. After discarding the supernatant, cells were stained for approximately 15' at RT in approximately 100 μ L of the relevant antibody unless otherwise stated.

Washing and centrifugation steps were repeated 3 times and, if necessary, staining was repeated with filtered secondary antibody followed by another 3 rounds of washing and centrifugation. Cells were then resuspended in a volume of PBS to provide a suitable density of cells for FACS - usually around 0.5 - 1 mL. Cells were kept on ice and sorted by Nigel Rust at the Sir William Dunn School of Pathology flow cytometry facility using a Beckman Coulter MoFlo® Astrios™.

The sorted cells were collected in 1 mL filtered FCS in 5 mL polypropylene round-bottom falcon tubes (with caps). Once the sorting was complete the cells were re-

suspended in pre-warmed RPMI + 10% FCS in 6-well plates at 37 °C with 10% CO₂.

2.13 Expression and purification of StrepTactin-SpyCatcher

Expression and purification of monovalent and tetravalent StrepTactin-SpyCatcher was performed by Dr Eleanor Denham and Dr Jesse Goyette as described previously (Denham (2016)).

2.14 Coupling of StrepTactin-SpyCatcher to CHO.K1 ligand cells

All steps of this procedure were performed in sterile conditions. The day prior to coupling, a v-bottomed plate was coated with 130 μ L/well of PBS 1% BSA, wrapped in plastic wrap and incubated overnight at 4 °C. On the day of coupling, ligand cells were harvested using TrypLE™ Express, resuspended in serum-containing DMEM and counted. They were washed twice with 10 mL PBS then resuspended in MES buffer (20 mM MES 140 mM NaCl 1% BSA pH 6.0) at 50×10^6 cells/mL. The v-bottomed plate was washed with 130 μ L/well MES buffer then the required volume of ligand cells were added to the appropriate wells.

The plate was centrifuged at $524 \times g$ for 5' and the buffer flicked off. The cells were resuspended in 160 μ L/well of MES buffer, pipetting up and down gently to ensure resuspension. The plate was centrifuged as before and the buffer flicked off.

Required dilutions and volumes of monovalent and/or tetravalent StrepTactin-SpyCatcher were made up in MES buffer. The ligand cells were resuspended in 20 μ L/well of the appropriate StrepTactin-SpyCatcher dilution, pipetting up and down gently to ensure resuspension and shaking for 2' on a plate shaker. The ligand cells were coupled for a total of 10' (including shaking) then 100 μ L/well PBS 1% BSA was added.

The plate was centrifuged at $524 \times g$ for 5' and the buffer flicked off. The cells were resuspended in 100 μ L/well of PBS 1% BSA, pipetting up and down gently to ensure resuspension. This washing step was repeated twice more before resuspending the cells in DMEM + 2 μ g/mL avidin at 20×10^6 cells/mL and pipetting up and down gently to ensure resuspension.

2.15 Assessment of coupling by flow cytometry

Coupled ligand cells in a v-bottomed plate were separated into two wells to compare unstained cells with cells stained with fluorescent biotin. The cells were washed with 100 μL /well PBA buffer, centrifuged at $524 \times g$ for 5' at 4 °C and the buffer was flicked off. This wash step was repeated twice before resuspending the cells in 50 μL of PBA (unstained) or 50 μL of a 3:1 mixture of 3 μM D-biotin:biotin-ATTO 647 (stained). After staining for 15' at RT the cells were washed three times with PBA as before and then fixed in 100 μL /well of PBS + 1% PFA before analysis using the FL4 channel on a FACS CaliburTM flow cytometer.

2.16 Stimulation of THP-1 receptor cells

2.16.1 Stimulation of THP-1 receptor cells with platebound antibody

All steps of this procedure were performed in sterile conditions. THP-1 cells were differentiated for 3 days prior to stimulation as per Section 2.8. On the day prior to the stimulation a Nunc Maxisorp 96-well plate was coated with 50 μL /well of relevant antibody mixtures or PBS + 1% BSA control. For any antibody mixtures containing multiple antibodies, the antibodies were mixed together prior to addition to the plate. The plate was wrapped in plastic wrap and refrigerated overnight at 4 °C.

On the day of the stimulation, the plate was washed three times with 200 μL PBS. The differentiated THP-1 cells were counted, washed three times in 10 mL PBS, and resuspended in complete RPMI at 1×10^6 cells/mL. A total of 100 μL of cells were added to each well and the plate was incubated for 20h at 37 °C and 5% CO₂. Stimulation was measured as detailed in Section 2.16.3.

2.16.2 Stimulation of THP-1 receptor cells with the two-ligand system

All steps of this procedure were performed in sterile conditions. Avidin (2 $\mu\text{g}/\text{mL}$) was added to stimulation media to eliminate any free biotin that would compete with Strep-tag II for binding to StrepTactin. THP-1 cells were differentiated for 3 days prior to stimulation as per Section 2.8. The day prior to the stimulation a flat-bottomed 96-well plate was blocked with 100 μL /well PBS + 1% BSA. The plate was wrapped in plastic wrap and refrigerated overnight at 4 °C.

On the day of the stimulation, the plate was washed three times with 200 μL PBS. The differentiated THP-1 cells were counted, washed three times in 10 mL PBS, and resuspended in DMEM + 2 $\mu\text{g}/\text{mL}$ avidin at 2×10^6 cells/mL. DMEM (40 μL) + 2 $\mu\text{g}/\text{mL}$ avidin was added to each well along with 50 μL of the appropriate THP-1 cells (total of 0.1×10^6 receptor cells/well). The plate was incubated at 37 °C and 10% CO_2 whilst the ligand cells were coupled as per Section 2.14.

A volume of 10 $\mu\text{L}/\text{well}$ of DMEM + 2 $\mu\text{g}/\text{mL}$ avidin or of the relevant ligand cells at 20×10^6 cells/mL (total of 0.2×10^6 ligand cells/well) were added to the plate and returned to the incubator for a 20h stimulation. Stimulation was measured as detailed in Section 2.16.3.

2.16.3 IL-8 ELISA to quantify THP-1 receptor cell activation

This procedure was performed with a Human IL-8 ELISA Ready-SET-Go! 2nd Generation kit according to the manufacturer's instructions with minor modifications, including extra washing steps prior to addition of substrate. The day before the ELISA, a non-sterile Nunc Maxisorp 96 well plate was coated with 100 $\mu\text{L}/\text{well}$ of IL-8 capture antibody diluted 1/250 in coating buffer, i.e. 48 μL capture antibody plus 12 mL of 1X coating buffer was prepared per plate and aliquotted out. The plate was sealed in plastic wrap and refrigerated overnight at 4 °C.

On the day of the ELISA, approximately 90 $\mu\text{L}/\text{well}$ of supernatant from THP-1 stimulation was transferred to a round-bottomed plate and then diluted as appropriate (usually 1/2) with 1X assay diluent in a separate round-bottom plate. Any unused supernatant was stored at -20 °C. The ELISA plate was aspirated and washed three times with 300 $\mu\text{L}/\text{well}$ PBS + 0.05% Tween-20 using a plate washer before blotting on absorbent paper to remove residual buffer.

The ELISA plate was blocked with 200 $\mu\text{L}/\text{well}$ of 1X assay diluent and incubated at RT for 1h before repeating the aspiration and washing procedure as before. Lyophilised human IL-8 standard was reconstituted by adding 1 mL dH_2O , gently mixing and leaving to sit for 15'. The reconstituted standard was diluted by adding 10 μL standard to 590 μL of 1X assay diluent to prepare the 250 pg/mL standard. A 2-fold serial dilution was performed using 1X assay diluent to generate the remaining standards down to 3.9 pg/mL.

A total of 100 μL of each sample or standard was added to the appropriate wells in duplicate, including two wells with 1X assay diluent alone. The plate was sealed and incubated at RT for 2h. The plate was then aspirated and washed six times with 300 $\mu\text{L}/\text{well}$ PBS + 0.05% Tween-20 using a plate washer.

The detection antibody was diluted by adding 48 μL of detection antibody to 12 mL of 1X assay diluent per plate. A volume of 100 μL /well of 1X detection antibody was added to the plate before sealing and incubating at RT for 1h. The plate was then aspirated and washed six times as before.

Avidin-HRP was diluted by combining 48 μL Avidin-HRP with 12 mL of 1X assay diluent per plate. A volume of 100 μL /well of 1X Avidin-HRP was added to the plate before sealing and incubating at RT for 30'. The plate was then aspirated and washed six times using a plate washer, once manually including a 1' soaking step, and then another six times using a plate washer.

A volume of 100 μL /well of substrate solution was added and incubated at RT for 15' or until samples appeared to be within the standard curve. To stop the reaction, 50 μL /well of 1 M phosphoric acid was added and the plate was read immediately at 450 nm using a SpectraMax M5 plate reader (Molecular Devices).

2.17 Stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells

2.17.1 Stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with platebound antibody

All steps of this procedure were performed in sterile conditions. The day prior to the stimulation a Nunc Maxisorp 96-well plate was coated with 50 μL /well of relevant antibody mixtures or PBS + 1% BSA control. For any antibody mixtures containing multiple antibodies, the antibodies were mixed together prior to addition to the plate. The plate was wrapped in plastic wrap and refrigerated overnight at 4 °C.

On the day of the stimulation, the plate was washed three times with 200 μL PBS. The Jurkat NF- κ B-eGFP/1G4 TCR cells were counted, washed three times in 10 mL PBS, and resuspended in complete RPMI at 1×10^6 cells/mL. 100 μL of the cells were then added to each well and the plate was incubated for 24h at 37 °C and 5% CO₂. Stimulation was measured as detailed in Section 2.17.3.

2.17.2 Stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with the two-ligand system

All steps of this procedure were performed in sterile conditions. Avidin (2 $\mu\text{g}/\text{mL}$) was added to stimulation media to eliminate any free biotin that would compete with

Strep-tag II for binding to StrepTactin. The day prior to the stimulation a flat-bottomed 96-well plate was blocked with 100 μL /well PBS + 1% BSA. The plate was wrapped in plastic wrap and refrigerated overnight at 4 °C.

On the day of the stimulation, the plate was washed three times with 200 μL PBS. The Jurkat NF- κ B-eGFP/1G4 TCR cells were counted, washed three times in 10 mL PBS, and resuspended in DMEM + 2 $\mu\text{g}/\text{mL}$ avidin at 2×10^6 cells/mL. A volume of 40 μL of DMEM + 2 $\mu\text{g}/\text{mL}$ avidin was added to each well along with 50 μL of the appropriate Jurkat NF- κ B-eGFP/1G4 TCR cells (total of 0.1×10^6 receptor cells/well). The plate was incubated at 37 °C and 10% CO₂ whilst the ligand cells were coupled as per Section 2.14.

NY-ESO-1 C9V peptide (sequence SLLMWITQV) was diluted to working stock concentrations using PBS. Either 5 μL of PBS or 5 μL of the relevant NYESO C9V peptide concentration was added to each well for exogenous loading of peptide onto the CHO.K1 SCD-based ligand cells. An additional 10 μL /well of DMEM + 2 $\mu\text{g}/\text{mL}$ avidin or of the relevant ligand cells at 20×10^6 cells/mL (total of 0.2×10^6 ligand cells/well) was added to the plate and returned to the incubator for a 24h stimulation. Stimulation was measured as detailed in Section 2.17.3.

2.17.3 Flow cytometry to quantify Jurkat NF- κ B-eGFP/1G4 TCR receptor cell activation

After stimulation of Jurkat NF- κ B-eGFP/1G4 TCR cells as per Sections 2.17.1 and 2.17.2, the cells were pipetted up and down gently to resuspend cells and transferred to a v-bottomed plate. The v-bottomed plate was then centrifuged for 5' at $524 \times g$ and 4 °C and the supernatant was flicked off. The cells were resuspended in 200 μL PBA to wash before repeating the centrifugation.

For Jurkat NF- κ B-eGFP/1G4 TCR cells stimulated with platebound antibody (Section 2.17.1), the washing and centrifugation steps were repeated before fixing in 100 μL /well of PBS + 1% PFA and analysing on the FACS CaliburTM flow cytometer.

For Jurkat NF- κ B-eGFP/1G4 TCR cells stimulated with the two-ligand system (Section 2.17.2), the samples were stained with 10 μL /well of approximately 2 $\mu\text{g}/\text{mL}$ (1/25 dilution) anti-hCD45-Alexa 647 (Table 2.2) for approximately 45' at 4 °C. The PBA washing and centrifugation steps were then repeated 3 times before fixing in 100 μL /well of PBS + 1% PFA and analysing on the FL1 (NF- κ B-eGFP) and FL4 (anti-hCD45-Alexa 647) channels on the FACS CaliburTM flow cytometer.

2.18 Data analysis and statistics

Flow cytometry data were analysed using FlowJo™ version 10.1r5 for Mac OS X (FlowJo, LLC, Ashland, OR), gating on live cells unless otherwise specified. For flow cytometric analyses of samples containing both Jurkat receptor cells and CHO.K1 ligand cells, the CHO.K1 ligand cells were removed from analyses by staining with anti-hCD45-Alexa 647 (Table 2.2) and then gating on cells positive for hCD45.

Data obtained from stimulation of THP-1 and Jurkat NF- κ B-eGFP/1G4 TCR receptor cells were analysed using GraphPad Prism™ version 6.0f for Mac OS X (GraphPad Software, Inc., La Jolla, CA). For comparison of stimulation of receptor cells with a single concentration of antibody/coupled ligand, a one-sample t test of normalised results compared to a specified value of 100 was used to test for a statistically significant difference to the positive control. For Figure 4.27, individual line of best fits were analysed for statistically significant inhibition using a null hypothesis of a slope of zero. Line of best fits were compared to each other with a comparison of fits using a null hypothesis of the same slope for both data sets. P-values for all statistical analyses were allocated statistical significance according to the following GraphPad Prism™ thresholds:

P-value > 0.05 = not statistically significant (n.s.)

P-value ≤ 0.05 = *

P-value ≤ 0.01 = **

P-value ≤ 0.001 = ***

Setting up a two-ligand system

3.1 Chapter Introduction

3.1.1 The challenge of studying NTR signalling

On the basis of several shared features, a group of more than 120 cell surface receptors have been categorised as NTRs (Dushek *et al.* (2012); Figure 1.1). These shared features are described in detail in Section 1.2.1 and include binding of surface-associated ligands with small receptor-ligand dimensions, having no intrinsic catalytic activity, and the presence of conserved tyrosine-based signalling motifs in their intracellular tails or in associated adaptor proteins.

These tyrosine-based motifs are phosphorylated by Src family kinases such as Lck and dephosphorylated by receptor-protein tyrosine phosphatases such as CD45. When phosphorylated, they recruit cytoplasmic proteins to the intracellular tail of the receptor to carry out downstream signalling. Despite extensive research on several NTRs, the mechanism of signalling and signal integration for the majority of these receptors remains poorly understood.

There are a number of proposed mechanisms for NTR triggering, with most fitting into one or more of the categories of aggregation, conformational change, or segregation, as described in detail in Section 1.4 of the General Introduction. One proposed model is the kinetic-segregation (KS) model (Davis & van der Merwe (1996); reviewed by Davis & van der Merwe (2006)), which focuses on the importance of small receptor-ligand dimensions for local segregation of the large phosphatase CD45 and/or CD148. The KS model can be tested by manipulating receptor-ligand dimensions to determine the importance of these small receptor-ligand dimensions for effective signalling.

Several investigations of NTR signalling have involved such manipulation of receptor-ligand dimensions (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009); Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2010); Yokosuka *et al.* (2012); Lim *et al.* (2015)). However, with over 120 receptors categorised as NTRs, it is impractical to study this group of receptors by individually elongating each receptor or its native ligand. On top of the sheer number of NTRs, some of these receptors, such as CD300e (Zenarruzabeitia *et al.* (2016)) and several LILR family members (Burshtyn & Morcos (2016)), have no known ligands so cannot currently be studied with their native ligand.

Therefore, in order to tackle this problem in a systematic manner, we set out to develop a model ligand system that can be used for investigation of signalling and signal integration by a range of NTRs.

3.1.2 Existing model ligand systems

A variety of model ligand systems have been developed to investigate immune signalling, in particular for studying T cell receptor signalling (reviewed by Turtle & Riddell (2010) and Eggermont *et al.* (2014)). One of the first cellular model ligand systems was the *Drosophila melanogaster*-based system expressing murine or human MHC and co-stimulatory molecules, for proliferation of murine and human cells (Cai *et al.* (1996); Sun *et al.* (1996)).

A more recently developed system is the T cell stimulator cell (TSC) system generated by Leitner and colleagues, which uses the murine thymoma cell line Bw5147 to activate human T cells via membrane-bound anti-CD3 (OKT3) single chain antibody fragments (Leitner *et al.* (2010)). Co-stimulatory or co-inhibitory ligands can also be expressed on the TSCs to analyse signal integration by a variety of immune receptors.

Another ligand-presentation system, referred to as engineered APCs (eAPCs), uses the human erythroleukemia cell line K562 to present immunodominant peptides on MHC class II to allergen-specific human CD4⁺ T cell lines (Roskopf *et al.* (2016)). eAPCs presenting either exogenously or endogenously loaded allergenic peptides resulted in antigen-dependent stimulation of T cells expressing allergen-specific TCRs. As with the TSCs described above, these eAPCs can present co-inhibitory or co-stimulatory ligands such as PD-L1/PD-L2 and CD80 to analyse signal integration (Roskopf *et al.* (2016)).

These model ligand-presentation systems are proving to be useful tools for studying co-stimulatory and co-inhibitory signal integration in T cells and offer potential

clinical applications such as investigation of immunomodulatory drugs. Two important differences between these systems and the system we are developing here are that we aim to generate a ligand that is 1) easily titratable, and therefore able to generate full dose response curves for studying signal integration, and 2) generic, so that it can be used to engage a range of NTRs. These features are further discussed below.

3.1.3 Desired features of a system for analysing NTR signalling

Ideal features of the ligand within a system for studying NTR signalling would include:

1. Membrane-presented
2. Titratable
3. Generic with a physiological affinity

1. Membrane-presented

When developing a system for studying NTR signalling it is important to consider that in a physiological setting cell surface receptors and their ligands are able to diffuse within the cell membrane. Additionally, many NTRs have been found to cluster on the cell surface, which may be important for their signalling, as discussed in Section 1.4.2. Hence, an ideal system for studying these receptors should allow such diffusion. Methods for allowing diffusion within a membrane include the use of cells or lipid bilayers, such as lipid bilayer-coated beads or planar lipid bilayers.

One drawback of presenting ligands using artificial lipid bilayers is that they are still very different to cell membranes. For example, lipid bilayers lack a glycocalyx and the mobility of the ligands and the mechanical properties of the membranes are also different. Therefore, considering these options, we chose to develop a cellular system using human cell lines as receptor cells and the Chinese Hamster Ovary (CHO.K1) cell line for ligand presentation.

2. Titratable

NTR signalling and signal integration may vary with ligand density, so it would be useful to be able to vary the amount of ligand presented. The ability to vary surface

density of ligands is particularly useful for analysing variations between NTRs and/or their ligands, such as different receptor-ligand dimensions or mutations to receptor signalling motifs, because differences in signalling may only be detected within a certain (and unknown) window of ligand density.

To vary the amount of a membrane-presented ligand, FACS could be used to sort cell populations with different expression levels of the ligand (e.g. low, medium, and high expression levels). Although feasible in theory, such an approach may be challenging in practise and would still only provide a limited number of ligand densities.

Blocking antibodies could potentially be used to titrate ligand; however, when analysing several cell lines expressing changeable or mismatched levels of ligands it is difficult to control for relative blocking levels between different cell lines as well as between experiments. Use of antibodies is also problematic when studying immune cells such as macrophages, NK cells, and B cells, which express Fc (Fragment, crystallisable) receptors that bind to the Fc region of antibodies. Soluble Fab (fragment, antibody-binding) could be used to avoid Fc receptor signalling, however this would still not allow for easy matching of relative blocking between different ligand cell lines and experiments.

To overcome these challenges and generate an easily titratable ligand, a split protein could be used with a cell surface-expressed component and a soluble component that would bind to the surface-associated component and reconstitute receptor binding sites on the surface of ligand cells. Titration of the soluble component of the ligand would then allow for a wide range of ligand densities to be presented to get full dose-response curves and control for relative coupling between cell lines as well as between experiments.

Howarth and colleagues have developed a suitable split protein for this purpose: SpyTag/SpyCatcher, which is based on *Streptococcus pyogenes* fibronectin binding protein, FbaB (reviewed by Reddington & Howarth (2015)). When split into two components and then mixed together again the two components form a spontaneous isopeptide bond. The covalent bond is formed between Lys31 and Asp117 in the second Ig domain, CnaB2, and is catalysed by the nearby Glu77 residue (Oke *et al.* (2010); Hagan *et al.* (2010)). Zakeri and colleagues split the domain into a short peptide (SpyTag) and protein partner (SpyCatcher), and demonstrated specific and stable covalent binding of the SpyTag and SpyCatcher in a range of conditions (Zakeri *et al.* (2012)). The system has been optimised (Li *et al.* (2014a)) and provides an appealing option as part of a titratable ligand system through coupling of a titration

of the soluble SpyCatcher protein to ligand cells expressing a membrane-presented SpyTag component.

3. Generic with a physiological affinity

Ideally this titratable ligand should be “generic” i.e. a ligand for a tag that can be easily added to any NTR of interest. For this aspect of the system we are using StrepTactin, a version of the tetrameric biotin-binding protein, Streptavidin, which has mutations to three key residues in the binding pocket. These mutations give StrepTactin a reportedly physiologically relevant binding affinity for the Strep-tag II peptide (WSHPQFEK) with $K_d \approx 1 \mu\text{M}$, while maintaining biotin binding ability (Voss & Skerra (1997)).

When fused to SpyCatcher, StrepTactin will form the generic ligand component of the system through addition of a soluble StrepTactin-SpyCatcher fusion protein to ligand cells expressing a cell surface SpyTag construct (Figure 3.1). StrepTactin-SpyCatcher coupled to ligand cells should then be able to act as a generic membrane-presented ligand for any Strep-tag II-tagged NTR.

Additional features

In addition to the features outlined above, use of StrepTactin in the system also enables variation of ligand valency from monovalent up to tetravalent, which may be useful for testing the effect of valency on signal integration. It also allows adjustment of affinity, with the potential for making additional mutations to the StrepTactin protein to further adjust its affinity for Strep-tag II.

Much of the work in setting up this StrepTactin-Strep-tag II-based generic ligand system is detailed in the thesis of Dr Eleanor Denham (Denham (2016)). The current thesis focuses on further developing the approach to analyse NTR signal integration in a two-ligand system. This requires a second ligand to provide an activating signal, which can then be used to analyse signal integration with either a co-stimulatory or co-inhibitory NTR engaged through the generic StrepTactin-Strep-tag II interaction (Figure 3.1).

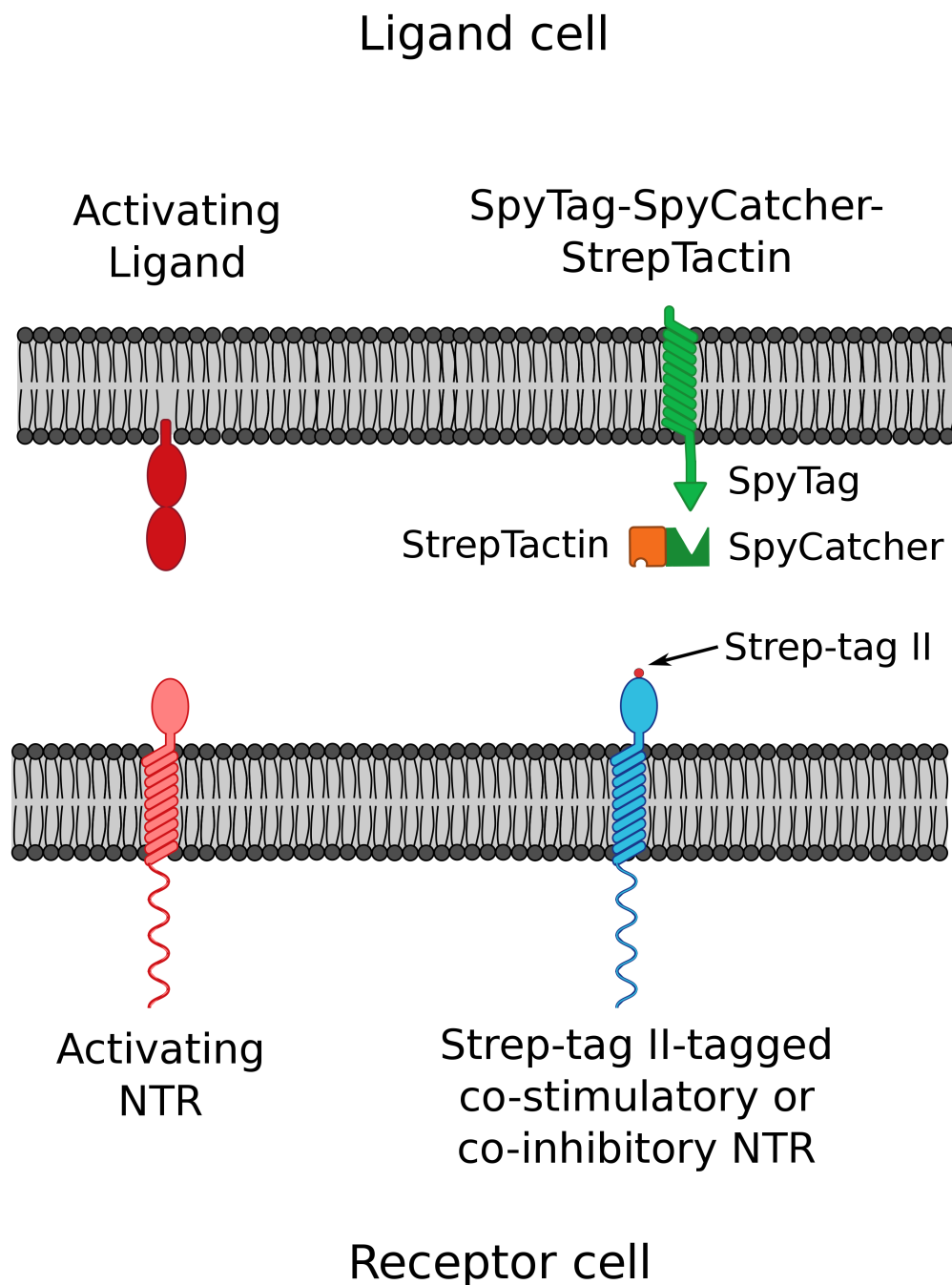


Figure 3.1: Schematic representation of the basic components of the proposed two-ligand system for analysis of NTR signal integration. The system includes ligand cells presenting an activating ligand, such as GPI-anchored CD48 (red), and a generic StrepTactin-based ligand (SpyTag-SpyCatcher-StrepTactin) to receptor cells expressing an activating NTR and a Strep-tag II-tagged co-stimulatory or co-inhibitory NTR. The soluble StrepTactin-SpyCatcher component can be coupled to the ligand cells by formation of a spontaneous isopeptide bond between the SpyCatcher and the surface-expressed SpyTag construct.

3.1.4 Activating NTRs for studying signal integration

In order to develop a system for studying NTR signal integration in different immune cell types we chose appropriate activating NTRs for both T lymphocytes and monocytes/ macrophages.

A candidate activating NTR for studying signal integration in monocytes and macrophages is CD64 - an activating Fc receptor expressed by a range of immune cells (Tan *et al.* (2003); Biburger *et al.* (2011)). Fc receptors such as CD64 bind to the Fc region of antibodies, stimulating immune responses such as antibody-mediated phagocytosis and antibody-dependent cell-mediated cytotoxicity against antibody-coated pathogens or infected cells (Murphy & Weaver (2016)).

We used a fusion protein including the mouse 2B4 ectodomain plus the transmembrane and intracellular regions of CD64, referred to as 2B4-CD64. Use of this fusion protein will allow specific engagement of the introduced CD64 construct (2B4-CD64) through the well-studied interaction between mouse 2B4 and CD48 (Brown *et al.* (1998); Velikovsky *et al.* (2007)).

To complement the use of CD64 as an activating NTR in monocytes and macrophages, the T cell receptor (TCR) can be used for studying signal integration in T cells. The 1G4 TCR is a particularly well-studied TCR (Chen *et al.* (1997); Jäger *et al.* (1998); Chen *et al.* (2000); Aleksic *et al.* (2010)), which can be engaged using HLA-A2 single chain dimer (SCD) loaded with cognate cancer-testis antigen NY-ESO-1 peptide variants, such as the C9V variant used in the current study.

3.1.5 A co-inhibitory NTR for studying signal integration

To study NTR signal integration, an appropriate co-stimulatory or co-inhibitory NTR is required in addition to the activating receptors introduced above. A candidate co-inhibitory NTR is PD-1, a member of the CD28 family, which inhibits T cell receptor signaling by recruiting cytoplasmic phosphatases such as SHP-2 (Latchman *et al.* (2001)).

PD-1 is an interesting receptor to study for a number of reasons. Firstly, it contains both an ITIM and ITSM but, unlike most other ITSM-containing receptors, PD-1 is reported to be unable to bind to SLAM-associated protein (SAP; Chemnitz *et al.* (2004)). It is also the focus of some of the emerging immunotherapies such as checkpoint inhibitors (as reviewed by Hatanishi *et al.* (2016)) and CARs (Kobold *et al.* (2015); Liu *et al.* (2016b)).

Several aspects of PD-1 signalling remain poorly understood, such as the importance of the length of its intracellular tail, which has potential implications for the design of CARs and other ACT-based therapies. However, enough is known about this inhibitory receptor to validate our results and to optimise the two-ligand system. For instance, previous studies have investigated the role of the PD-1 ITIM and ITSM, the two phosphorylatable motifs in the intracellular tail of PD-1, and have shown that the ITSM is most important for the inhibitory function of PD-1 (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012)). These studies will be utilised as a comparison for results obtained from the two-ligand system.

This chapter covers the development of the basic components of a generic cellular two-ligand system for studying NTR signal integration using PD-1 as an example of a co-inhibitory NTR.

3.2 Chapter Aim

The aim of this chapter is to develop a generic cellular two-ligand system and suitable stimulation assays for analysis of signal integration by NTRs.

3.3 Results

3.3.1 Generation of receptor cell lines

Lenitviral vectors encoding activating NTR constructs were as described in Section 2.7 and shown in Table 3.1.

Receptor construct (abbreviated name)	Source of construct DNA	Tags
mouse 2B4 ectodomain-human CD64 transmembrane and intracellular tail (2B4-CD64)	Provided by Dr Jesse Goyette	-
1G4 wildtype T cell receptor (1G4 TCR)	Provided by Dr Andre Cohnen and Prof. Oreste Acuto	C-terminal HA (β chain); C-terminal FLAG tag (α chain)
human PD-1 wildtype control (PD-1 control)	Cloned from cDNA [†]	-
human PD-1 with Strep-tag II (PD-1 Strep-tag II)	Cloned from cDNA [†]	N-terminal Strep-tag II

Table 3.1: Details of receptor constructs for use in the two-ligand system for studying NTR signal integration. [†]I.M.A.G.E. clone 30915198

Monocytic cell line

A well-studied human monocytic cell line is the THP-1 cell line, derived from an acute monocytic leukemia patient (Tsuchiya *et al.* (1980)). THP-1 cells can be activated through Fc receptors, such as CD64, and are suitable for lentiviral transduction. Production of cytokines, such as the inflammatory chemokine IL-8 (Baggiolini *et al.* (1989)), can be used as a measure of activation of these cells.

T lymphocyte cell line

A well-studied human T lymphocyte cell line is the Jurkat cell line, originally derived from a T cell leukaemia (Schneider *et al.* (1977)). A Jurkat JE6.1 reporter cell

line expressing an NF- κ B-eGFP reporter has been developed for studying T cell signalling (Ratzinger *et al.* (2014)). These reporter cells were subsequently lentivirally transduced with the 1G4 TCR (Table 3.1) plus CD8 co-receptor (pHR-SIN-BX-CD8- β -IRES- α ; Dr Wolfgang Paster, personal communication) and were kindly provided for use in the current work. The NF- κ B-eGFP reporter can be used to measure activation of the Jurkat cells by flow cytometry (Ratzinger *et al.* (2014)).

As demonstrated in Figure 3.2, these Jurkat NF- κ B-eGFP/1G4 TCR cells express endogenous PD-1, which is upregulated upon activation such as after 24h platebound antibody stimulation with 0.5 μ g/mL anti-CD3 (UCHT1; Figure 3.2). In order to specifically target transduced PD-1 constructs but not the endogenous PD-1 expressed by these cells we can use a tag on the transduced PD-1 constructs, such as the N-terminal Strep-tag II tag used in the current study.

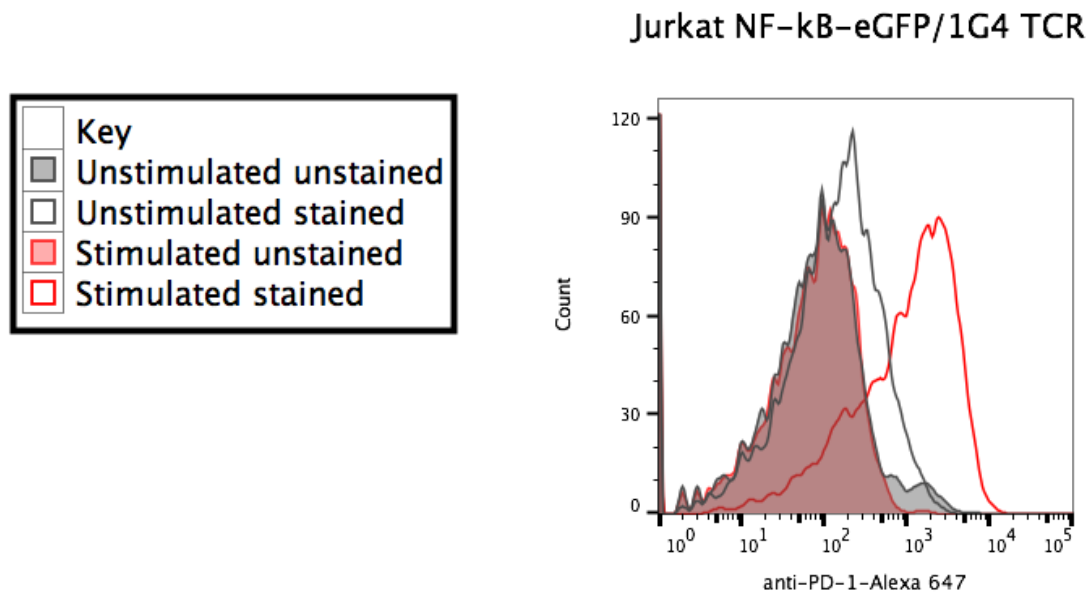


Figure 3.2: Flow cytometry results showing endogenous PD-1 expression of Jurkat NF- κ B-eGFP/1G4 TCR cells either unstimulated (grey) or stimulated for 24h with 0.5 μ g/mL platebound anti-CD3 (UCHT1; red). The cells were unstained (filled histograms) or stained with anti-PD-1-Alexa 647 (empty histograms).

Generation of receptor cell lines expressing NTRs of interest

Lentiviral transduction was used to generate THP-1 and Jurkat receptor cell lines expressing activating and co-inhibitory NTRs of interest as detailed in Table 3.2. This includes THP-1 cells transduced with PD-1 alone, THP-1 cells co-transduced with PD-1 plus 2B4-CD64 for activation with ligand cells expressing GPI-anchored CD48-based constructs, and Jurkat NF- κ B-eGFP/1G4 TCR reporter cells transduced with PD-1. These Jurkat cells also express the CD8 co-receptor and can be activated by ligand cells expressing HLA-A2 SCD loaded with cognate peptide.

In addition to the PD-1 construct tagged with Strep-tag II (PD-1 Strep-tag II), PD-1 WT without the tag (PD-1 control) was also transduced into the THP-1 and Jurkat receptor cells as a control. As seen in Figure 3.3, staining transduced receptor cells with anti-Strep-tag II followed by anti-mouse IgG1-Alexa 647 results in specific staining of the PD-1 Strep-tag II construct and not the PD-1 control.

Following the lentiviral transductions, the receptor cell lines were sorted using antibody-coated Dynabeads and/or FACS as indicated in Table 3.2. Flow cytometry results of the sorted receptor cells are shown in Figures 3.4 - 3.5, with well-matched expression levels of the various constructs in the PD-1 control and PD-1 Strep-tag II cells of each type.

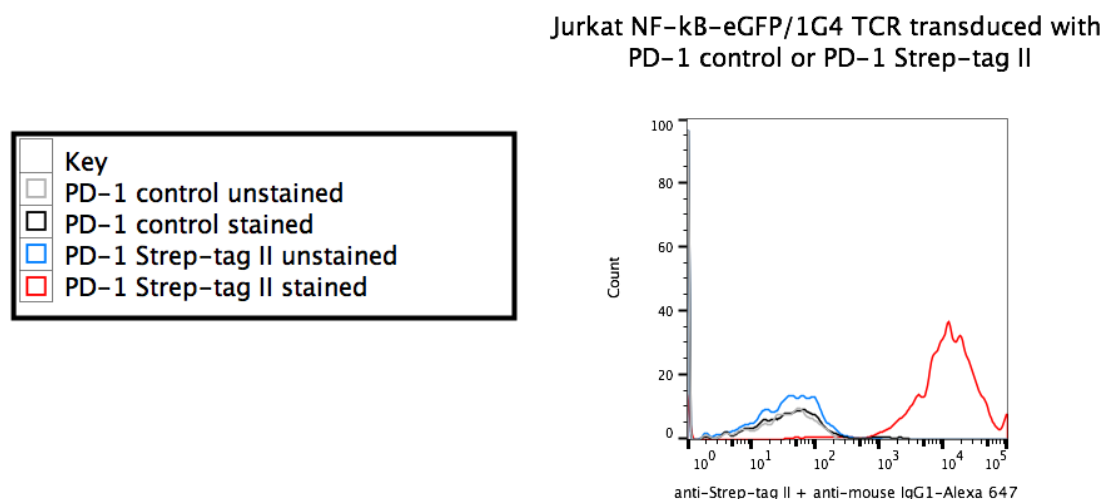


Figure 3.3: Flow cytometry results showing anti-Strep-tag II staining of Jurkat NF- κ B-eGFP/1G4 TCR cells lentivirally transduced with pHR-SIN-BX-PD-1 control or pHR-SIN-BX-PD-1 Strep-tag II. Unstained samples of the PD-1 control cells and PD-1 Strep-tag II cells are shown in grey and blue, respectively, and samples of the PD-1 control cells and PD-1 Strep-tag II cells stained with anti-Strep-tag II followed by anti-mouse IgG1-Alexa 647 are shown in black and red, respectively.

Cell type	Transduced activating NTR	Transduced co-inhibitory NTR	Sorting method/s
THP-1	nil	PD-1 control	FACS
THP-1	nil	PD-1 Strep-tag II	anti-Strep-tag II bead-sorted
THP-1	2B4-CD64	PD-1 control	FACS
THP-1	2B4-CD64	PD-1 Strep-tag II	anti-Strep-tag II bead-sorted, anti-2B4 bead-sorted and FACS
Jurkat NF- κ B-eGFP	1G4 TCR	PD-1 control	FACS
Jurkat NF- κ B-eGFP	1G4 TCR	PD-1 Strep-tag II	FACS

Table 3.2: *THP-1 and Jurkat receptor cell lines for use in the two-ligand system. The cells were lentivirally transduced with the indicated NTRs and sorted by FACS and/or bead-sorting with antibody-coated Dynabeads.*

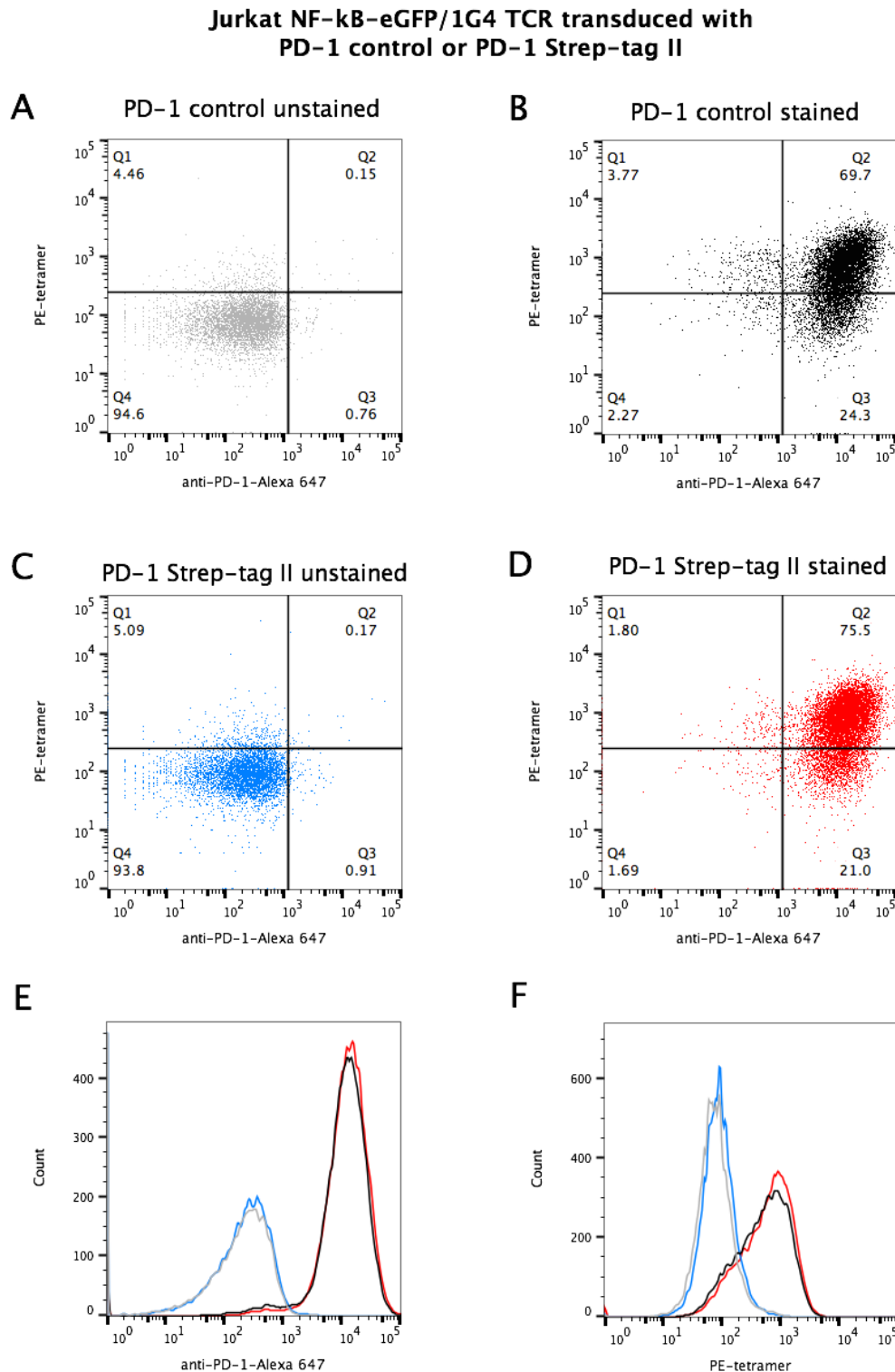


Figure 3.4: Flow cytometry results showing Jurkat NF κ B-eGFP/1G4 TCR cells lentivirally transduced with pHR-SIN-BX-PD-1 control or pHR-SIN-BX-PD-1 Strep-tag II. Panels A - D show dotplot representations of the PD-1 control cells unstained (A) or stained with PE-tetramer (HLA-A2-C9V; ligand for the 1G4 TCR) and anti-PD-1-Alexa 647 (B), and the PD-1 Strep-tag II cells unstained (C) or stained with PE-tetramer and anti-PD-1-Alexa 647 (D). Histograms of the results are displayed in panels E and F. Unstained samples of the PD-1 control cells and PD-1 Strep-tag II cells are shown in grey and blue, respectively, and samples of the PD-1 control cells and PD-1 Strep-tag II cells stained with anti-PD-1 Alexa 647/PE-tetramer are shown in black and red, respectively.

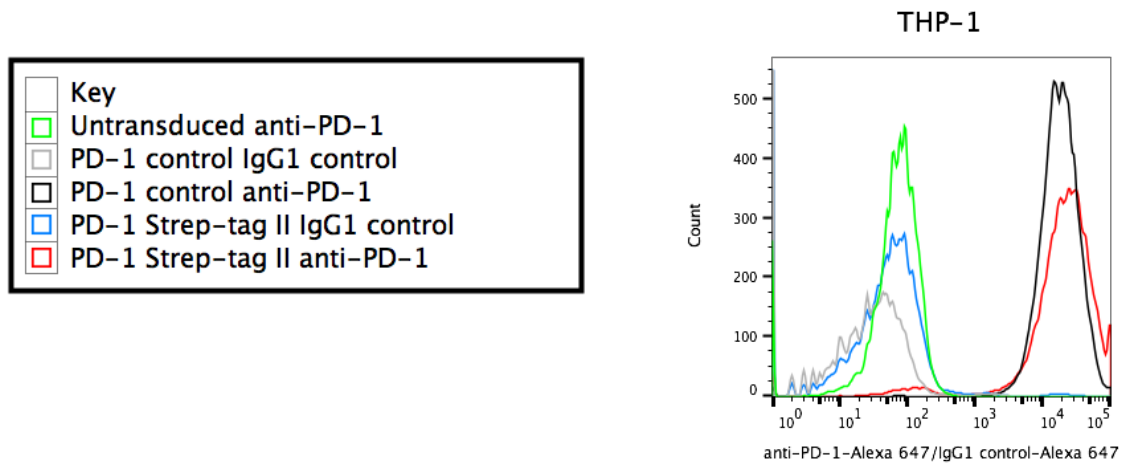


Figure 3.5: Flow cytometry results showing untransduced THP-1 cells (green) and THP-1 cells lentivirally transduced with *pHR-SIN-BX-PD-1* control (black) or *pHR-SIN-BX-PD-1* Strep-tag II (red) stained with anti-PD-1-Alexa 647. Samples stained with an IgG1-Alexa 647 isotype control antibody are shown for the PD-1 control cells (grey) and PD-1 Strep-tag II cells (blue).

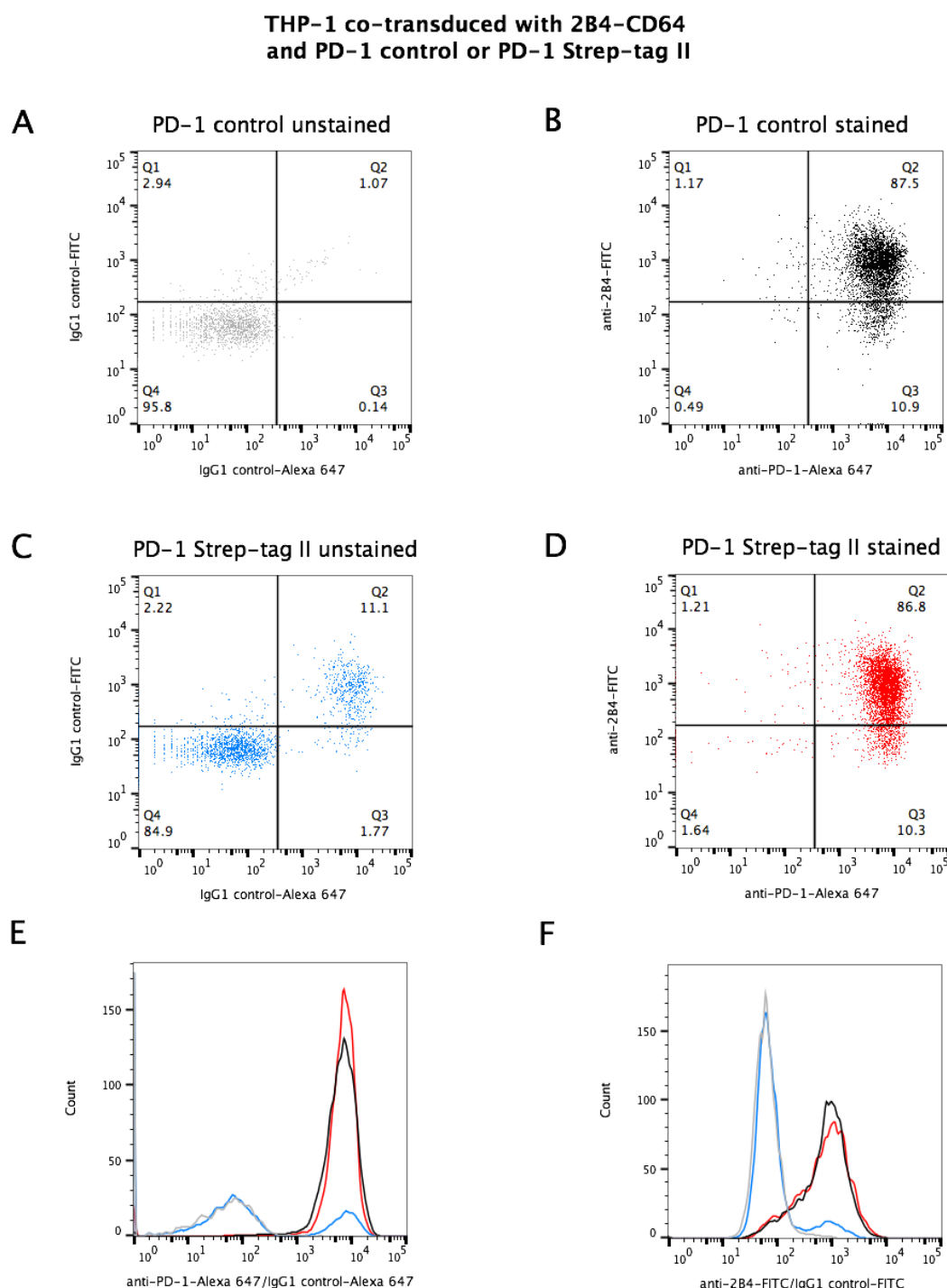


Figure 3.6: Flow cytometry results showing THP-1 cells lentivirally co-transduced with *pHR-SIN-BX-2B4-CD64* plus either *pHR-SIN-BX-PD-1* control or *pHR-SIN-BX-PD-1* Strep-tag II. Panels A - D show dotplot representations of the PD-1 control cells stained with IgG1-Alexa 647 and IgG1-FITC isotype control antibodies (A) or anti-PD-1-Alexa 647 and anti-2B4-FITC (B) and the PD-1 Strep-tag II cells stained with IgG1-Alexa 647 and IgG1-FITC isotype control antibodies (C) or anti-PD-1-Alexa 647 and anti-2B4-FITC (D). Histograms of the results are displayed in panels E and F. Samples of the PD-1 control cells and PD-1 Strep-tag II cells stained with IgG1-Alexa 647 and IgG1-FITC isotype control antibodies are shown in grey and blue, respectively, and samples of the PD-1 control cells and PD-1 Strep-tag II cells stained with anti-PD-1 Alexa 647/anti-2B4-FITC are shown in black and red, respectively.

3.3.2 Generation of ligand cell lines

In order to create a generic ligand system, ligand cells expressing different ligands are required to stimulate the receptor cells detailed above. Since being subcloned from the Chinese Hamster Ovary (CHO) cell line (Tjio & Puck (1958)), the CHO.K1 cell line has been used extensively in biological research due to its suitability for DNA transfection and expression of cell surface proteins.

CHO.K1 cells are useful as ligand-expressing cells for this system as they do not express many ligands for human NTRs but some cell adhesion interactions should be maintained such as through ICAM-1 and LFA-1 (Johnston *et al.* (1990); Xu *et al.* (2011)). In addition to facilitating adhesion, use of an intact mammalian cellular membrane provides a more physiological setting compared to presenting ligands via a phospholipid bilayer.

3.3.2.1 Avi tag approach

Our initial approach for creating a generic ligand system started out with the expression of biotin protein ligase BirA-ER along with a surface expressed protein with an Avi-tag for biotinylation. In theory this would allow addition of multivalent Strep-Tactin to the surface of the ligand cells, leaving free binding sites for engagement of Strep-tag II-tagged NTRs on receptor cells.

The construct designed for this purpose, referred to as CD80-Avi, consisted of an Ig κ signal sequence, the Avi tag, and an HA tag followed by the stalk, transmembrane and intracellular regions of mouse CD80 in a pcDNA3 vector.

Many different transfection techniques including Amaxa, PEI, and FuGENE were trialled and optimised to try to generate CHO.K1 ligand cells expressing the CD80-Avi construct. Transient expression of the CD80-Avi construct was evident in HEK 293T cells but only very low transfection efficiencies were obtained for the CHO.K1 cells (data not shown).

In an attempt to improve the transfection efficiency, the Ig κ signal sequence was replaced with the *Gaussia* luciferase signal sequence, which is reported to be ideal for use in CHO.K1 cells (Knappskog *et al.* (2007)). Several different transfection techniques were once again trialled along with subsequent selection using hygromycin to select for positive cells, however the transfection efficiency and expression of the Avi-tagged construct in the CHO.K1 cells remained limited (Figures 3.7 and 3.8).

As well as the poor transfection efficiency using these constructs, results from other laboratory members showed that the biotinylated constructs were not effective

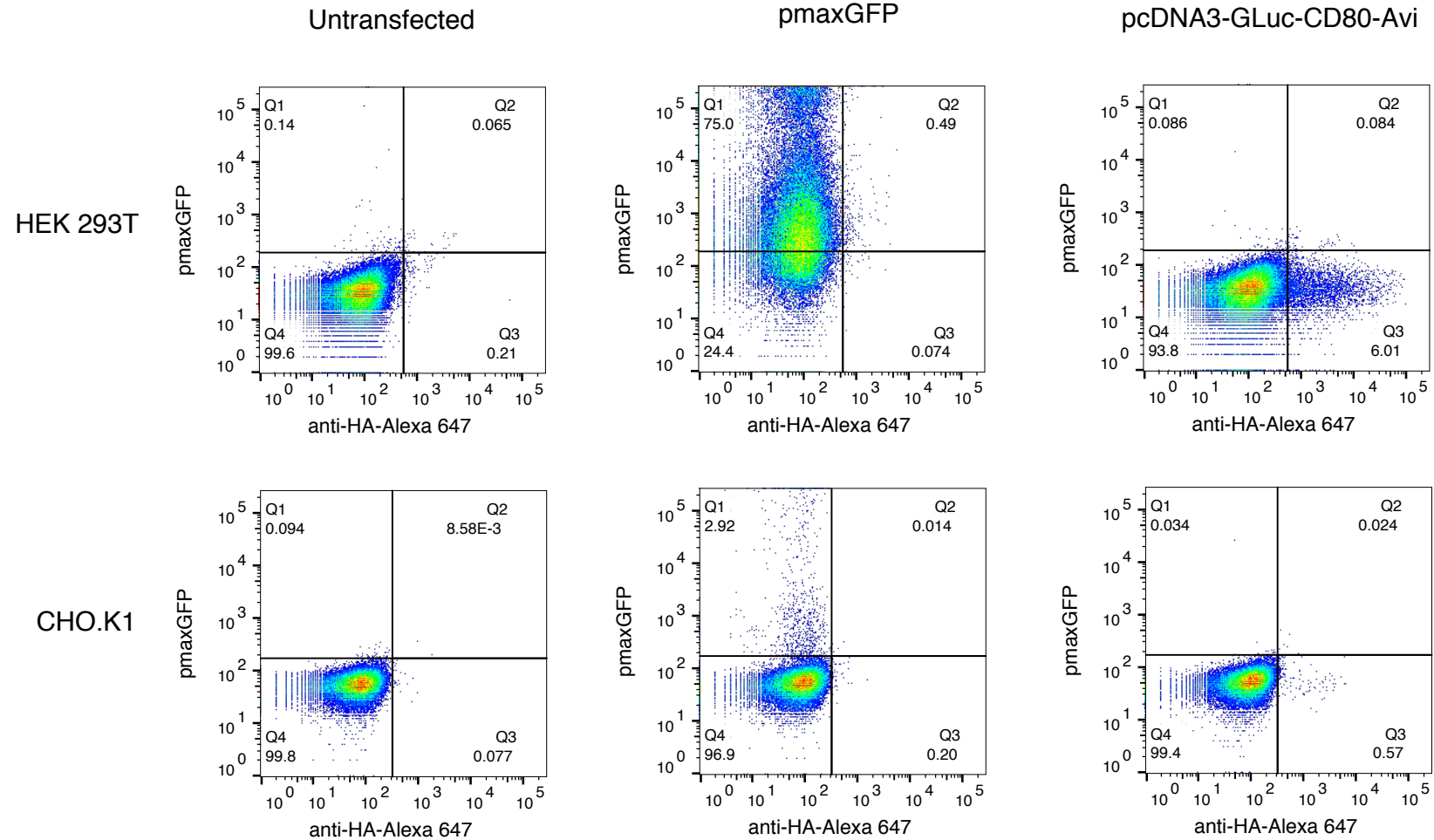


Figure 3.7: Representative flow cytometry results showing results from FuGENE transfection of HEK 293T cells (top row) and CHO.K1 cells (bottom row). Cells were untransfected (left column), or transfected using the FuGENE reagent with a pmax-GFP positive control (middle column) or pcDNA3-GLuc-CD80-Avi (right column). Cells were stained with anti-HA-Alexa 647 against the CD80-Avi construct.

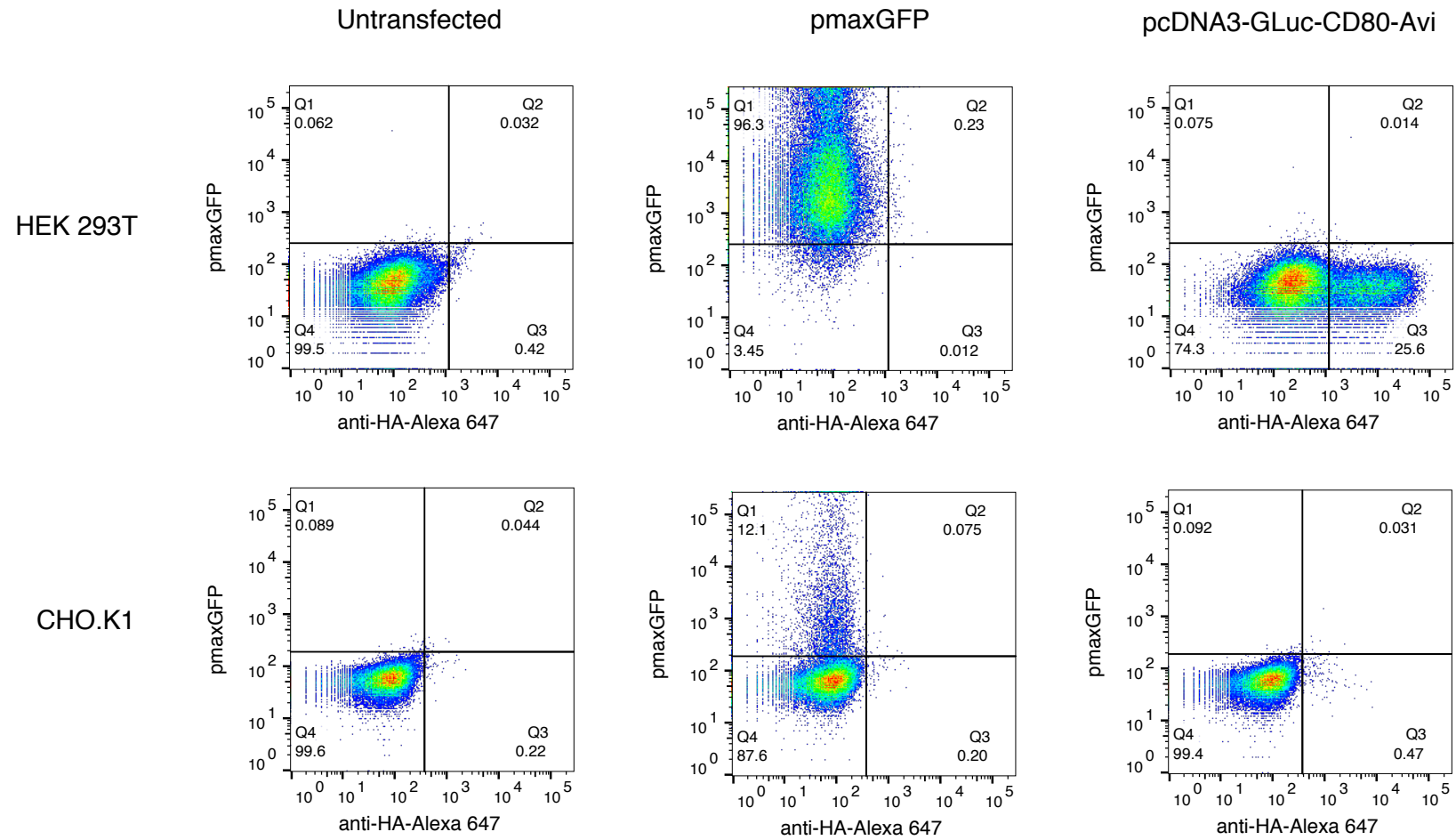


Figure 3.8: Representative flow cytometry results showing results from PEI transfection of HEK 293T cells (top row) and CHO.K1 cells (bottom row). Cells were untransfected (left column), transfected using PEI with a pmax-GFP positive control (middle column) or pcDNA3-GLuc-CD80-Avi (right column). Cells were stained with anti-HA-Alexa 647 against the CD80-Avi construct.

ligands for Strep-tag II-tagged receptors; adding bivalent StrepTactin did not result in available binding sites for Strep-tag II (Denham (2016)). This was possibly due to the StrepTactin binding multivalently to the biotinylated ligands and leaving insufficient accessible binding sites free for binding to the Strep-tag II. Considering these results, this approach was not pursued any further and the SpyTag approach described below was developed.

3.3.2.2 SpyTag approach

This approach uses CHO.K1 ligand cells transfected with a construct encoding the SpyTag peptide (sequence AHIVMVDAYKPTK) followed by the short extracellular stalk, transmembrane and cytoplasmic regions of mouse CD80. This surface expressed construct will be referred to as "Spy". A soluble StrepTactin-SpyCatcher fusion protein can then be coupled to the ligand cells expressing the Spy construct through formation of a covalent bond between the SpyTag and SpyCatcher (Zakeri *et al.* (2012)). The StrepTactin should be able to act as a ligand for NTRs tagged with Strep-tag II (Figure 3.1).

The ligand cells were also transfected with ligands including mouse CD48 and HLA-A2 SCD to provide activating signals to the receptor cells through binding to 2B4-CD64 and 1G4 TCR in THP-1 and Jurkat receptor cells respectively. The activating ligands, CD48 and SCD, as well as the Spy construct were generated as described in Section 2.2.2.

After trialling several different vectors, transfection methods, and subsequent selection and sorting approaches, the FuGENE transfection method (Section 2.4.1) and subsequent sorting approaches described in Section 2.6 were implemented to generate CHO.K1 ligand cell lines for use in the two-ligand system. As seen in Figures 3.9 and 3.10, the ligand cells showed expression of the desired ligands well above the background of untransfected CHO.K1 cells and the autofluorescence observed in the unstained ligand cells.

Despite several rounds of FACS and continuous selection (25 μ M MSX + 1 mg/mL G418), a moderate population of the SCD/Spy ligand cells and a small population of the CD48/Spy ligand cells remained negative for anti-HA staining against the Spy construct (Figures 3.9 and 3.10).

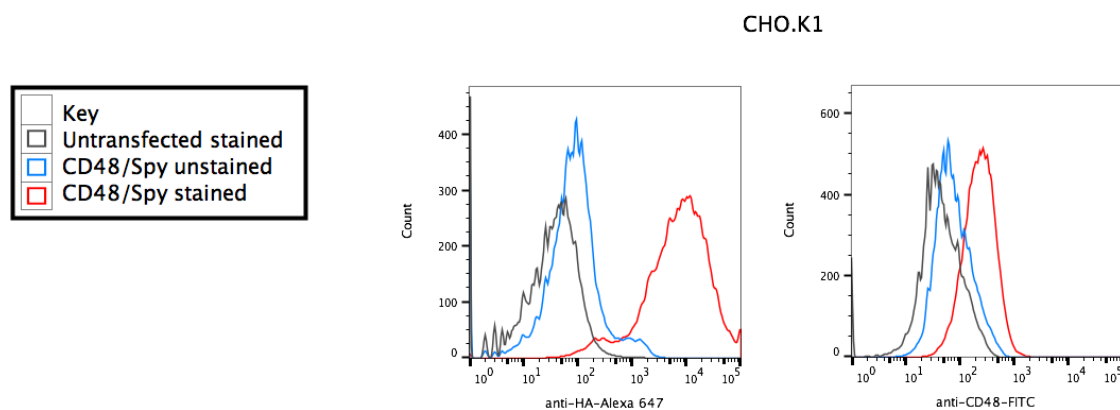


Figure 3.9: Flow cytometry results showing untransfected CHO.K1 cells (grey) and CHO.K1 CD48/Spy ligand cells (red) stained with anti-HA-Alexa 647 (left panel) and anti-CD48-FITC (right panel). Unstained samples of the CHO.K1 CD48/Spy ligand cells are shown in blue.

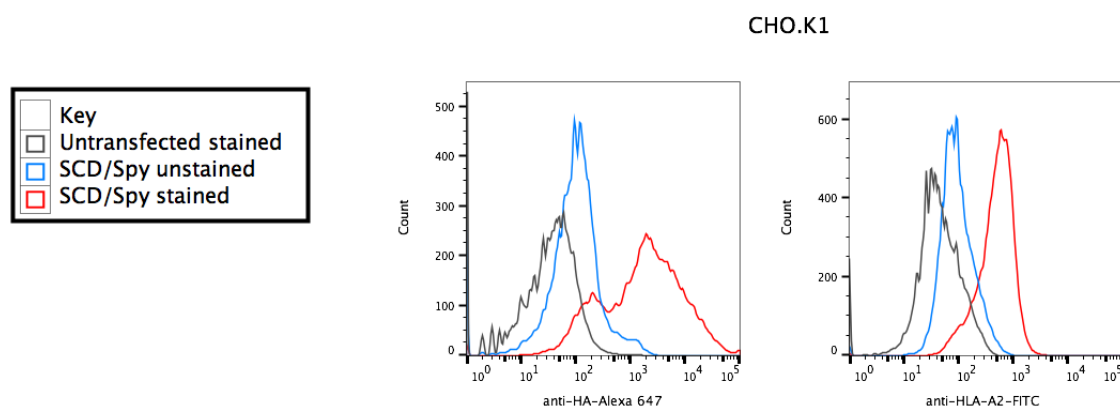


Figure 3.10: Flow cytometry results showing untransfected CHO.K1 cells (grey) and CHO.K1 SCD/Spy ligand cells (red) stained with anti-HA-Alexa 647 (left panel) and anti-HLA-A2-FITC (right panel). Unstained samples of the CHO.K1 SCD/Spy ligand cells are shown in blue.

3.3.3 Coupling of titratable ligand to CHO.K1 ligand cells

Expression of the Spy construct on the CHO.K1 ligand cells can be analysed by flow cytometry with anti-HA (Figures 3.9 and 3.10); however, we also need a measure of the relative number of binding sites available for binding to Strep-tag II once the ligand cells have been coupled with the soluble StrepTactin-SpyCatcher fusion protein.

Since biotin also binds to StrepTactin (Voss & Skerra (1997)), staining of the ligand cells with fluorescently-labelled biotin, such as biotin-ATTO 647, can be used as a surrogate measure for the relative number of available binding sites for Strep-tag II. A preliminary experiment was performed as described in Sections 2.14 and 2.15 to determine the concentration of biotin-ATTO 647 required to efficiently saturate the coupled StrepTactin-SpyCatcher. Briefly, CHO.K1 SCD/Spy ligand cells were coupled with 20 μM (predicted to be saturating levels) of either monovalent or tetravalent StrepTactin-SpyCatcher and then stained with various concentrations of biotin-ATTO 647.

As seen in Figure 3.11, biotin-ATTO 647 staining of the CHO.K1 ligand cells coupled with monovalent StrepTactin was saturated, with no increase in staining from 200 nM to 1 μM biotin-ATTO 647. In contrast, Figure 3.12 shows that for the tetravalent StrepTactin the fluorescence intensity actually decreased as the concentration of biotin-ATTO 647 was increased. The most likely explanation for this is that the close proximity of the ATTO 647 fluorophores when bound to tetravalent StrepTactin resulted in quenching (Strömqvist *et al.* (2011)).

Quenching can be minimised by staining the ligand cells with a 3:1 mixture of D-biotin:biotin-ATTO 647 to give an average of 1 fluorescent biotin per tetravalent StrepTactin molecule. As seen in Figures 3.13 and 3.14, staining with 3 μM of a 3:1 mixture of D-biotin:biotin-ATTO 647 results in an increase in staining with increased coupling of tetravalent StrepTactin-SpyCatcher to the ligand cells.

This staining approach of 3 μM of a 3:1 mixture of D-biotin:biotin-ATTO 647 was used throughout the current study to determine the relative number of binding sites available for binding to Strep-tag II-tagged NTRs. It was also used to test the concentration of monovalent and tetravalent StrepTactin-SpyCatcher required for saturation of the SpyTag sites for coupling of StrepTactin-SpyCatcher onto the CHO.K1 CD48/Spy ligand cells. Approximately 1 μM of the tetravalent StrepTactin-SpyCatcher was sufficient to saturate the binding sites (Appendix A.1). This was in contrast to the monovalent form of the ligand, which still increased in staining from coupling of 4 μM to 8 μM of the monovalent StrepTactin-SpyCatcher (Appendix

A.2). These results were used to inform the design of stimulation experiments using the two-ligand system.

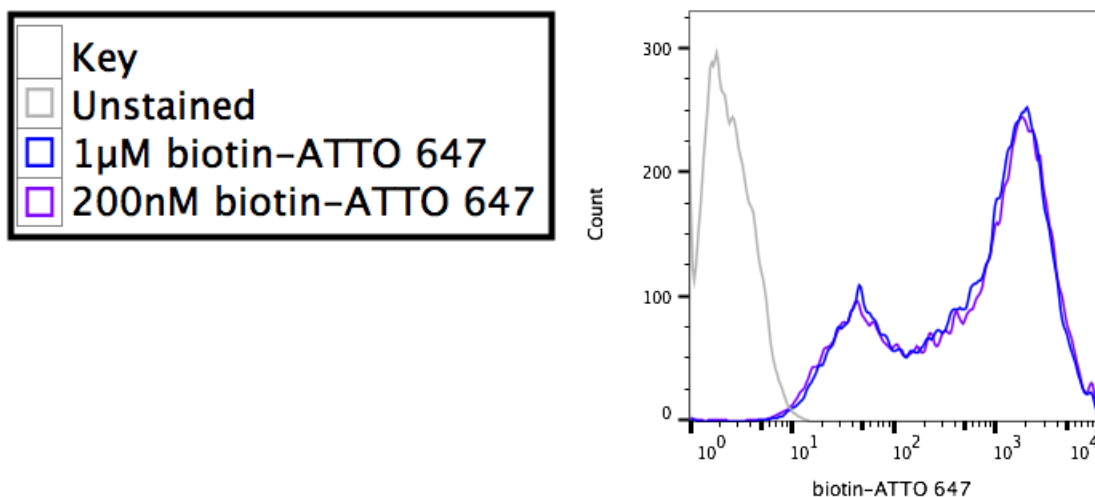


Figure 3.11: Flow cytometry results showing *CHO.K1 SCD/Spy* ligand cells coupled with $20\ \mu\text{M}$ monovalent *StrepTactin-SpyCatcher* and either unstained (grey) or stained with $1\ \mu\text{M}$ (blue) or $200\ \text{nM}$ (purple) of biotin-ATTO 647.

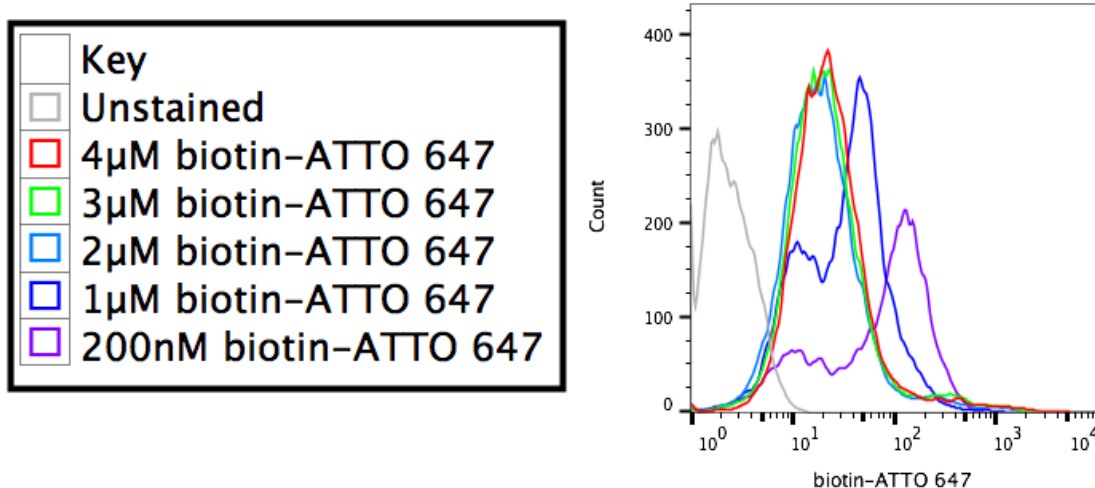


Figure 3.12: Flow cytometry results showing *CHO.K1 SCD/Spy* ligand cells coupled with $20\ \mu\text{M}$ tetravalent *StrepTactin-SpyCatcher* and either unstained (grey) or stained with the indicated concentration of biotin-ATTO 647.

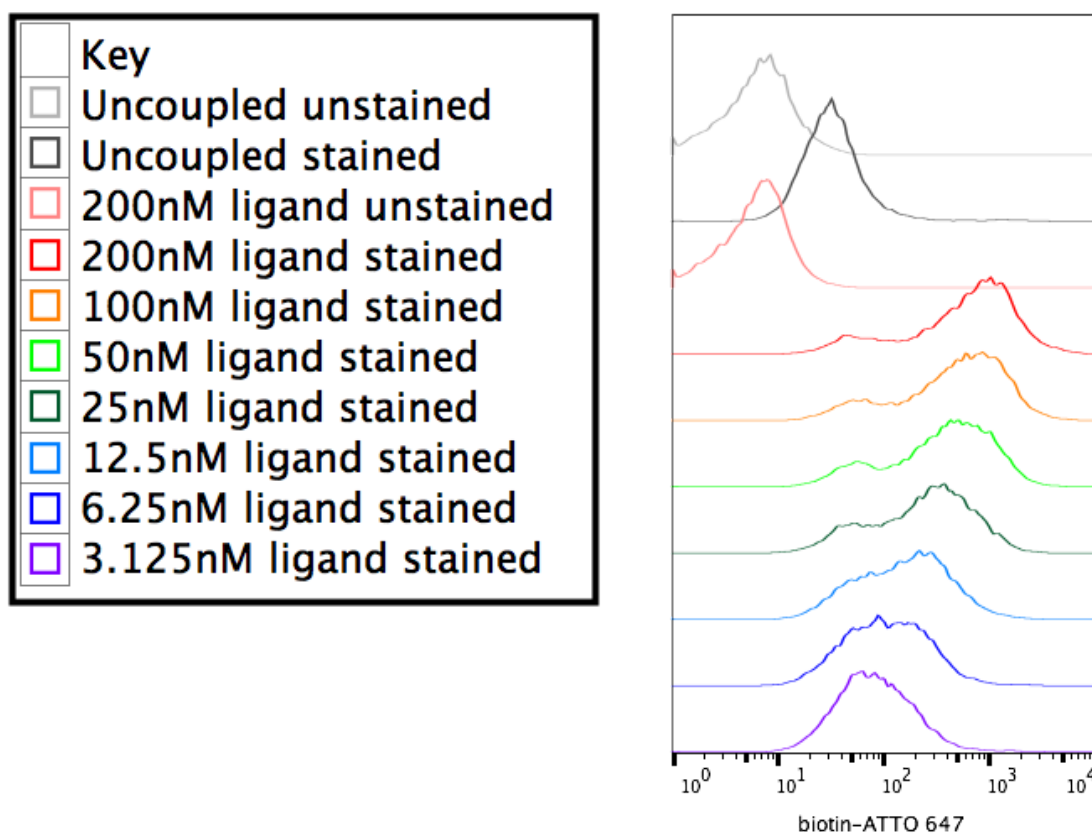


Figure 3.13: Flow cytometry results of CHO.K1 SCD/Spy ligand cells coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647, along with several controls including unstained uncoupled cells (light grey), stained uncoupled cells (dark grey), and unstained coupled cells (pink).

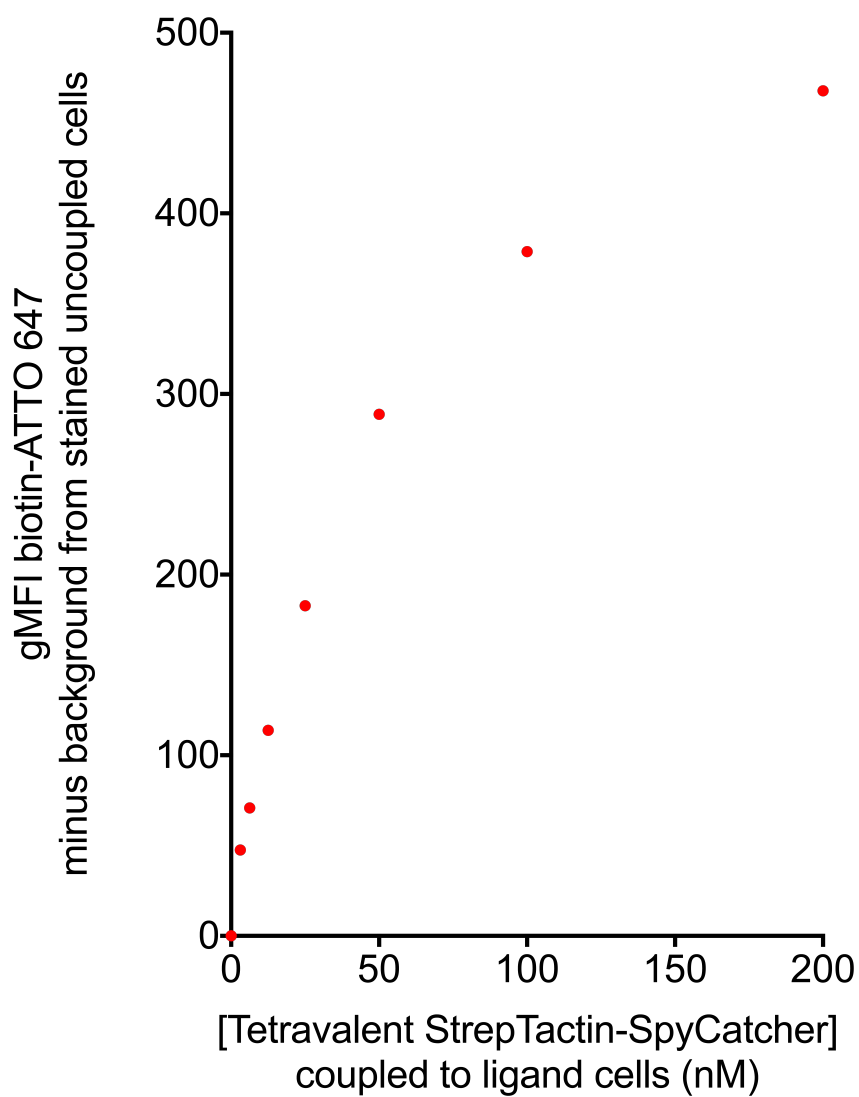


Figure 3.14: Quantification of flow cytometry results from Figure 3.13, showing the gMFI of CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647.

3.3.4 Stimulation assay development

To investigate NTR signal integration using the two-ligand system, appropriate stimulation assays are required to measure activation of the THP-1 and Jurkat receptor cells.

3.3.4.1 Stimulation of THP-1 receptor cells

A commonly used measure of activation of monocytic cells is production of the pro-inflammatory chemokine, IL-8 (Baggiolini *et al.* (1989)). The monocyte-like THP-1 cell line produces low levels of IL-8 upon stimulation, but differentiation with vitamin D towards a more macrophage-like phenotype has been shown to increase IL-8 production by these cells (Daigneault *et al.* (2010)). Therefore, differentiation of the THP-1 receptor cells with 100 nM vitamin D for 3 days prior to stimulation was used to improve IL-8 production levels, which can then be measured by IL-8 ELISA.

Platebound antibody stimulation of THP-1 receptor cells

THP-1 cells can be stimulated with platebound antibody against endogenous CD64. It is important to note that for antibody-based stimulation of THP-1 cells it is crucial to keep total IgG levels constant using isotype control antibodies to control for any stimulation via Fc receptors.

Stimulation of vitamin D-differentiated THP-1 cells with a level of 2.5 $\mu\text{g}/\text{mL}$ of anti-CD64 plus 10 $\mu\text{g}/\text{mL}$ IgG1 isotype control resulted in substantial IL-8 production above background levels as measured by IL-8 ELISA (Figure 3.15). Stimulation with culture plate surfaces coated with 2.5 $\mu\text{g}/\text{mL}$ of anti-CD64 plus 10 $\mu\text{g}/\text{mL}$ anti-Strep-tag II to engage the PD-1 Strep-tag II resulted in statistically significant inhibition of IL-8 production in the THP-1 2B4-CD64/PD-1 Strep-tag II cells but not the PD-1 control cells ($P = 0.0006$ and $P = 0.3400$ respectively; Figure 3.15). These results indicate that PD-1 Strep-tag II can be specifically engaged through the Strep-tag II tag and is able to inhibit CD64-mediated IL-8 production in this system.

Platebound antibody stimulations of the THP-1 receptor cells will be used throughout the current study as controls and also to complement the cell-cell stimulation assays.

Cell-cell stimulation of THP-1 receptor cells with the two-ligand system

To test cell-cell stimulation of THP-1 receptor cells in the two-ligand system, THP-

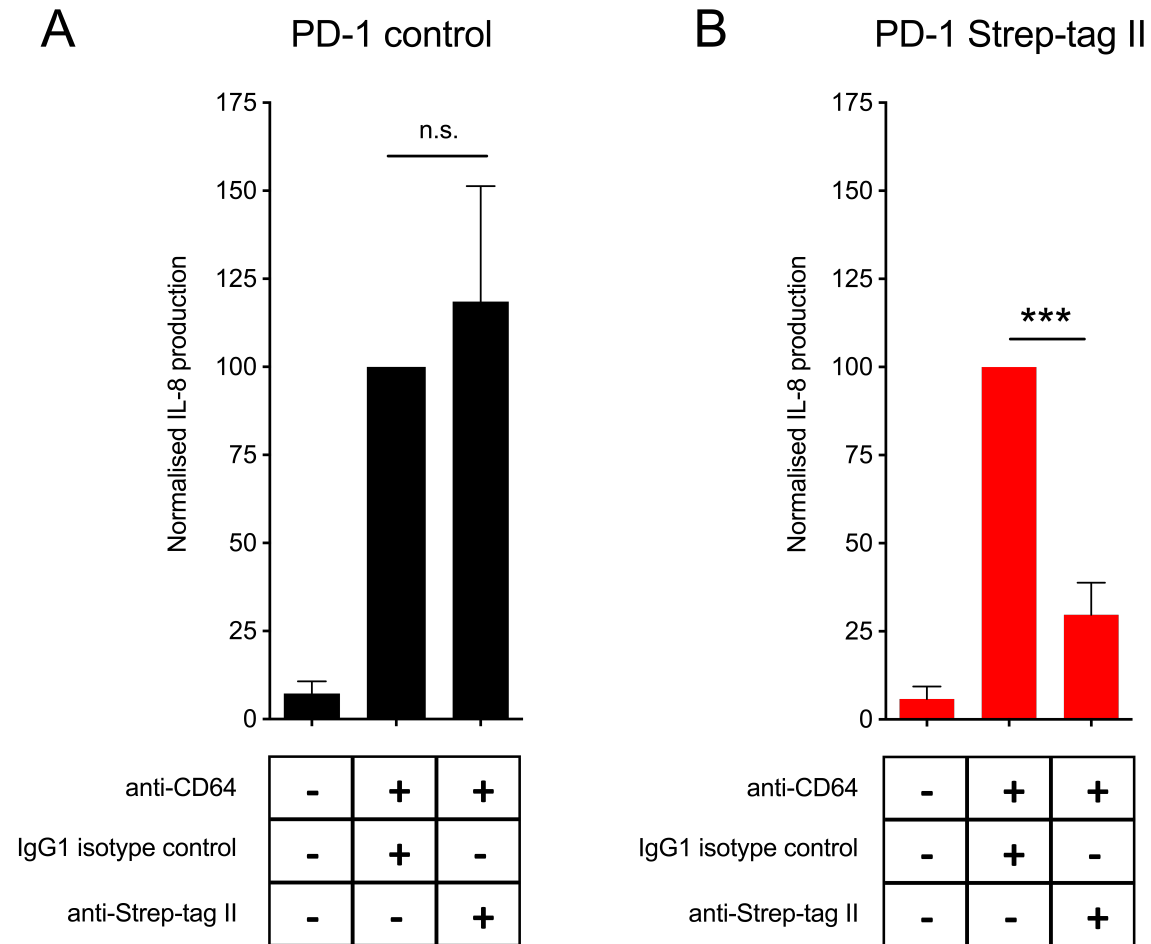


Figure 3.15: *IL-8 production from stimulation of vitamin D-differentiated THP-1 receptor cells expressing 2B4-CD64 and either PD-1 control (panel A) or PD-1 Strep-tag II (panel B). The THP-1 cells were unstimulated (PBS + 1% BSA), or stimulated in wells coated with 2.5 µg/mL anti-CD64 plus 10 µg/mL of either IgG1 isotype control or anti-Strep-tag II as indicated. IL-8 levels in supernatant were measured by ELISA and normalised to anti-CD64 stimulation without anti-Strep-tag II. Results show mean plus standard deviation from four independent experiments, each performed with biological replicates of each experimental condition. n.s. = $P > 0.05$; *** = $P \leq 0.001$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.*

1 receptor cells expressing 2B4-CD64 and PD-1 Strep-tag II or PD-1 control were stimulated with CHO.K1 ligand cells expressing CD48 and the Spy construct for coupling of soluble StrepTactin-SpyCatcher.

THP-1 cell activation was measured by assessing the supernatant for IL-8 by ELISA. As seen in Figure 3.16, the THP-1 2B4-CD64/PD-1 receptor cells were successfully activated by the CHO.K1 CD48/Spy ligand cells, with a substantial increase in IL-8 production compared to culturing with untransfected CHO.K1 cells.

Coupling of 200 nM tetravalent StrepTactin-SpyCatcher to the CHO.K1 CD48/Spy ligand cells to engage the PD-1 Strep-tag II resulted in a trend of inhibition of IL-8 production (panel B, Figure 3.16). This trend of inhibition was not observed with coupling of up to 7 μ M monovalent StrepTactin-SpyCatcher, despite successful coupling of the ligand as confirmed by staining with biotin-ATTO 647 (Appendix A.3).

Stimulation of THP-1 2B4-CD64/PD-1 control cells with CHO.K1 ligand cells coupled with either monovalent or tetravalent StrepTactin-SpyCatcher did not result in inhibition of IL-8 production (panel A, Figure 3.16), indicating that the trend of inhibition observed with the PD-1 Strep-tag II cells is acting specifically through the Strep-tag II-tagged PD-1. Importantly, as with the stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II cells with monovalent StrepTactin-SpyCatcher, there was an apparent increase in IL-8 production upon stimulation of the THP-1 2B4-CD64/PD-1 control cells using ligand cells coupled with either monovalent or tetravalent StrepTactin-SpyCatcher (panel A, Figure 3.16). Although the exact mechanism for this effect is not currently known, this non-specific increase in activation upon presentation of StrepTactin-SpyCatcher may be a factor in the only modest amount of inhibition observed with engagement of the PD-1 Strep-tag II with tetravalent ligand. This highlights an area for further investigation and development of the system.

Overall these cell-cell results provide preliminary evidence that PD-1 Strep-tag II can be engaged via the Strep-tag II tag to inhibit CD64-mediated activation in the two-ligand system. This approach will be used in subsequent chapters to confirm whether the modest inhibition observed here is statistically significant and for further investigation of NTR signal integration in the THP-1 receptor cells. Due to the lack of inhibition observed with coupling of monovalent StrepTactin, we will focus on use of the tetravalent StrepTactin-SpyCatcher to provide inhibitory signals via the Strep-tag II-tagged PD-1.

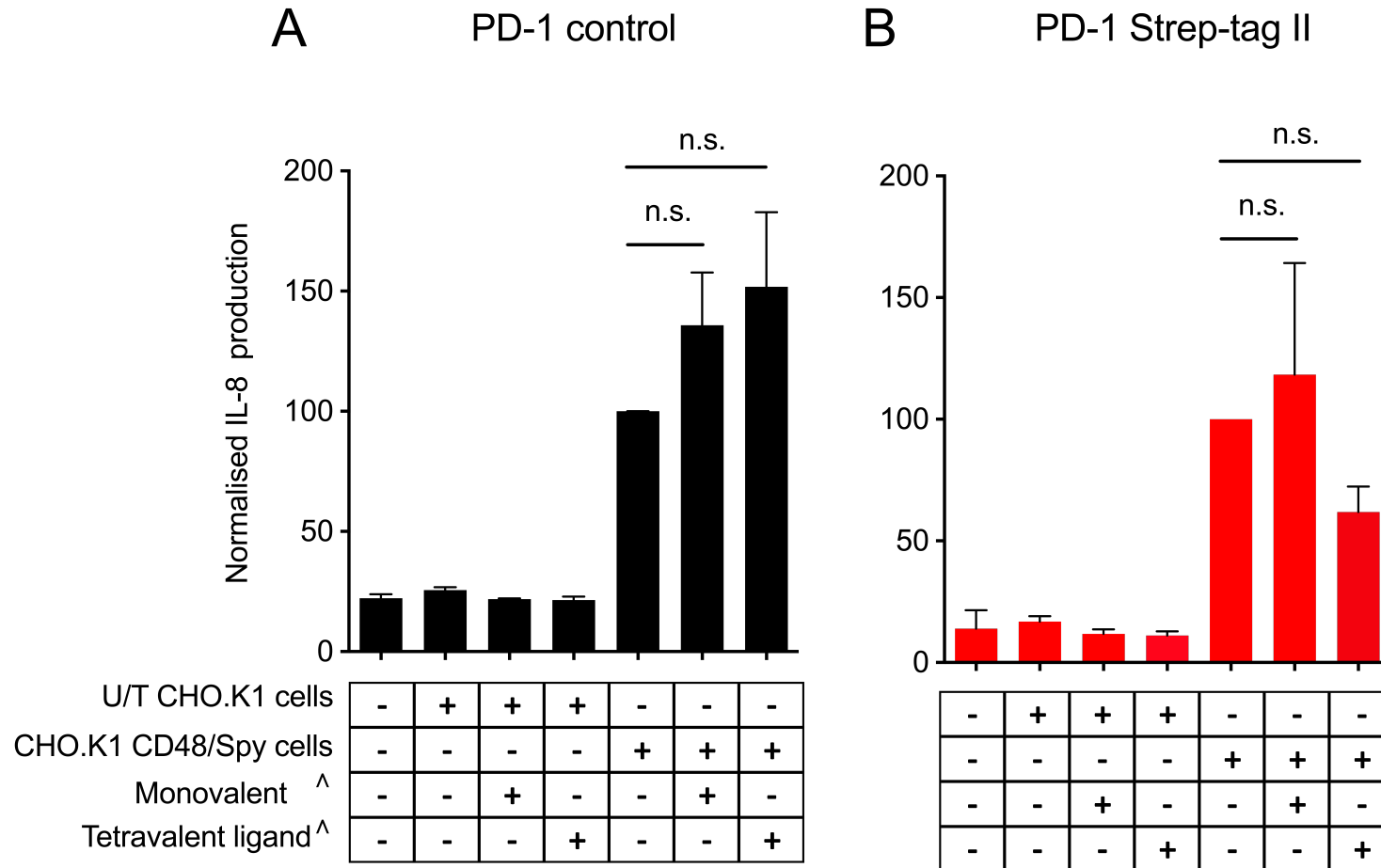


Figure 3.16: *IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 2B4-CD64/PD-1 receptor cells in the two-ligand system. Cells expressing PD-1 control (panel A) or PD-1 Strep-tag II (panel B) were unstimulated (PBS + 1% BSA), or stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48/Spy ligand cells, either uncoupled or coupled with 7 μ M monovalent StrepTactin-SpyCatcher or 200 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in supernatant were measured by ELISA. Results show mean plus standard deviation from two independent experiments, each performed with biological replicates of each experimental condition. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100. [^]Ligand = StrepTactin-SpyCatcher.*

3.3.4.2 Stimulation of Jurkat receptor cells

Activation of T cells such as the Jurkat receptor cells used in the current work can be measured by quantifying upregulation of transcriptional regulators (Jutz *et al.* (2016)). One such regulator is NF- κ B, a protein complex that upon T cell activation relocates to the nucleus and acts as a transcription factor for a range of genes required for T cell proliferation, survival, and cytokine production (Li & Verma (2002)). A Jurkat cell line with an NF- κ B-eGFP reporter, which upregulates eGFP expression upon activation of NF- κ B, was recently generated and was kindly provided for the current work (Ratzinger *et al.* (2014)). Activation of these Jurkat cells can be measured by analysing NF- κ B-eGFP expression by flow cytometry. In addition to the NF- κ B-eGFP reporter, the Jurkat reporter cells used here also express the 1G4 TCR and the CD8 co-receptor.

As shown in Figure 3.2, these Jurkat NF- κ B-eGFP/1G4 TCR cells upregulate expression of endogenous PD-1 upon activation, such as after 24h platebound antibody stimulation with 0.5 μ g/mL anti-CD3. Therefore, to specifically engage the transduced PD-1 constructs for analysis of signal integration we can engage them via the N-terminal Strep-tag II tag. This may be achieved through platebound antibody stimulation using anti-CD3 plus anti-Strep-tag II or using the cellular two-ligand system.

Platebound antibody stimulation of Jurkat receptor cells

Platebound antibody stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR receptor cells showed that approximately 0.5 - 1 μ g/mL of platebound anti-CD3 (UCHT1) is a suitable concentration for stimulation of these cells (Appendix A.4).

Based on this, Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with or without expression of the PD-1 Strep-tag II construct were stimulated with 0.5 μ g/mL anti-CD3 plus 10 μ g/mL IgG1 isotype control or 10 μ g/mL anti-Strep-tag II. Analysis of NF- κ B-eGFP upregulation by flow cytometry 24h later showed strong eGFP upregulation upon anti-CD3 stimulation (Figure 3.17). Addition of anti-Strep-tag II to engage the PD-1 Strep-tag II construct resulted in statistically significant inhibition of this NF- κ B-eGFP upregulation (panel B, Figure 3.17; $P = 0.0119$), indicating that PD-1 Strep-tag II is able to inhibit CD3-mediated activation in this system. This inhibition was not observed with Jurkat NF- κ B-eGFP/1G4 TCR reporter cells without expression of the PD-1 Strep-tag II construct (panel A, Figure 3.17).

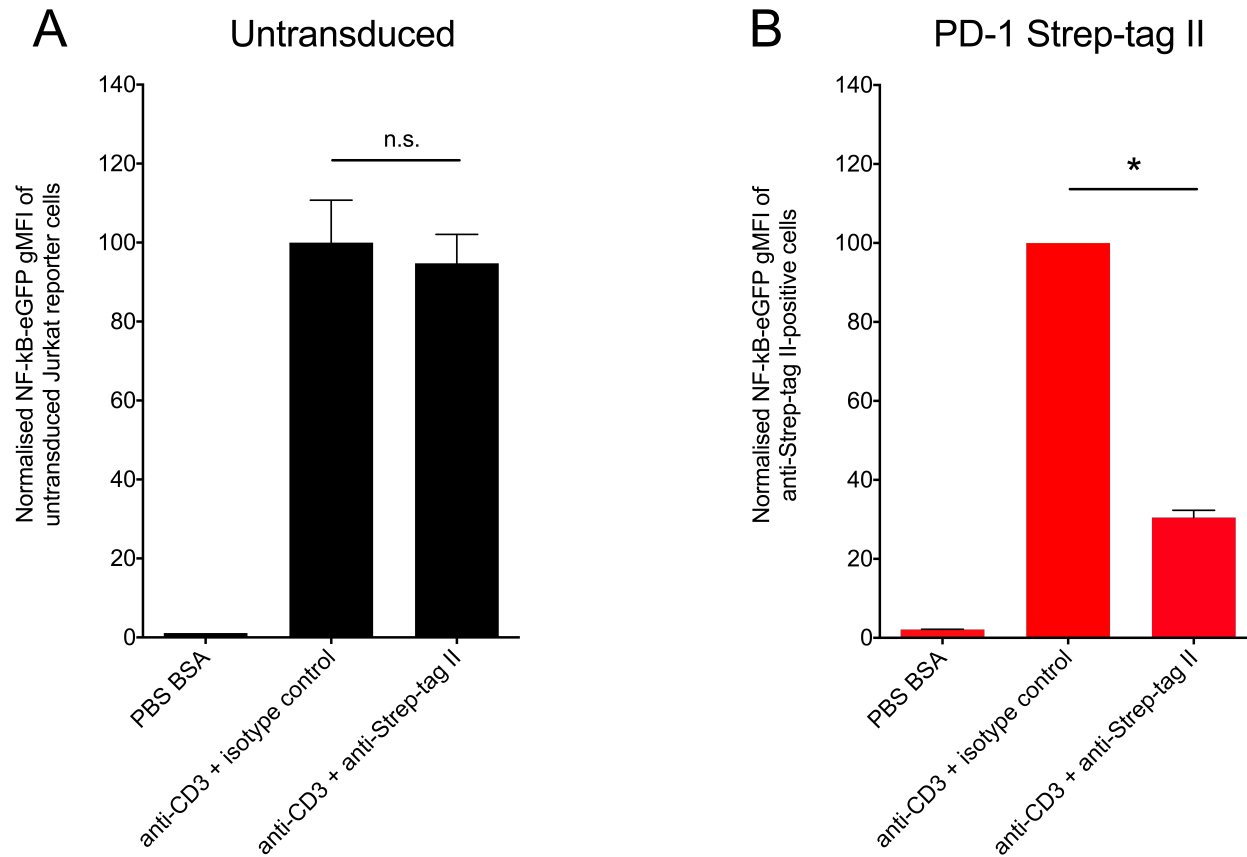


Figure 3.17: Quantification of flow cytometry results showing NF- κ B-eGFP gMFI from platebound antibody stimulation of Jurkat NF- κ B-eGFP/1G4 TCR reporter cells. Untransduced Jurkat NF- κ B-eGFP/1G4 TCR reporter cells (panel A) or Jurkat NF- κ B-eGFP/1G4 TCR reporter cells transduced with PD-1 Strep-tag II (panel B) were unstimulated (PBS + 1% BSA), or stimulated with 0.5 μ g/mL anti-CD3 plus 10 μ g/mL IgG1 isotype control or 10 μ g/mL anti-Strep-tag II as indicated. Results show mean plus standard deviation from two independent experiments, each performed with biological replicates of each experimental condition. n.s. = $P > 0.05$; * = $P \leq 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

As with the THP-1 receptor cells, platebound antibody stimulations of the Jurkat cells will be used as controls and also to complement the cell-cell stimulation assays using the two-ligand system.

Cell-cell stimulation of Jurkat receptor cells with the two-ligand system

For cell-cell stimulation of the Jurkat receptor cells, CHO.K1 ligand cells expressing the HLA-A2 SCD were exogenously loaded with cognate peptide (NY-ESO-1 C9V; SLLMWITQV) by addition of peptide to the culture media to activate the Jurkat cells through engagement of the 1G4 TCR. When analysing the cells by flow cytometry to assess NF- κ B-eGFP upregulation, the CHO.K1 ligand cells were excluded by staining with anti-human CD45-Alexa 647 and gating on CD45-positive cells.

It was determined that approximately 10 - 100 nM of exogenously loaded NY-ESO-1 C9V peptide is a suitable concentration for stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR reporter cells (Appendix A.5). A concentration of 10 nM of C9V peptide was then used to load CHO.K1 SCD/Spy cells for stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 control or PD-1 Strep-tag II.

After 24h stimulation, the NF- κ B-eGFP gMFI of the Jurkat receptor cells stimulated with peptide-loaded CHO.K1 ligand cells was greatly increased compared to incubation with untransfected CHO.K1 cells (Figure 3.18). Coupling of tetravalent StrepTactin-SpyCatcher to the CHO.K1 ligand cells to engage the PD-1 Strep-tag II through the Strep-tag II resulted in statistically significant inhibition of NF- κ B-eGFP upregulation (panel B, Figure 3.18; $P = 0.0109$). Stimulation of the Jurkat receptor cells with CHO.K1 ligand cells coupled with monovalent StrepTactin-SpyCatcher resulted in an apparent increase in NF- κ B-eGFP gMFI compared to stimulation with uncoupled CHO.K1 ligand cells (panel B, Figure 3.18).

Stimulation of Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 control cells with CHO.K1 ligand cells coupled with either monovalent or tetravalent StrepTactin-SpyCatcher did not result in inhibition of NF- κ B-eGFP gMFI (panel A, Figure 3.18). This once again shows that the apparent inhibition observed for the PD-1 Strep-tag II cells is acting specifically through engagement of the Strep-tag II-tagged PD-1.

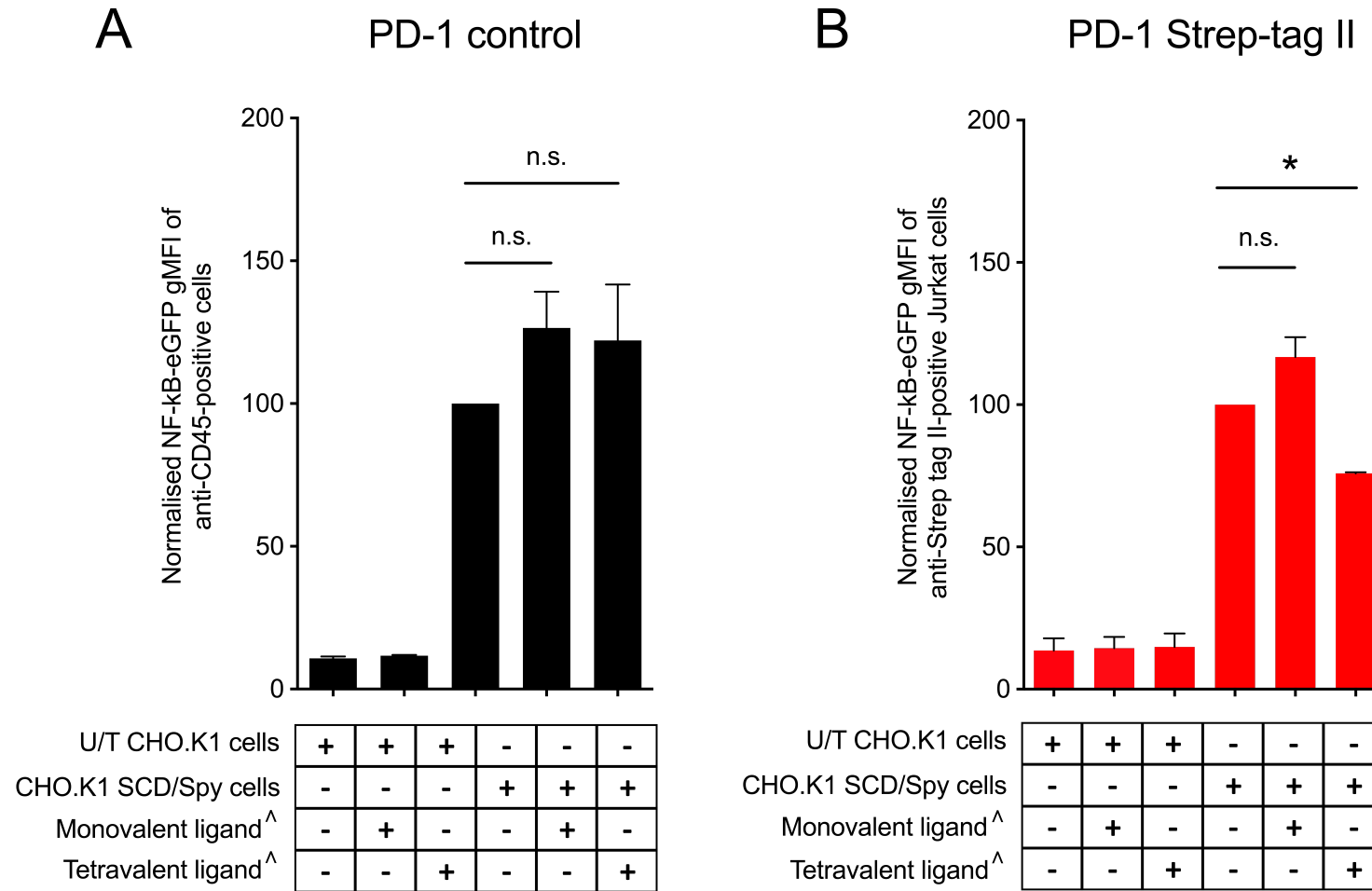


Figure 3.18: Quantification of flow cytometry results showing NF-κB-eGFP gMFI from cell-cell stimulation of Jurkat NF-κB-eGFP/1G4 TCR reporter cells in the two-ligand system. Jurkat NF-κB-eGFP/1G4 TCR reporter cells transduced with PD-1 control (panel A) or PD-1 Strep-tag II (panel B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells loaded with 10 nM NY-ESO-1 C9V peptide and either uncoupled or coupled with 7 μM monovalent StrepTactin-SpyCatcher or 200 nM tetravalent StrepTactin-SpyCatcher as indicated. PD-1 control cells were gated on using anti-human CD45-Alexa 647; PD-1 Strep-tag II cells (unsorted) were gated on using anti-Strep-tag II plus goat anti-mouse IgG1-Alexa 647. Results show mean plus standard deviation from two independent experiments, each performed with biological replicates of each experimental condition. n.s. = $P > 0.05$; * = $P \leq 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100. [^]Ligand = StrepTactin-SpyCatcher.

As with the results from the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells stimulated with monovalent StrepTactin-SpyCatcher (Figure 3.18), there was an apparent increase in NF- κ B-eGFP gMFI upon culturing the PD-1 control cells with ligand cells coupled with StrepTactin-SpyCatcher (panel A, Figure 3.18). This also reflects the results found with the cell-cell stimulation of the THP-1 receptor cells with the CHO.K1 CD48/Spy ligand cells (Figure 3.16).

Overall these cell-cell results provide preliminary evidence that PD-1 Strep-tag II can be specifically engaged to inhibit 1G4 TCR-mediated activation in the two-ligand system. This approach will be used in subsequent chapters for further investigation of NTR signal integration in the Jurkat receptor cells.

3.4 Chapter Discussion

This chapter outlines the development of the foundations of a generic two-ligand system for analysis of signal integration by a group of immune cell-surface receptors known as NTRs. Key features of this system that were deemed important for its utilisation include having membrane-presented ligands with physiological affinities, with one ligand for activation and a second ligand that is both generic and titratable.

The titratable feature of the generic ligand was developed using the innovative SpyTag-SpyCatcher tool developed by Zakeri *et al.* and further optimised by Li and colleagues (Zakeri *et al.* (2012); Li *et al.* (2014a)). By fusing the SpyCatcher component of the system to StrepTactin, ligand cells can be coupled with a titration of this generic ligand for Strep-tag II-tagged NTRs.

Both monocyte-like THP-1 receptor cell lines and T lymphocyte Jurkat receptor cell lines expressing NTRs of interest were generated along with CHO.K1 ligand cell lines for stimulation of the receptor cells (Figures 3.5 - 3.10). The ligand cells can be coupled with various amounts of the soluble StrepTactin-SpyCatcher and the relative number of Strep-tag II binding sites available on ligand cells can be measured by flow cytometry with biotin-ATTO 647 (Figures 3.13 and 3.14). A 3:1 mixture of 3 μ M D-biotin:biotin-ATTO 647 was used to minimise the quenching effects observed from binding of multiple fluorescent biotin molecules to tetravalent StrepTactin-SpyCatcher (Figure 3.12).

Platebound antibody and cell-cell stimulation assays were tested and optimised for both the THP-1 and Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 control or PD-1 Strep-tag II. These initial experiments show a trend of modest but specific inhibition upon engagement of the PD-1 Strep-tag II with either platebound

anti-Strep-tag II or CHO.K1 ligand cells coupled with StrepTactin-SpyCatcher (Figures 3.15 - 3.18). These results provide preliminary evidence that PD-1 Strep-tag II is functional in this system in both the THP-1 and Jurkat receptor cells.

These initial experiments will be followed up in Chapter 4 and 5 to determine if the observed inhibition in the two-ligand system is statistically significant and to investigate signal integration in different conditions e.g. with mismatched receptor-ligand dimensions or PD-1 variants with intracellular tails of different lengths.

Interestingly, only stimulation using CHO.K1 ligand cells coupled with tetravalent but not monovalent StrepTactin-SpyCatcher showed a trend of inhibition of IL-8 production and NF- κ B-eGFP gMFI in the THP-1 and Jurkat receptor cells respectively (Figures 3.16 and 3.18). This suggests that higher avidity and/or clustering may be required for the observed PD-1-mediated inhibitory effect and highlights an area for future investigation and development of the system. Alternatively, there may be steric effects that prevent or reduce binding of the Strep-tag II-tagged receptor to the monovalent StrepTactin.

Considering these results and due to time constraints we will focus on use of the tetravalent StrepTactin-SpyCatcher to provide inhibitory signals via the Strep-tag II-tagged PD-1. Nonetheless, future development of the system could attempt to increase the level of PD-1-mediated inhibition by improving the coupling of the soluble ligand component or increasing the affinity of the generic ligand-receptor interaction. Possible approaches for this work, such as use of a twin-Strep-tag II tag, are discussed in Section 6.4.

Investigating the importance of receptor-ligand dimensions for NTR signal integration

4.1 Chapter Introduction

4.1.1 The immunological synapse and extracellular dimensions

When the plasma membranes of immune cells such as T cells and antigen-presenting cells come into close apposition they can form a specialised structure known as the immune synapse or immunological synapse (IS; Grakoui (1999)). The formation of such a synapse between T cells and APCs was hypothesised by Norcross (Norcross (1984)), with supporting evidence coming from the work of Monks *et al.* (Monks *et al.* (1998)) and Dustin *et al.* (Dustin *et al.* (1998)), who showed the organisation of molecules into concentric rings at the immune cell-cell interface.

The organisation of molecules within and around the IS has been a topic of active interest for many immunologists for decades. It was first proposed by Springer that cell surface molecules in close contact areas between cells may be segregated according to the size of their extracellular domains (Springer (1990)). This hypothesis was based on the relatively large extracellular dimensions estimated for adhesion molecules such as the integrin LFA-1 bound to its ligand ICAM-1 (≈ 40 nm; Staunton *et al.* (1990)) and other large glycocalyx components including CD43 and the abundant receptor tyrosine phosphatase CD45 (Springer (1990)).

In line with this, it has recently been shown that the shortest isoform of CD45, referred to as CD45RO, has rigid extracellular domains with a length of ≈ 22 nm

(Chang *et al.* (2016)). This distance extends beyond that of TCR-antigen complexes and results in exclusion from areas of close contact (Chang *et al.* (2016)).

A crystal structure has not yet been determined for the phosphatase CD148, however, the combination of 8 - 10 fibronectin domains along with an 80 amino acid N-terminal mucin-like region points to extracellular dimensions of ≈ 55 nm (van der Merwe *et al.* (2000)). These dimensions are in agreement with size-based exclusion of CD45 and CD148 from areas of close contact in the IS, such as is proposed by the KS model introduced in Section 1.4.3.2.

4.1.2 Receptor-ligand dimensions and NTR signalling

In the context of the IS, NTRs have relatively small and strikingly similar receptor-ligand dimensions of 10 - 15 nm, pointing to a functional relevance of this shared feature (Dushek *et al.* (2012)). For instance, studies have determined the intercellular dimensions of several NTRs bound to their ligands, including the TCR (≈ 12 nm; Garboczi *et al.* (1996); Garcia *et al.* (1996)), various NK cell receptors (10 - 15 nm; Fan *et al.* (2001); Li *et al.* (2001); McCann *et al.* (2003); Li *et al.* (2011); Vivian *et al.* (2011)), PD-1 (≈ 11 nm; Zak *et al.* (2015)); CTLA-4 (≈ 15 nm; Stamper *et al.* (2001)), and CD28 (≈ 15 nm; estimated based on Stamper *et al.* (2001) and Evans *et al.* (2005)). The binding of these molecules to ligands on apposing membranes therefore forms close contact regions of around 15 nm (Shaw & Dustin (1997); Dushek *et al.* (2012)).

The role of these small receptor-ligand dimensions has been investigated for several NTRs by manipulating the dimensions of the NTR-ligand complex (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009); Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2012); Lim *et al.* (2015)), or of RPTPs (Irles *et al.* (2002); Lin & Weiss (2003); Cordoba *et al.* (2013)), as described below. Many of these studies have increased receptor-ligand dimensions relative to RPTPs through elongation of ligand to increase the intermembrane distance at sites of receptor binding, or by truncation of the ectodomain of RPTPs. These approaches prevent size-based segregation of RPTPs such as CD45 and CD148, which are then able to dephosphorylate the NTRs and proximal signalling components to inhibit signalling (Irles *et al.* (2002); Lin & Weiss (2003); Cordoba *et al.* (2013)). In general, results from these studies have demonstrated the importance of receptor-ligand dimensions for signalling of these NTRs (reviewed by Dushek *et al.* (2012)).

4.1.3 An activating NTR: the T cell receptor

In the case of the TCR, it has been shown that the tyrosine phosphatases CD45 and CD148 are excluded from areas of ligated TCR (Bunnell *et al.* (2002); Varma *et al.* (2006)). Both elongation of receptor-ligand dimensions (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009)) as well as truncation of the CD45 or CD148 ectodomains (Irles *et al.* (2002); Lin & Weiss (2003); Cordoba *et al.* (2013)) have been shown to prevent segregation of these phosphatases and abrogate TCR signalling. Substituting the CD45 or CD148 ectodomain with the similarly large and bulky ectodomain of the glycoprotein CD43 (leukosialin) rescued TCR signalling compared to the truncated versions (Irles *et al.* (2002); Cordoba *et al.* (2013)). This indicates that exclusion of CD45 and CD148 from areas of close contact and subsequent TCR signalling is dependent on RPTPs having a large ectodomain.

The importance of receptor-ligand dimensions has also been shown for chimeric receptors such as chimeric antigen receptors (CARs) and bispecific T cell engagers (BiTEs), with epitope position (e.g. membrane distal or proximal) having a marked impact on the efficiency of receptor-mediated T cell activation (Hombach *et al.* (2007); Bluemel *et al.* (2010); reviewed by Srivastava & Riddell (2015)).

4.1.4 A co-stimulatory NTR: CD28

As with the TCR, elongation of the CD28 ligand CD80 abrogated segregation of the phosphatase CD45 from CD80 at cell-cell contacts (Lim *et al.* (2015)). Ligand elongation also reduced CD28-mediated co-stimulation, but this was not due to mismatched receptor-ligand dimensions as demonstrated by a trans co-stimulation assay. It was shown that mutation of the CD28 YxNM signalling motif to prevent its phosphorylation reduced the sensitivity of CD28 to elongation of CD80, suggesting that in this case ligand elongation is affecting YxNM-mediated signalling (Lim *et al.* (2015)).

4.1.5 Co-inhibitory NTRs: NK cell receptors and PD-1

Studies of signal integration between activating and co-inhibitory NTRs have highlighted the requirement of receptor co-localisation for effective signal integration for some NTR pairs, with this co-localisation being dependent on well-matched receptor-ligand dimensions (Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2012)), as discussed in further detail below. These results are in agreement with membrane proximal NTR signalling, whereby recruited molecules such as cytoplasmic kinases and phosphatases act on nearby membrane proximal substrates. Such membrane

proximal signal integration would require close co-localisation of the NTRs and could be important for directional immune responses such as release of cytotoxic granules (reviewed by Dustin & Long (2010)).

NK cell receptors

A strong case for the importance of matched receptor-ligand dimensions for NTR signal integration has been provided for the human activating NK cell receptor NKG2D and the co-inhibitory KIR2DL1 (Köhler *et al.* (2010)). The function of both of these receptors was abrogated by insertion of inert immunoglobulin spacers into their ligands, despite ligand binding not being affected. Moreover, it was shown that similar receptor-ligand dimensions were required for co-localisation and signal integration between NKG2D and KIR2DL1. Interestingly, when ligands for both receptors were elongated to the same extent, co-localisation and signal integration were partially restored (Köhler *et al.* (2010)). This provides strong support for the importance of well-matched receptor-ligand dimensions and co-localisation for signal integration between these receptors.

Results from investigation of the equivalent murine activating and inhibitory NK cell receptors, NKG2D and Ly49C, also showed a requirement for small receptor-ligand dimensions for optimal signalling of these receptors (Brzostek *et al.* (2010)).

PD-1

Similar studies have been carried out for the inhibitory receptor PD-1, a member of the CD28 family of receptors. PD-1 is expressed on a range of immune cells including B and T cells, natural killer cells, dendritic cells and monocytes, and is upregulated on activated B and T cells (Agata *et al.* (1996); Keir *et al.* (2008)). It has been shown to inhibit proximal signalling through the BCR and TCR upon binding of its ligands PD-L1 and PD-L2 (Freeman *et al.* (2000); Latchman *et al.* (2001); Okazaki *et al.* (2001); Sheppard *et al.* (2004)).

A crystal structure of human PD-1 bound to PD-L1 confirms the characteristic small dimensions shared by many NTRs, with the PD-1-PD-L1 complex spanning approximately 11 nm (Zak *et al.* (2015)). Elongation of murine PD-1 by insertion of between one and five Ig domains from human CD22 resulted in size-dependent exclusion of the elongated PD-1 from TCR microclusters, with PD-1 elongated by four or five Ig domains being almost completely segregated from TCR microclusters (Yokosuka *et al.* (2012)). Unlike the shorter PD-1 chimeras, the two tallest chimeras

remained unphosphorylated and were unable to recruit the phosphatase SHP-2 or inhibit TCR-mediated signalling. The inhibitory function of the two tallest chimeras was not restored by fusion of the phosphatases SHP-1 or SHP-2 directly to the intracellular tail, indicating that the lack of inhibitory function was due to more than simply a lack of phosphatase recruitment (Yokosuka *et al.* (2012)).

It has not yet been determined whether simultaneous elongation of the TCR and PD-1 receptor-ligand dimensions can rescue signal integration as was found with the NK cell receptors described above (Brzostek *et al.* (2010); Köhler *et al.* (2010)). Such an experiment would provide information as to whether ligand elongation impacts PD-1 signal integration for reasons other than mismatched receptor-ligand dimensions.

4.1.6 Use of the two-ligand system for studying signal integration between NTRs

Overall, the studies described above provide evidence for the KS model for a number of NTRs. However, with more than 120 receptors classified as NTRs, much more investigation is required to determine whether or not the KS model applies to a wide range of these receptors. It is also of interest to determine whether signal integration between NTRs, such as between activating and co-inhibitory or co-stimulatory NTRs, requires close co-localisation and therefore well-matched receptor-ligand dimensions. As discussed in Section 1.7, we propose that this is important for proximal signal integration between NTRs, many of which have been found to recruit cytoplasmic molecules to their intracellular tail to act locally (i.e. at the plasma membrane) on other NTRs and/or proximal signalling components. This may be important for the development of emerging immunotherapies involving NTRs, which may be affected by receptor-ligand dimensions and the mechanism/s of signal integration for different receptors.

Based on the evidence from the signal integration studies described above, we hypothesise that many NTRs require well-matched receptor-ligand dimensions for co-localisation and optimal signal integration (“hypothesis 1”; Figure 1.3). The two-ligand system described in Chapter 3 will be further developed so that it can be utilised for studies of receptor-ligand dimensions to test this hypothesis. For the current work we will focus on the co-inhibitory NTR PD-1 so that we can extend our understanding of this receptor while also referring to results from previous studies to confirm that the two-ligand system is functional. For example, considering the results of Yokosuka and colleagues, we would expect elongation of the PD-1 receptor-ligand

dimensions by 4 or more Ig domains to abrogate its inhibitory function (Yokosuka *et al.* (2012)).

To manipulate NTR receptor-ligand dimensions, either the receptor or its ligand can be elongated. In the case of the generic two-ligand system presented here, we manipulated the ligand dimensions so that one set of ligands and ligand cell lines can be used to study a variety of NTRs.

Elongation can be achieved by insertion of Ig domains from IgSF members such as CD2, CD4, or CD22. This approach has been used previously to study a number of NTRs (Wild *et al.* (1999); Choudhuri *et al.* (2005); Choudhuri *et al.* (2009); Brzostek *et al.* (2010); Köhler *et al.* (2010); Yokosuka *et al.* (2012); Lim *et al.* (2015)). The Ig domains are usually inserted into the unstructured stalk region of the receptor, joining the transmembrane domain to the ectodomain, to preserve structural integrity. Insertion of CD2 (two Ig domains) or CD4 (four Ig domains) is predicted to increase the length by approximately 7 nm or 12 nm respectively, as estimated from crystal structures (Garrett *et al.* (1993); Bodian *et al.* (1994); Wu *et al.* (1997)).

This approach of using Ig domains from CD2 or CD4 as spacers was applied here to generate a set of ligands with matched and mismatched dimensions for use with the THP-1 and Jurkat receptor cells, with an example shown in Figure 4.1. The stimulation assays developed in Chapter 3 were then used to determine the impact of different receptor-ligand dimensions on NTR signal integration in the two-ligand system.

4.2 Chapter Aim

The aim of this chapter is to use the two-ligand system to investigate the importance of receptor-ligand dimensions for NTR signal integration.

4.3 Results

4.3.1 Generation of CHO.K1 ligand cell lines expressing ligands with matched and mismatched dimensions

Mutations were made to the human CD2 and CD4 Ig domains to prevent binding to CD58 and MHC class II respectively (Piatier-Tonneau *et al.* (1991); van der Merwe *et al.* (1995)). The Ig domains were then inserted into the CD48 and Spy constructs and cloned into the pDisplay and pEE14 vectors respectively as shown in Table 4.1. Primers used for the cloning process are detailed in Table 2.3.

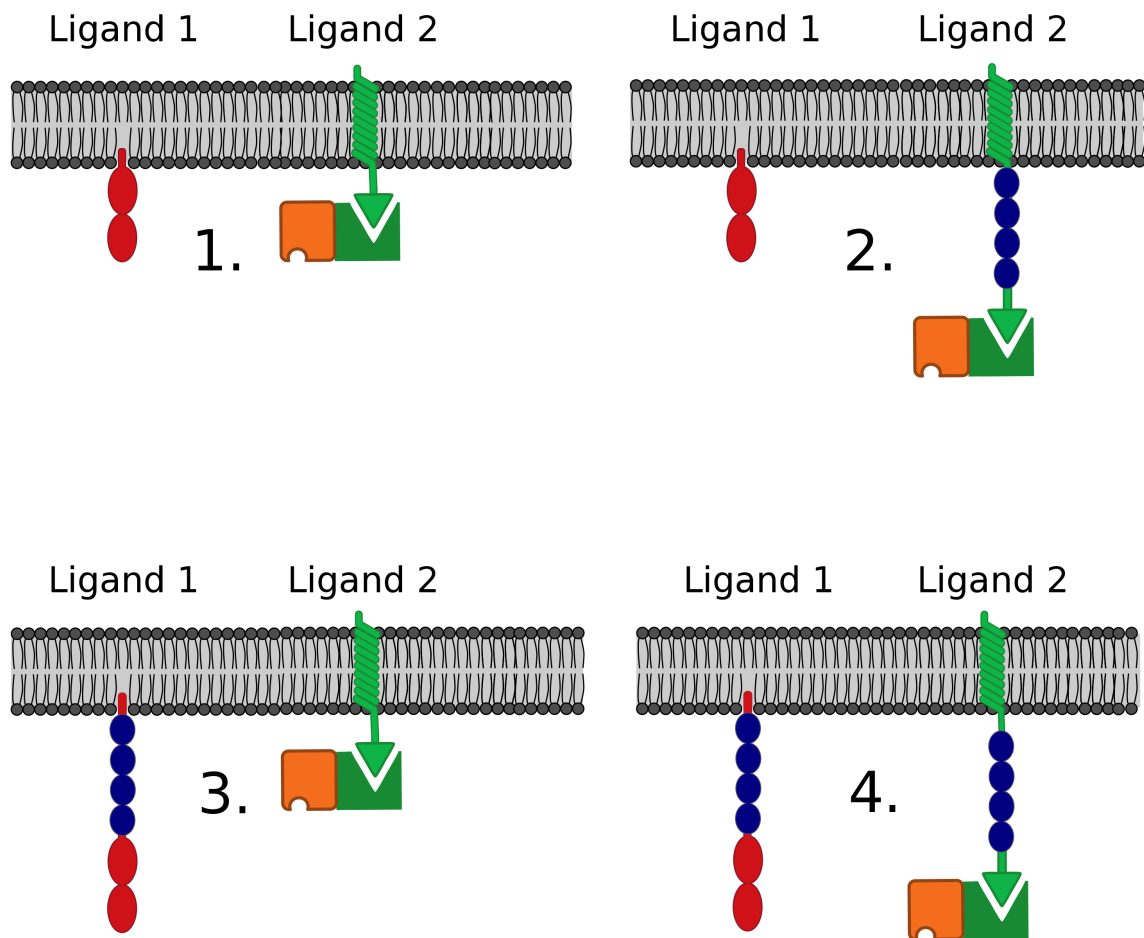


Figure 4.1: Schematic representation of four ligand cell lines expressing ligands with matched or mismatched dimensions. Inert Ig spacers are shown in blue. The four ligand cell lines shown are expressing 1. Ligand 1 = normal length activating ligand e.g. CD48 plus Ligand 2 = normal length Spy; 2. Ligand 1 = normal length activating ligand e.g. CD48 plus Ligand 2 = elongated Spy e.g. Spy-CD4; 3. Ligand 1 = elongated activating ligand e.g. CD48-CD4 plus Ligand 2 = normal length Spy; 4. Ligand 1 = elongated activating ligand e.g. CD48-CD4 plus Ligand 2 = elongated Spy e.g. Spy-CD4. NB: the CD48 constructs are GPI-anchored and the Spy-based constructs can be coupled with a soluble StrepTactin-SpyCatcher component (orange and green fusion) to act as a ligand for Strep-tag II-tagged NTRs.

Only normal-length HLA-A2 SCD was used since insertion of Ig domains into SCD renders it non-functional, possibly because elongation disrupts peptide loading (Dr Keith Gould, personal communication).

The set of normal length and elongated ligands was used to generate the ligand cell lines set out in Table 4.1. Due to variability in the effectiveness of transfection and selection procedures, the elongated Spy construct used with the CD48 constructs when stimulating the THP-1 receptor cells was Spy-CD2-CD2, whereas a co-transfection of the CHO.K1 cells with the SCD and Spy-CD4 constructs resulted in high expression levels and these cells were used to stimulate the Jurkat receptor cells. Therefore, possible differences between ligands elongated with different spacers should be considered when interpreting results.

To obtain sufficient and reasonably well-matched expression levels of the ligands in the different CHO.K1 ligand cell lines, an approach was developed that includes consecutive transfection, selection and sorting of the ligand cells (Sections 2.4.1 and 2.6). Figures 4.2 - 4.5 show the expression levels of the different ligand constructs after the full selection and sorting process.

As seen in Figures 4.2 and 4.4, the Spy expression levels were not very well matched in either the CD48-based or SCD-based ligand cell lines, despite the extensive sorting and continuous co-selection of the ligand cells using both MSX and G418.

Additionally, the expression levels of the different constructs were found to vary from week to week and to decrease over time, sometimes resulting in poorly matched expression levels. Differences in the expression levels of the Spy-based constructs can be partly overcome by titrating the amount of soluble StrepTactin-SpyCatcher coupled to the cells, however this is an area of the two-ligand system that would benefit from further development.

In contrast to the Spy expression levels, the expression levels of the activating ligands were quite well matched for both the CD48-based and SCD ligand cells, as seen in Figures 4.3 and 4.5. The expression levels of these constructs also remained quite well-matched between experiments (data not shown).

Ligand 1 (pDisplay vector)	Ligand 1 estimated receptor-ligand dimensions	Ligand 2 (pEE14 vector)	Ligand 2 estimated receptor-ligand dimensions	Relatively matched [^] or mismatched [†] dimensions
CD48	12 nm	Spy	≈ 15 nm	Matched
CD48	12 nm	Spy-CD2-CD2	≈ 29 nm	Mismatched
CD48-CD4	24 nm	Spy	≈ 15 nm	Mismatched
CD48-CD4	24 nm	Spy-CD2-CD2	≈ 29 nm	Matched
SCD	12 nm	Spy	≈ 15 nm	Matched
SCD	12 nm	Spy-CD4	≈ 27 nm	Mismatched

Table 4.1: *Ligands expressed by CHO.K1 ligand cells for testing the importance of receptor-ligand dimensions for NTR signal integration. Estimated receptor-ligand dimensions are shown for the receptors that each ligand will be used with in the two-ligand system, i.e. 2B4-CD64, 1G4 TCR, and PD-1 as receptors for the CD48-, SCD-, and Spy-based ligands respectively. SCD = HLA-A2 single chain dimer. [^]Matched = estimated to be less than 5 nm difference in receptor-ligand dimensions. [†]Mismatched = estimated to be more than 9 nm difference in receptor-ligand dimensions. Dimensions have been estimated based on known crystal structures and related evidence for 1G4-HLA-A2 (Chen et al. (2005)), CD48-2B4 (Velikovsky et al. (2007)), CD2 (Bodian et al. (1994)), CD4 (Garrett et al. (1993); Wu et al. (1997)), SpyTag-SpyCatcher-StrepTactin-PD-1 (Hendrickson et al. (1989) (Streptavidin); Oke et al. (2010) (SpyTag-SpyCatcher); Zhang et al. (2004), Cheng et al. (2013), and Zak et al. (2015) (PD-1)).*

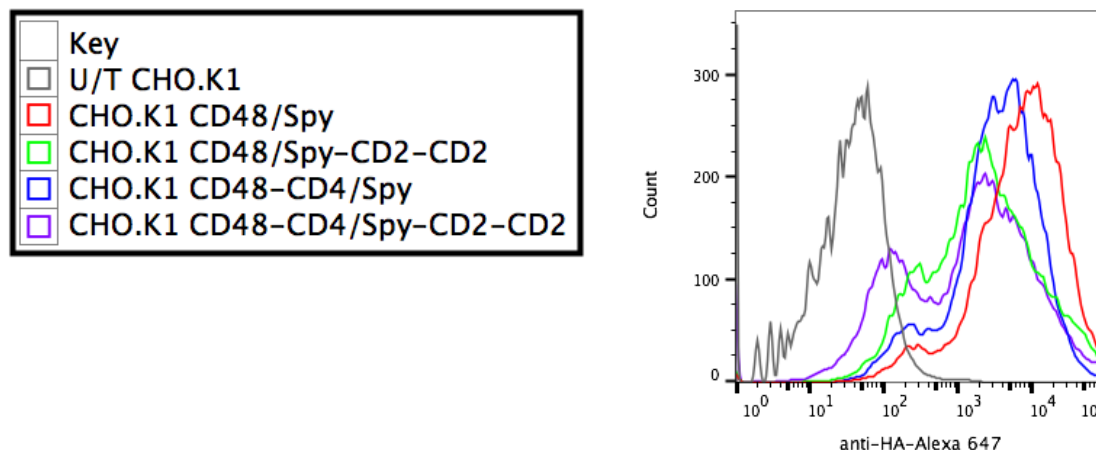


Figure 4.2: Flow cytometry results showing untransfected (U/T) CHO.K1 cells and CHO.K1 CD48-based ligand cells stained with anti-HA-Alexa 647 to detect expression of the Spy construct. Samples include U/T CHO.K1 cells (grey), CHO.K1 CD48/Spy (red), CHO.K1 CD48/Spy-CD2-CD2 (green), CHO.K1 CD48-CD4/Spy (blue) and CHO.K1 CD48-CD4/Spy-CD2-CD2 (purple) ligand cells. Results for the U/T CHO.K1 and CHO.K1 CD48/Spy cells were shown previously in Figure 3.9.

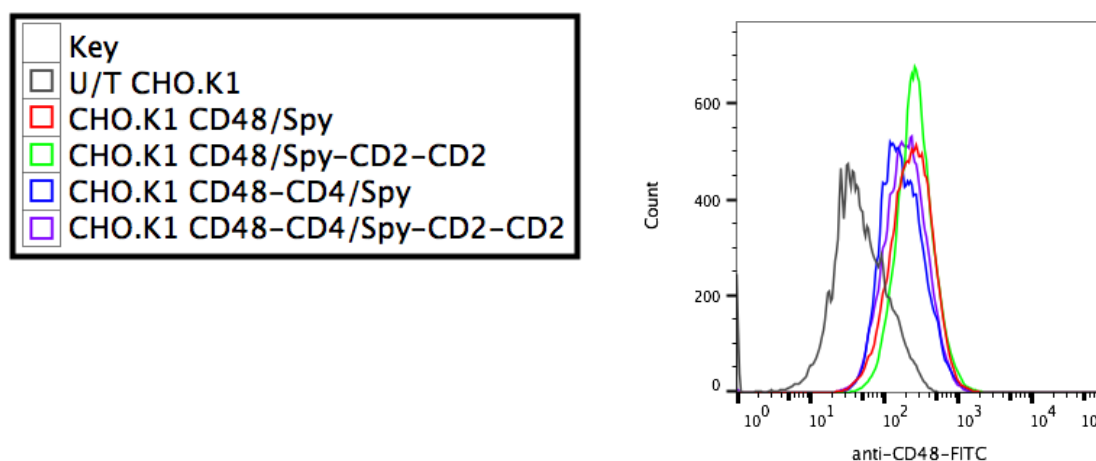


Figure 4.3: Flow cytometry results showing untransfected (U/T) CHO.K1 cells and CHO.K1 CD48-based ligand cells stained with anti-CD48-FITC. Samples include U/T CHO.K1 cells (grey), CHO.K1 CD48/Spy (red), CHO.K1 CD48/Spy-CD2-CD2 (green), CHO.K1 CD48-CD4/Spy (blue) and CHO.K1 CD48-CD4/Spy-CD2-CD2 (purple) ligand cells. Results for the U/T CHO.K1 and CHO.K1 CD48/Spy cells were shown previously in Figure 3.10.

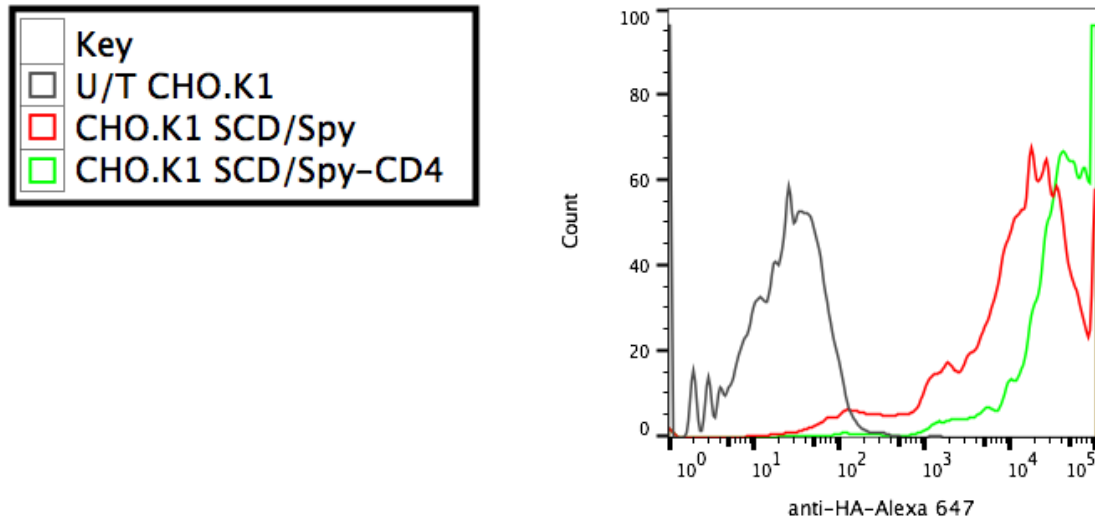


Figure 4.4: Flow cytometry results showing untransfected (U/T) CHO.K1 cells and CHO.K1 SCD-based ligand cells stained with anti-HA-Alexa 647 to detect expression of the Spy construct. Samples include U/T CHO.K1 cells (grey), CHO.K1 SCD/Spy (red), and CHO.K1 SCD/Spy-CD4 (green) ligand cells.

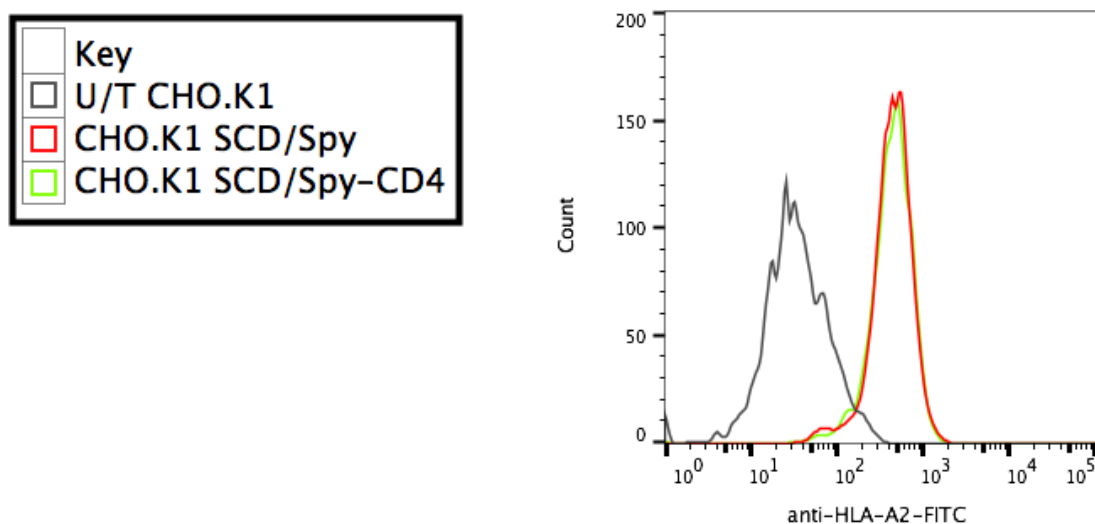


Figure 4.5: Flow cytometry results showing untransfected (U/T) CHO.K1 cells and CHO.K1 SCD-based ligand cells stained with anti-HLA-A2-FITC. Samples include U/T CHO.K1 cells (grey), CHO.K1 SCD/Spy (red), and CHO.K1 SCD/Spy-CD4 (green) ligand cells.

4.3.2 Stimulation of THP-1 receptor cells with ligand cells expressing ligands with matched and mismatched dimensions

To test the importance of receptor-ligand dimensions for PD-1 signal integration in the THP-1 two-ligand system, the THP-1 receptor cells expressing 2B4-CD64 and either PD-1 Strep-tag II or PD-1 control were stimulated using the CHO.K1 ligand cells expressing CD48 constructs and Spy constructs of different lengths as detailed in Table 4.1. Pilot experiments showed no further inhibition with concentrations higher than 60 nM tetravalent StrepTactin-SpyCatcher coupled to the CHO.K1 CD48/Spy ligand cells (Appendix B.1), so a titration from 60 nM down to 14 nM was used to try to get full dose response curves.

Coupling of the tetravalent StrepTactin-SpyCatcher to the CD48-based ligand cells was found to vary between days and also between the different ligand cell lines (Figures 4.6 - 4.11). Coupling levels were particularly poor for the second THP-1 stimulation experiment (Figure 4.8) and for the two Spy-CD2-CD2-based ligand cell lines for all three THP-1 stimulation experiments (Figures 4.6 - 4.11). These poor coupling levels were despite continuous co-selection of the ligand cells and several rounds of FACS to sort ligand cells with high and well-matched expression levels. Obtaining more consistent and more effective coupling of the StrepTactin-SpyCatcher to ligand cells is clearly an aspect of the two-ligand system that requires further development in order to be able to compare stimulation of the THP-1 receptor cells with the different CD48-based ligand cell lines.

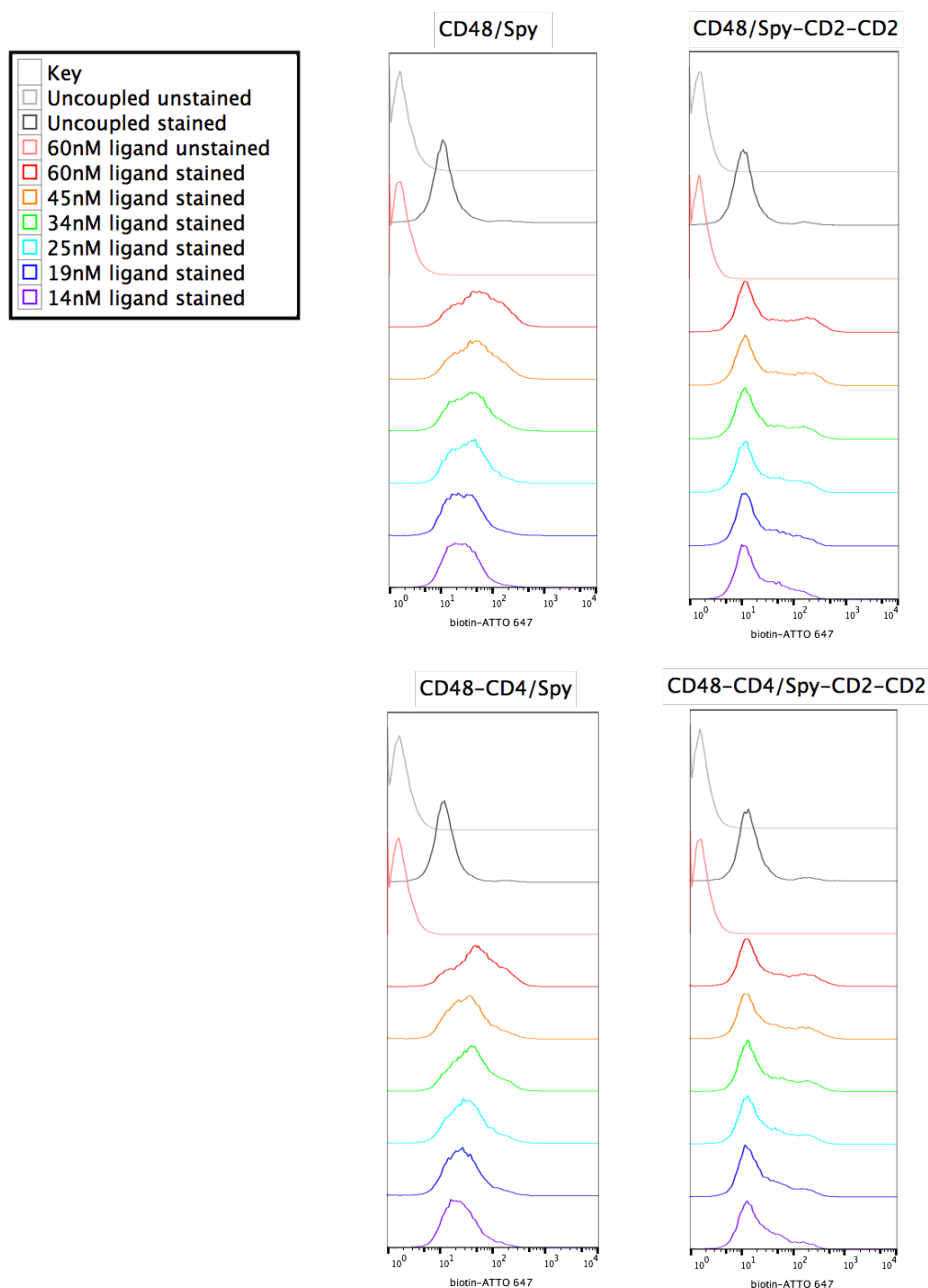


Figure 4.6: Analysis of coupling levels for the first THP-1 stimulation experiment showing flow cytometry results of different CHO.K1 CD48-based ligand cells. Ligand cells were coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher and stained with $3 \mu\text{M}$ of a 3:1 mixture of D-biotin:biotin-ATTO 647 along with several controls including unstained uncoupled cells (light grey), stained uncoupled cells (dark grey), and unstained coupled cells (pink).

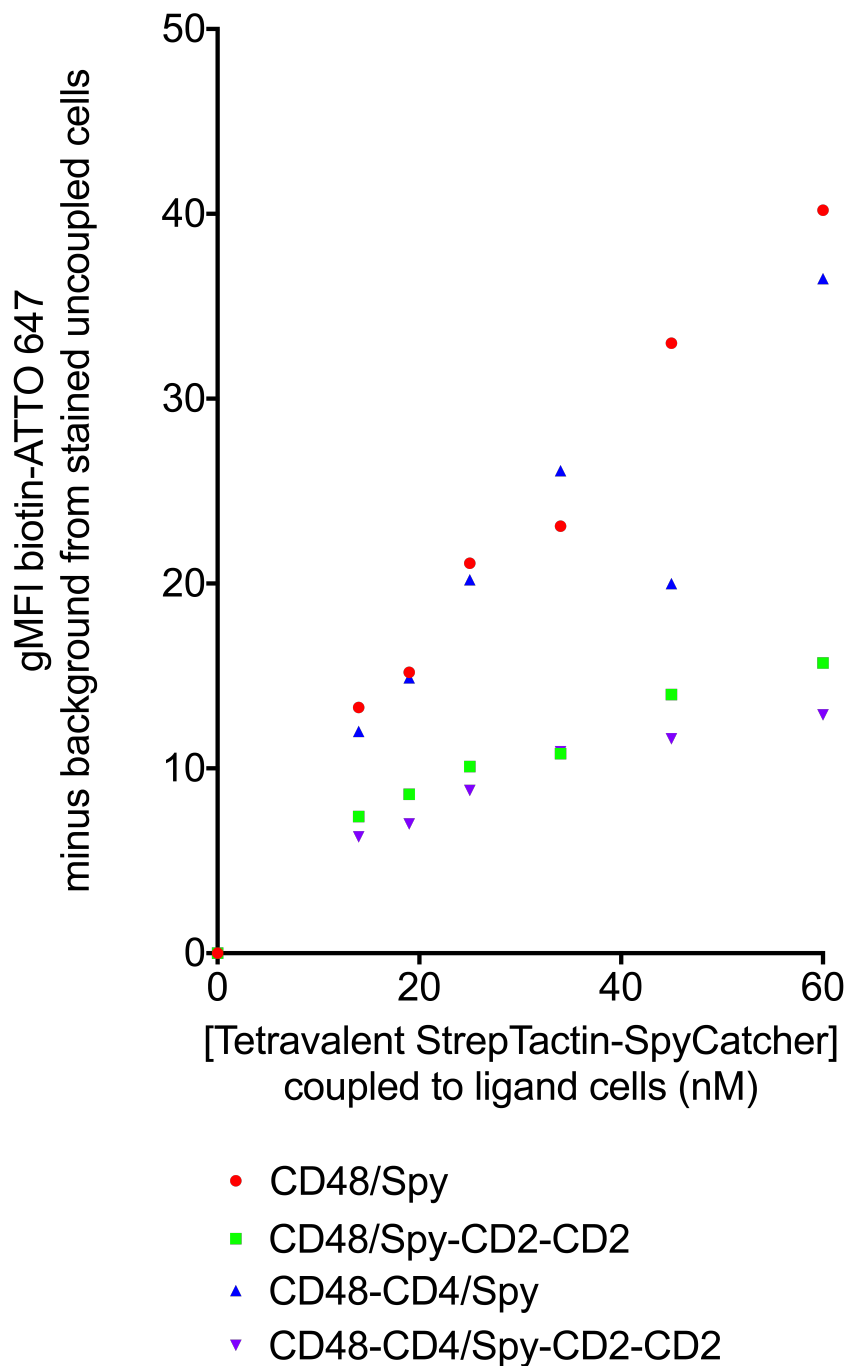


Figure 4.7: Quantification of flow cytometry results from Figure 4.6, showing the gMFI of CHO.K1 CD48/Spy (red), CD48/Spy-CD2-CD2 (green), CD48-CD4/Spy (blue) and CD48-CD4/Spy-CD2-CD2 (purple) ligand cells coupled with tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647.

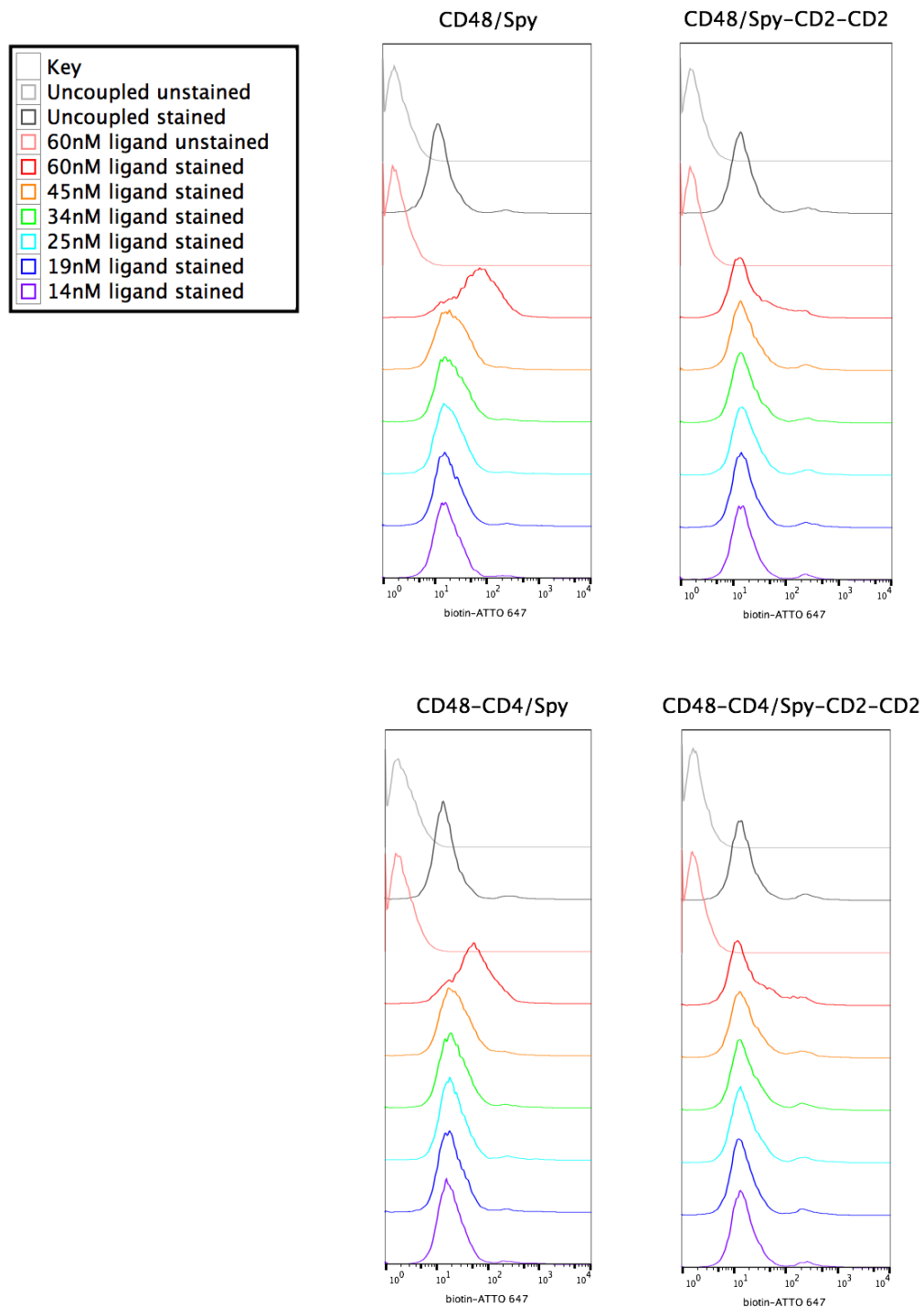


Figure 4.8: Analysis of coupling levels for the second THP-1 stimulation experiment showing flow cytometry results of different CHO.K1 CD48-based ligand cells. Ligand cells were coupled with the indicated concentration of tetraivalent StrepTactin-SpyCatcher and stained with $3 \mu\text{M}$ of a 3:1 mixture of D-biotin:biotin-ATTO 647 along with several controls including unstained uncoupled cells (light grey), stained uncoupled cells (dark grey), and unstained coupled cells (pink).

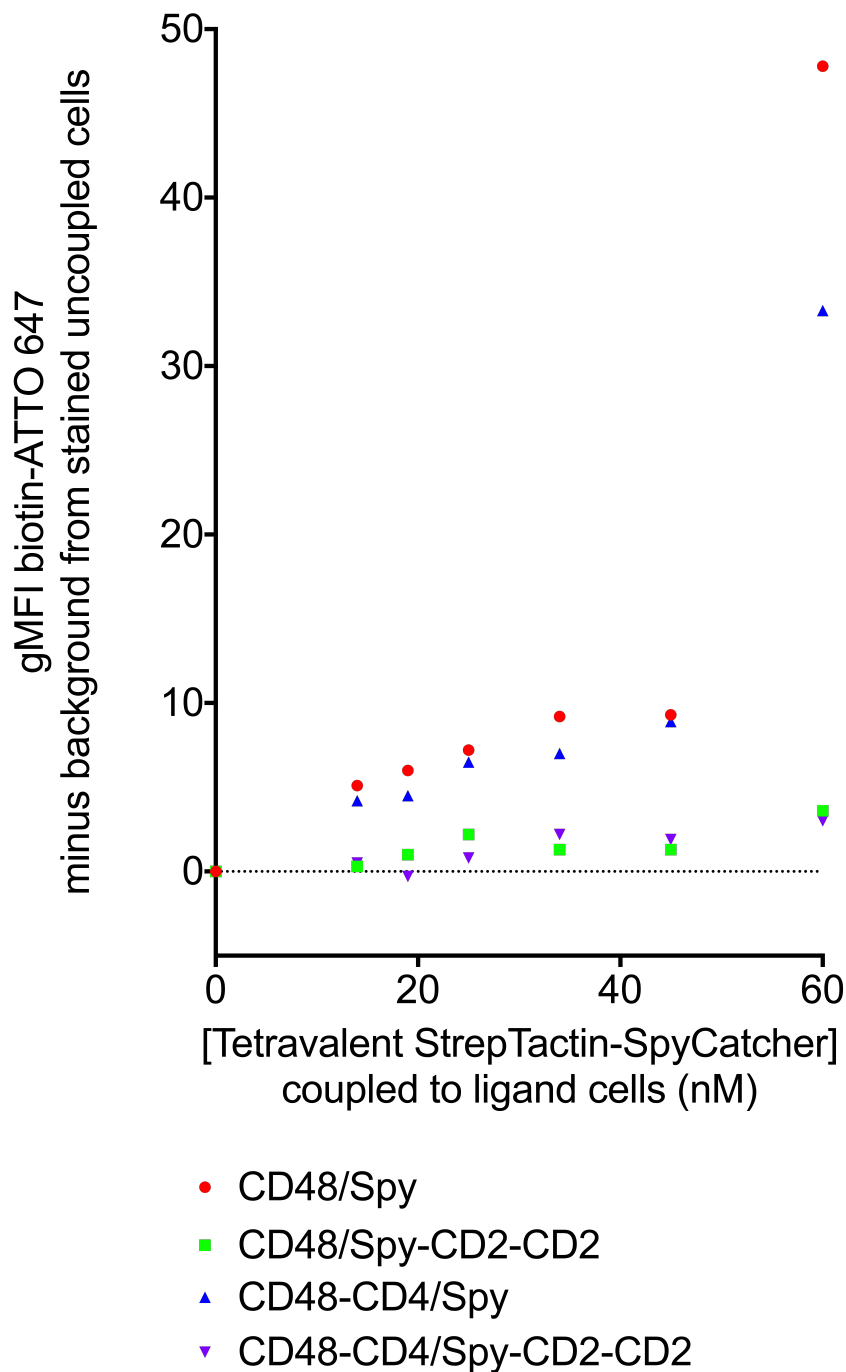


Figure 4.9: Quantification of flow cytometry results from Figure 4.8, showing the gMFI of CHO.K1 CD48/Spy (red), CD48/Spy-CD2-CD2 (green), CD48-CD4/Spy (blue) and CD48-CD4/Spy-CD2-CD2 (purple) ligand cells coupled with tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647.

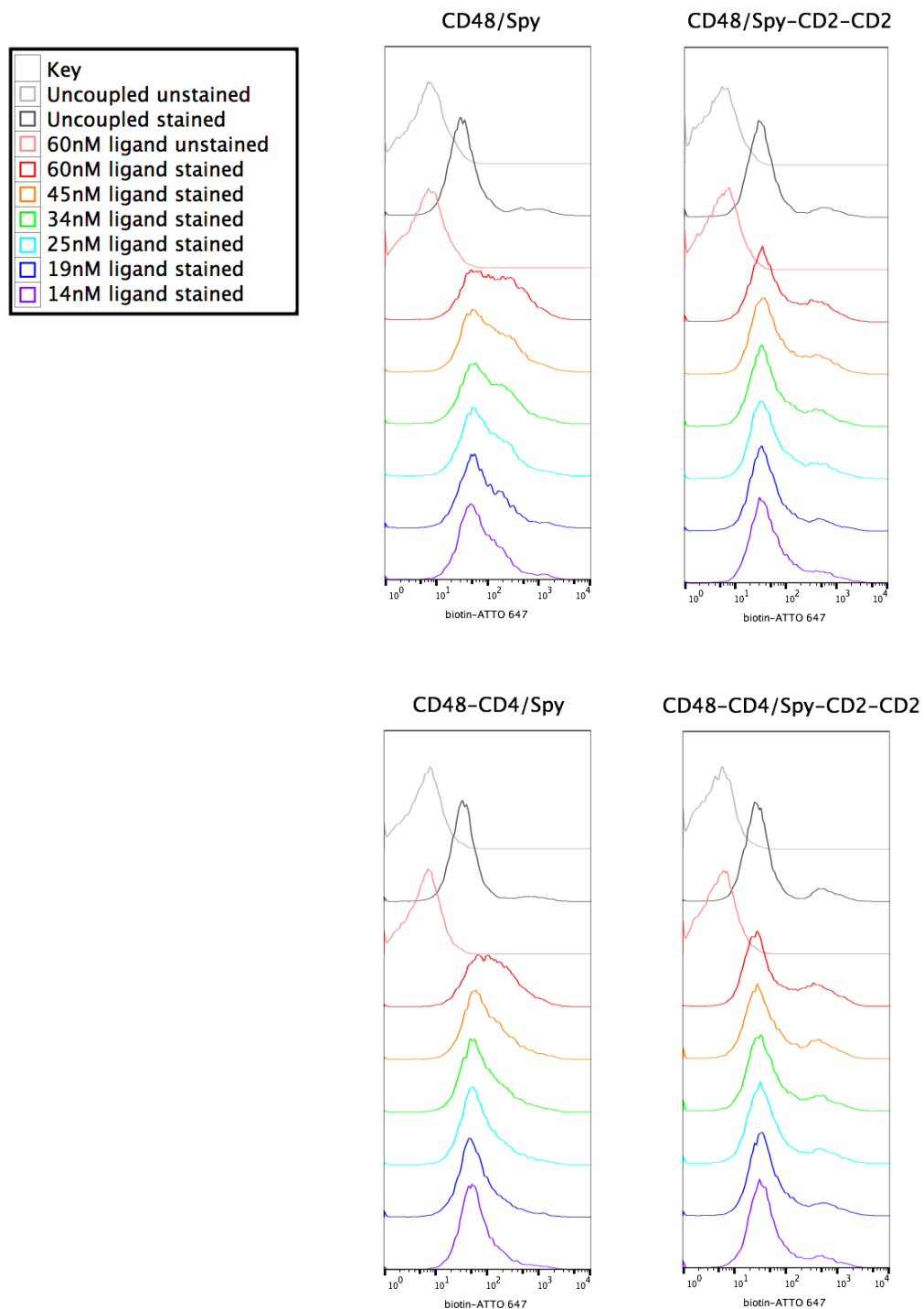


Figure 4.10: Analysis of coupling levels for the third THP-1 stimulation experiment showing flow cytometry results of different CHO.K1 CD48-based ligand cells. Ligand cells were coupled with the indicated concentration of tetraivalent StrepTactin-SpyCatcher and stained with $3 \mu\text{M}$ of a 3:1 mixture of D-biotin:biotin-ATTO 647 along with several controls including unstained uncoupled cells (light grey), stained uncoupled cells (dark grey), and unstained coupled cells (pink).

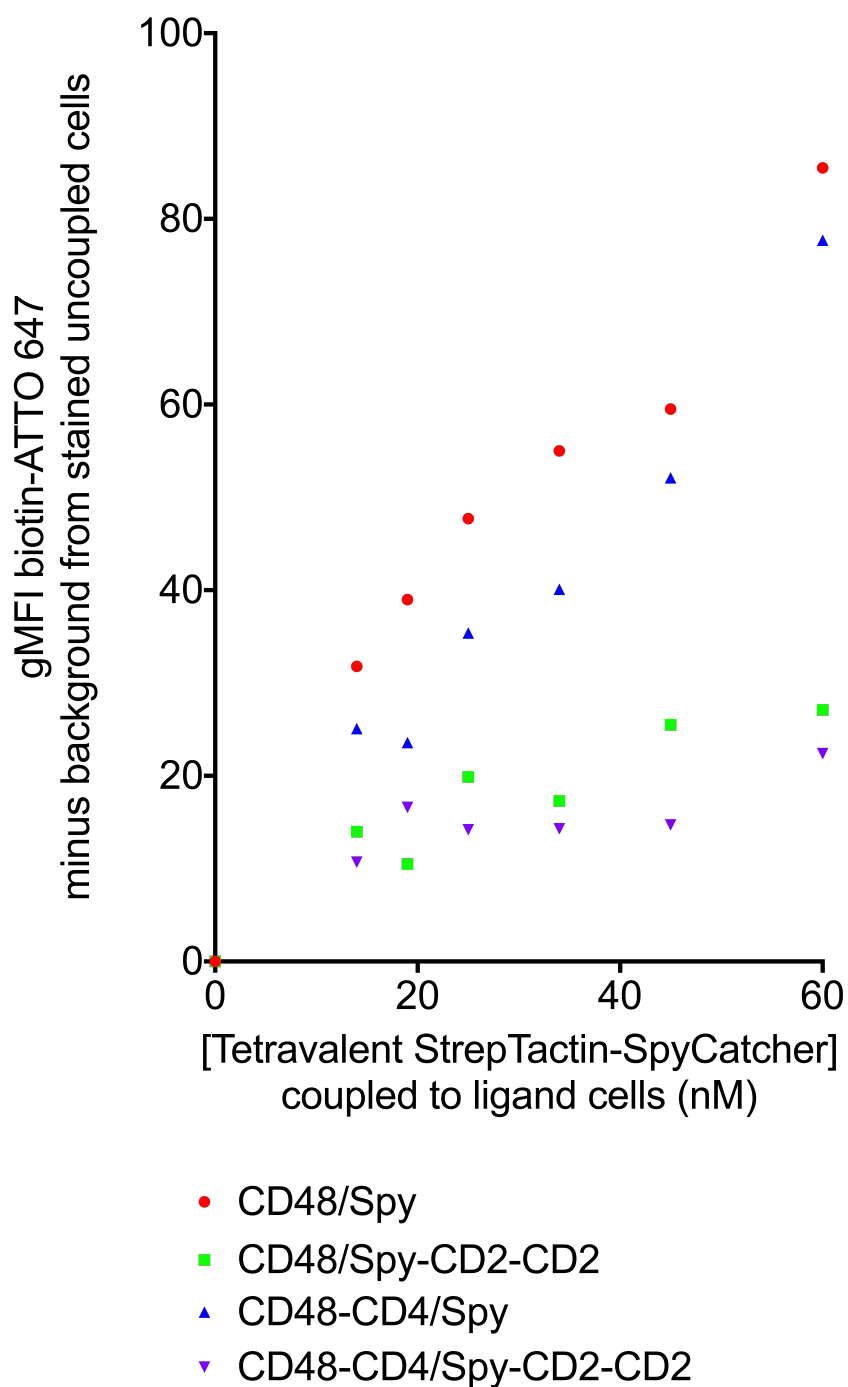


Figure 4.11: Quantification of flow cytometry results from Figure 4.10, showing the gMFI of CHO.K1 CD48/Spy (red), CD48/Spy-CD2-CD2 (green), CD48-CD4/Spy (blue) and CD48-CD4/Spy-CD2-CD2 (purple) ligand cells coupled with tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647.

Despite poor coupling of the StrepTactin-SpyCatcher to the CD48-based ligand cells, we proceeded to test these ligand cells in the two-ligand system to determine whether they were capable of stimulating the THP-1 receptor cells and whether any PD-1-mediated inhibition could be observed upon stimulation with ligand cells coupled with tetravalent StrepTactin-SpyCatcher. Results from three independent experiments were combined by normalising IL-8 production to levels from stimulation with uncoupled CHO.K1 ligand cells. Normalised results are shown in Figures 4.12-4.19, with raw IL-8 levels for each of the three independent experiments shown in Appendices B.2 - B.4.

Stimulation with CHO.K1 CD48/Spy cells

Stimulation of the THP-1 receptor cells using the CHO.K1 CD48/Spy ligand cells resulted in substantial IL-8 production above the non-specific IL-8 levels from culturing with U/T CHO.K1 cells (Figure 4.12). There was not a statistically significant difference in IL-8 levels between THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells stimulated with CHO.K1 CD48/Spy cells coupled with 60 nM of tetravalent StrepTactin-SpyCatcher compared to those stimulated with uncoupled CHO.K1 CD48/Spy cells ($P = 0.0685$; panel A, Figure 4.12). Titration of tetravalent StrepTactin-SpyCatcher showed a modest trend of inhibition with increasing concentrations of the tetravalent StrepTactin-SpyCatcher (Figure 4.13).

There was no difference observed in IL-8 levels from stimulation of THP-1 2B4-CD64/PD-1 control cells with either uncoupled or coupled CHO.K1 CD48/Spy ligand cells ($P = 0.4245$; panel B, Figure 4.12). This provides support for the specificity of the modest trend of inhibition observed from stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II cells with coupled CHO.K1 CD48/Spy ligand cells.

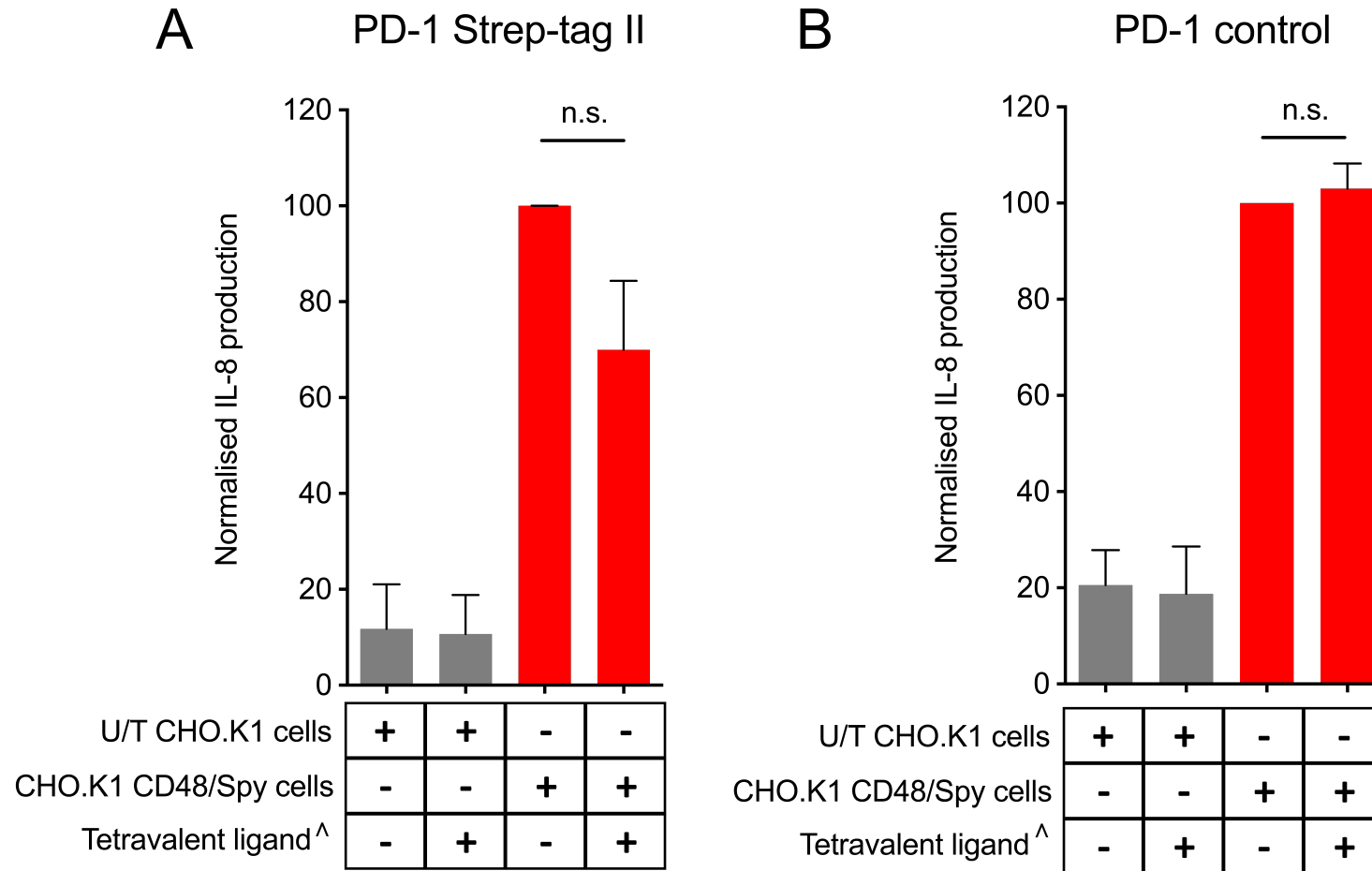


Figure 4.12: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48/Spy cells. THP-1 2B4-CD64/PD-1 Strep-tag II (A) or PD-1 control (B) receptor cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48/Spy ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48/Spy cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

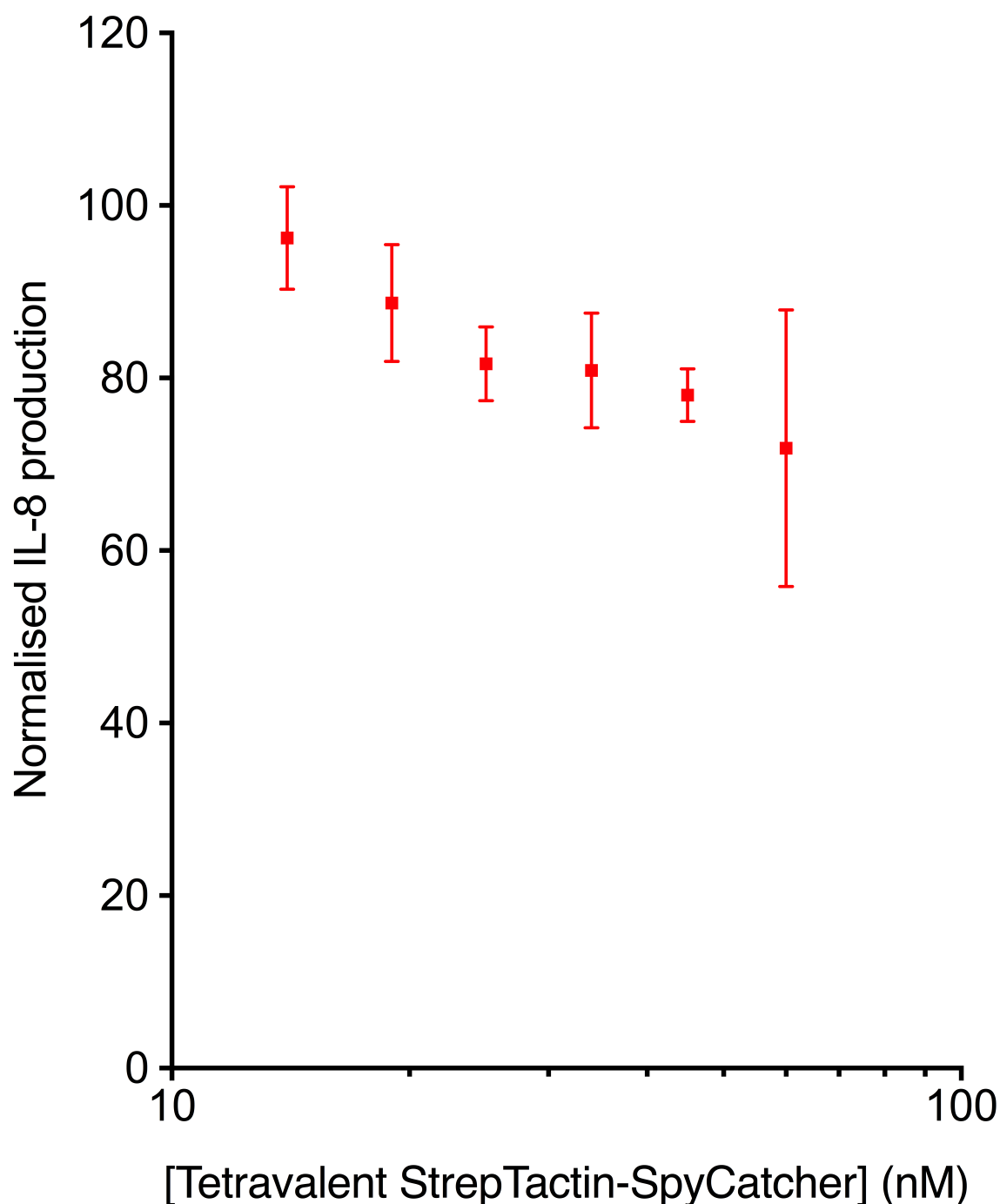


Figure 4.13: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 2B4-CD64/PD-1 receptor cells with CHO.K1 CD48/Spy cells. The THP-1 receptor cells were stimulated with CHO.K1 CD48/Spy ligand cells either uncoupled or coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48/Spy cells, showing mean plus standard deviation from three independent experiments performed with biological replicates.

Stimulation with CHO.K1 CD48/Spy-CD2-CD2 cells

As with the CHO.K1 CD48/Spy ligand cells, stimulation of the THP-1 receptor cells using the CHO.K1 CD48/Spy-CD2-CD2 ligand cells resulted in substantial IL-8 production above background levels (Figure 4.14). There was not a statistically significant difference between IL-8 levels from stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with CHO.K1 CD48/Spy-CD2-CD2 cells coupled with 60 nM of tetravalent StrepTactin-SpyCatcher compared to stimulation with uncoupled CHO.K1 CD48/Spy-CD2-CD2 cells ($P = 0.2250$; panel A, Figure 4.14). Despite poor coupling of the StrepTactin-SpyCatcher to these ligand cells (Figures 4.6 - 4.11), there was a possible trend of IL-8 inhibition from low to intermediate coupling levels but no further inhibition at higher levels of coupling (Figure 4.15).

Interestingly, there was a statistically significant increase in IL-8 levels from stimulation of THP-1 2B4-CD64/PD-1 control cells with CHO.K1 CD48/Spy-CD2-CD2 ligand cells coupled with 60 nM StrepTactin-SpyCatcher compared to uncoupled ligand cells ($P = 0.0199$; panel B, Figure 4.14). As discussed in Section 3.3.4.1, this may be due to a non-specific increase in IL-8 production in the presence of StrepTactin-SpyCatcher coupled to the ligand cells.

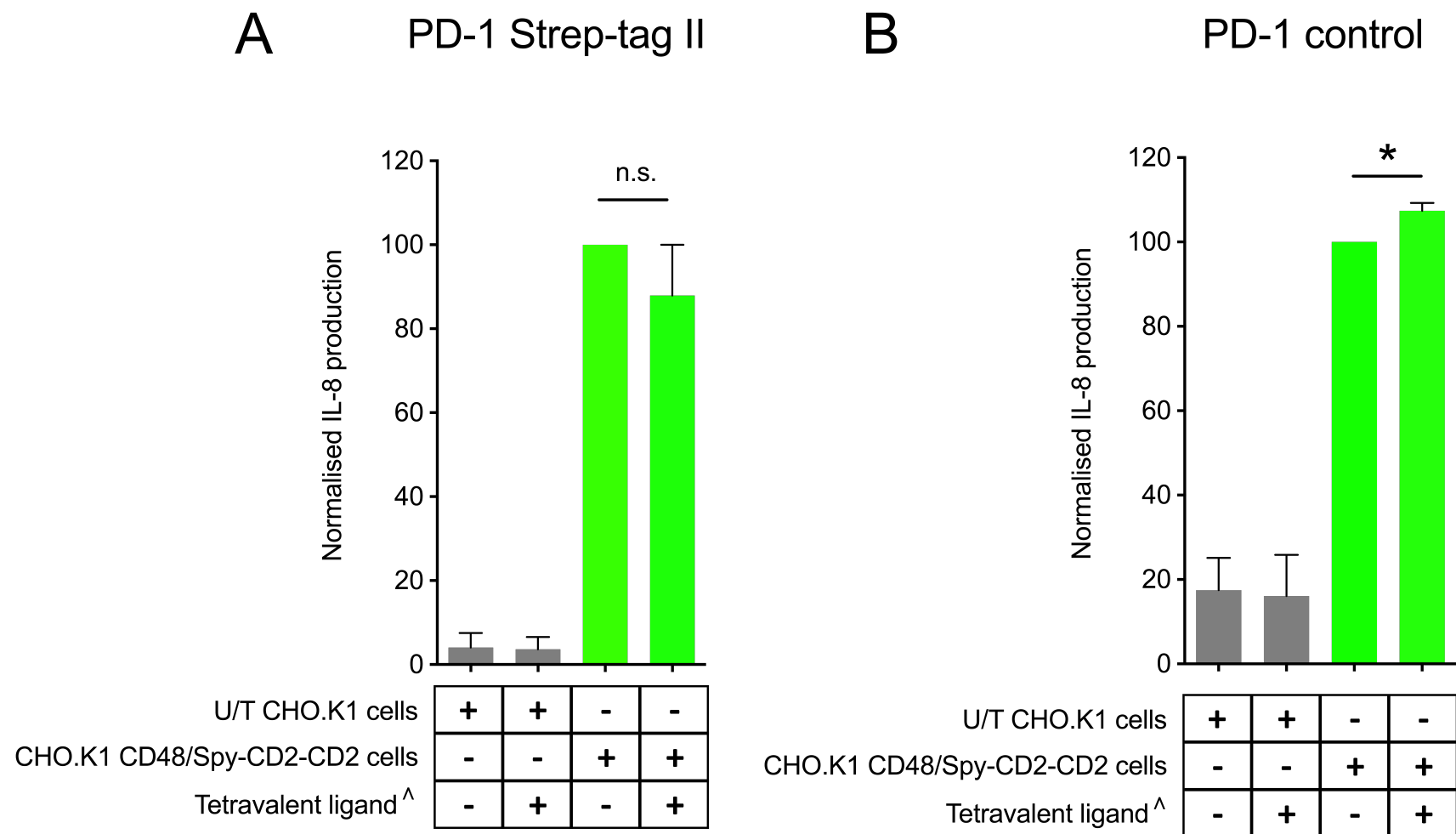


Figure 4.14: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48/Spy-CD2-CD2 cells. THP-1 2B4-CD64/PD-1 Strep-tag II (A) or PD-1 control (B) receptor cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48/Spy-CD2-CD2 ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48/Spy-CD2-CD2 cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; * = $P \leq 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

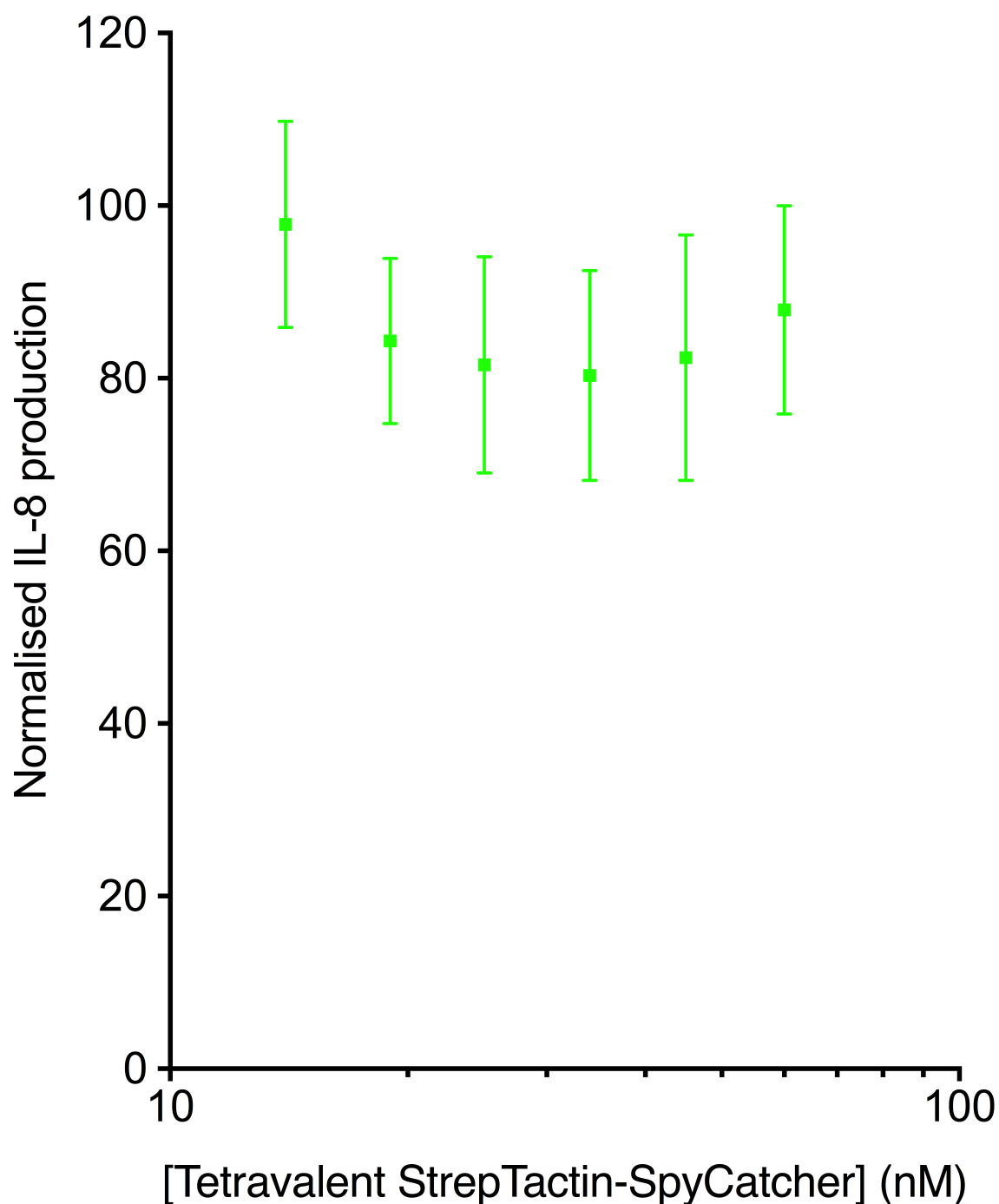


Figure 4.15: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 2B4-CD64/PD-1 receptor cells with CHO.K1 CD48/Spy-CD2-CD2 cells. The THP-1 receptor cells were stimulated with CHO.K1 CD48/Spy-CD2-CD2 ligand cells either uncoupled or coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48/Spy-CD2-CD2 cells, showing mean plus standard deviation from three independent experiments performed with biological replicates.

Stimulation with CHO.K1 CD48-CD4/Spy cells

Use of the CHO.K1 CD48-CD4/Spy ligand cells to stimulate the THP-1 receptor cells resulted in IL-8 production above that measured with U/T CHO.K1 cells, but not to the same level measured with the CHO.K1 CD48/Spy or CHO.K1 CD48/Spy-CD2-CD2 ligand cells (Appendices B.2 - B.4). There was no statistically significant difference in IL-8 levels for either the THP-1 2B4-CD64/PD-1 Strep-tag II or THP-1 2B4-CD64/PD-1 control cells when stimulated with CHO.K1 CD48-CD4/Spy ligand cells coupled with 60 nM of tetravalent StrepTactin-SpyCatcher compared to stimulation with uncoupled CHO.K1 CD48-CD4/Spy cells ($P = 0.6140$ and $P = 0.8875$ for PD-1 Strep-tag II and PD-1 control receptor cells respectively; Figure 4.16).

IL-8 production from stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with CHO.K1 CD48-CD4/Spy ligand cells coupled with a titration of the StrepTactin-SpyCatcher showed no clear trend of inhibition (Figure 4.17).

Stimulation with CHO.K1 CD48-CD4/Spy-CD2-CD2 cells

Stimulation of the THP-1 receptor cells using the CHO.K1 CD48-CD4/Spy-CD2-CD2 ligand cells resulted in IL-8 production above that measured from culturing with U/T CHO.K1 cells and to similar levels as those from stimulation with the CHO.K1 CD48-CD4/Spy ligand cells (Appendices B.2 - B.4). As with the CHO.K1 CD48-CD4/Spy cells, there was no difference in IL-8 production by either the THP-1 2B4-CD64/PD-1 Strep-tag II or THP-1 2B4-CD64/PD-1 control cells stimulated with CHO.K1 CD48-CD4/Spy-CD2-CD2 cells coupled with 60 nM of tetravalent StrepTactin-SpyCatcher compared to stimulation with uncoupled CHO.K1 CD48-CD4/Spy-CD2-CD2 cells ($P = 0.0617$ and $P = 0.4705$ for PD-1 Strep-tag II and PD-1 control receptor cells respectively; Figure 4.18).

Additionally, IL-8 production from stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with the CHO.K1 CD48-CD4/Spy-CD2-CD2 ligand cells coupled with a titration of the StrepTactin-SpyCatcher showed no apparent inhibition (Figure 4.19).

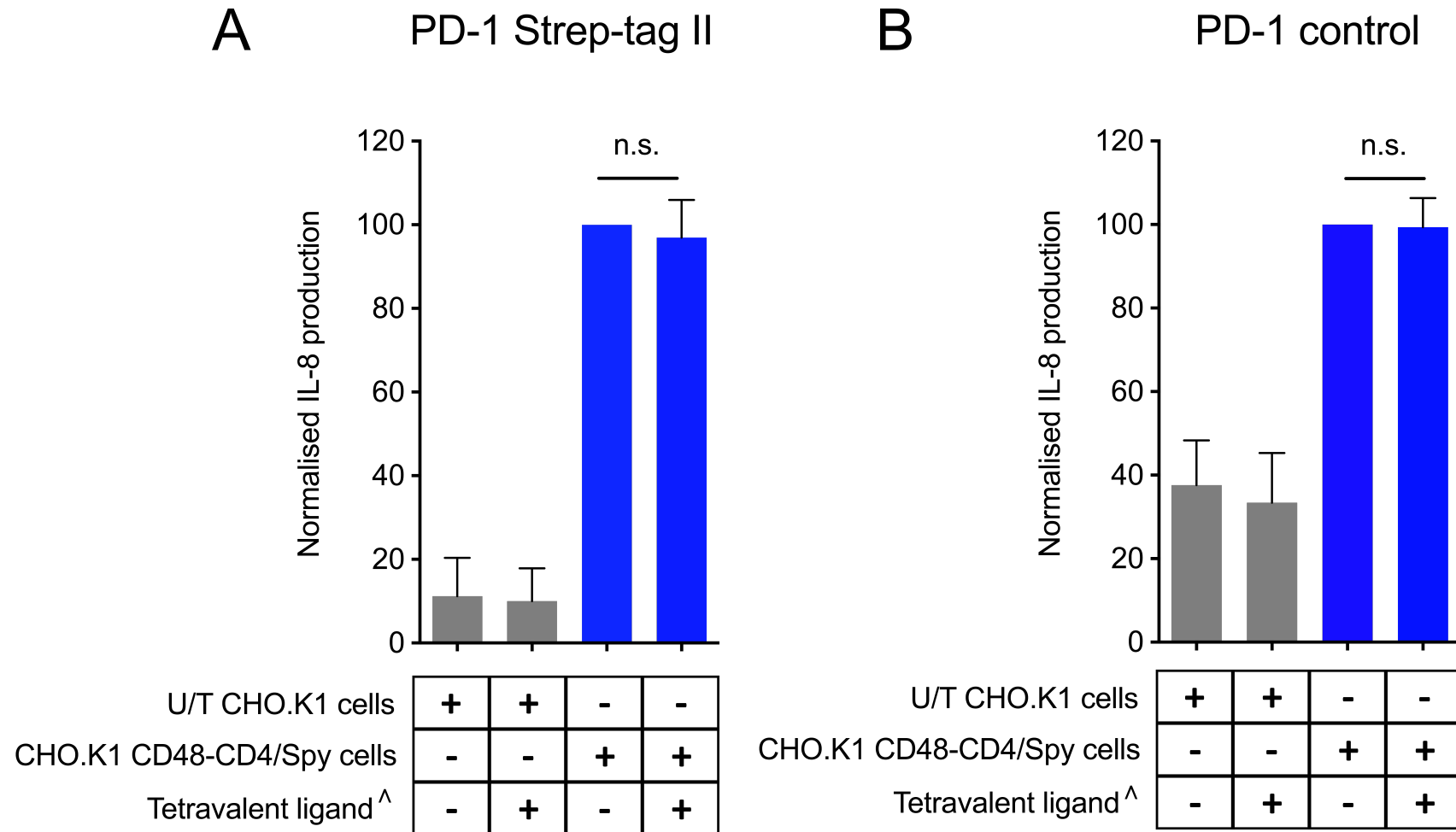


Figure 4.16: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48-CD4/Spy cells. THP-1 2B4-CD64/PD-1 Strep-tag II (A) or PD-1 control (B) receptor cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48-CD4/Spy ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48-CD4/Spy cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100..

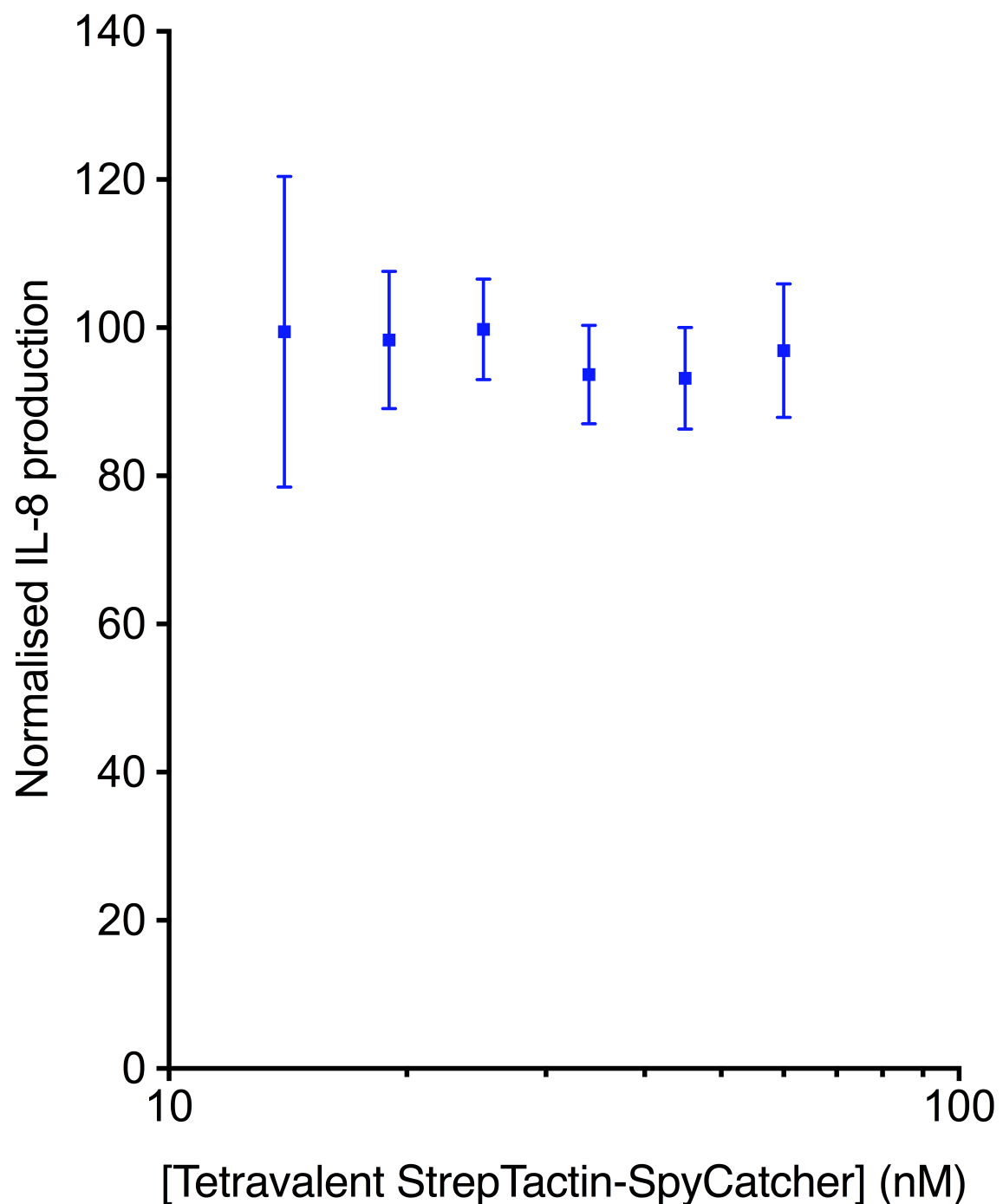
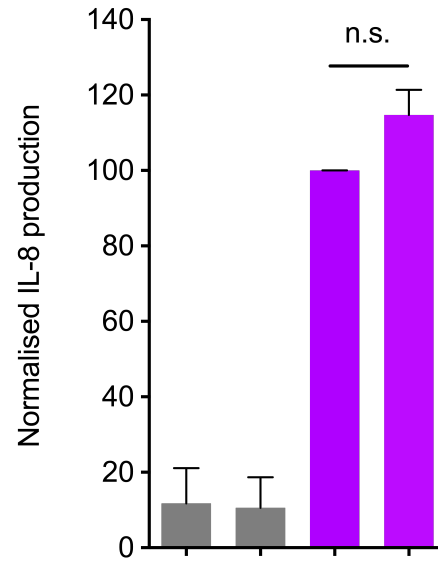


Figure 4.17: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 2B₄-CD64/PD-1 receptor cells with CHO.K1 CD48-CD4/Spy cells. The THP-1 receptor cells were stimulated with CHO.K1 CD48-CD4/Spy ligand cells either uncoupled or coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48-CD4/Spy cells, showing mean plus standard deviation from three independent experiments performed with biological replicates.

A PD-1 Strep-tag II



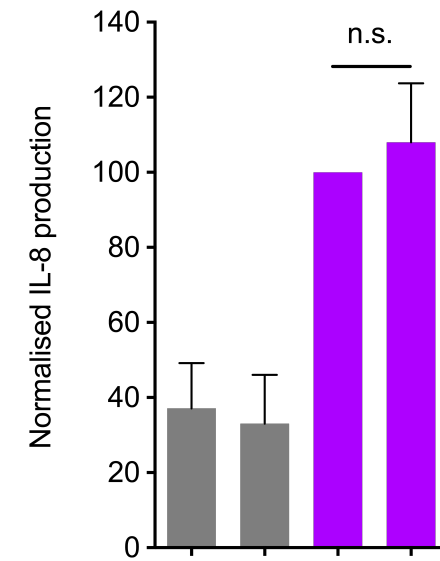
U/T CHO.K1 cells

CHO.K1 CD48-CD4/Spy-CD2-CD2 cells

Tetravalent ligand ^

+	+	-	-
-	-	+	+
-	+	-	+

B PD-1 control



U/T CHO.K1 cells

CHO.K1 CD48-CD4/Spy-CD2-CD2 cells

Tetravalent ligand ^

+	+	-	-
-	-	+	+
-	+	-	+

Figure 4.18: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48-CD4/Spy-CD2-CD2 cells. THP-1 2B4-CD64/PD-1 Strep-tag II (A) or PD-1 control (B) receptor cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48-CD4/Spy-CD2-CD2 ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48-CD4/Spy-CD2-CD2 cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. ^Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

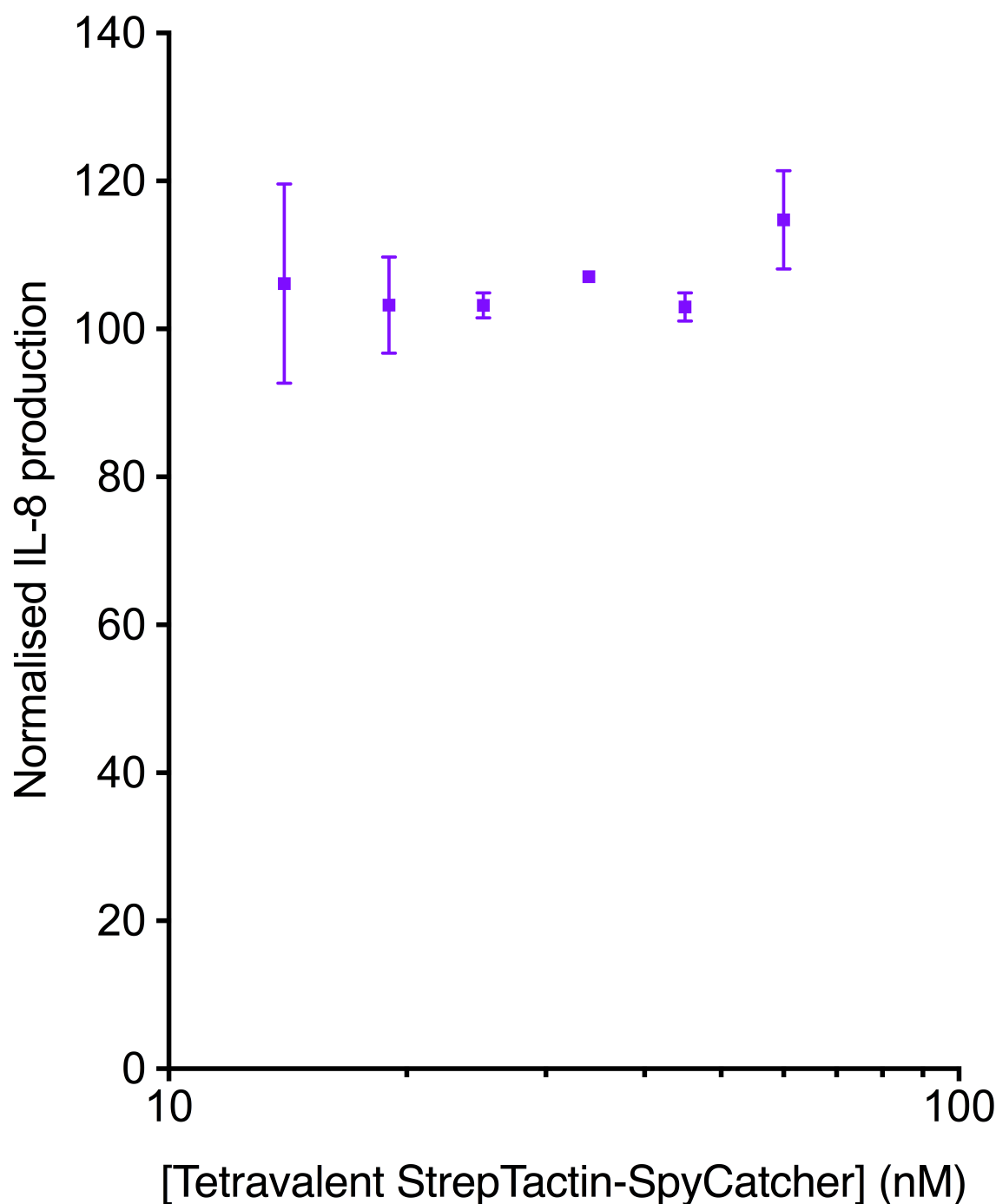


Figure 4.19: Normalised IL-8 production from cell-cell stimulation of vitamin D-differentiated THP-1 2B₄-CD64/PD-1 receptor cells with CHO.K1 CD48-CD4/Spy-CD2-CD2 cells. The THP-1 receptor cells were stimulated with CHO.K1 CD48-CD4/Spy-CD2-CD2 ligand cells either uncoupled or coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher. IL-8 levels in supernatant were measured by ELISA and normalised to stimulation with uncoupled CHO.K1 CD48-CD4/Spy-CD2-CD2 cells, showing mean plus standard deviation from three independent experiments performed with biological replicates.

4.3.3 Stimulation of Jurkat receptor cells with ligand cells expressing ligands with matched and mismatched dimensions

To test the importance of receptor-ligand dimensions for PD-1 signal integration in the Jurkat two-ligand system, the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II and PD-1 control receptor cells were stimulated using the CHO.K1 ligand cells expressing SCD and Spy constructs of different lengths coupled with monovalent or tetravalent StrepTactin-SpyCatcher.

Coupling of the StrepTactin-SpyCatcher to the SCD-based ligand cells was much more effective and consistent than the CD48-based ligand cells. The coupling levels were similar across seven independent experiments with higher levels of the monovalent StrepTactin-SpyCatcher coupling to the CHO.K1 SCD/Spy-CD4 cells compared to the CHO.K1 SCD/Spy cells and comparable levels of the tetravalent StrepTactin-SpyCatcher coupling to the CHO.K1 SCD/Spy and CHO.K1 SCD/Spy-CD4 cells (Figures 4.20 - 4.22). Since the coupling of StrepTactin-SpyCatcher to the CHO.K1 SCD/Spy-CD4 cells, which have mismatched receptor-ligand dimensions, is similar if not higher than coupling to the CHO.K1 SCD/Spy cells, any abrogation of PD-1-mediated inhibition from stimulation using the CHO.K1 SCD/Spy-CD4 cells cannot be due to lower coupling of the StrepTactin-SpyCatcher to those cells.

Throughout these experiments there was a small population of the CHO.K1 SCD/Spy cells that did not couple (Figures 4.20 and 4.21), which may result in diminishment of any inhibitory effects from stimulation with these ligand cells as uncoupled ligand cells would only provide activating signals to the receptor cells. However, if the PD-1-mediated inhibitory signals are strong enough we should still be able to test our hypothesis that matched receptor-ligand dimensions are required for optimal NTR signal integration.

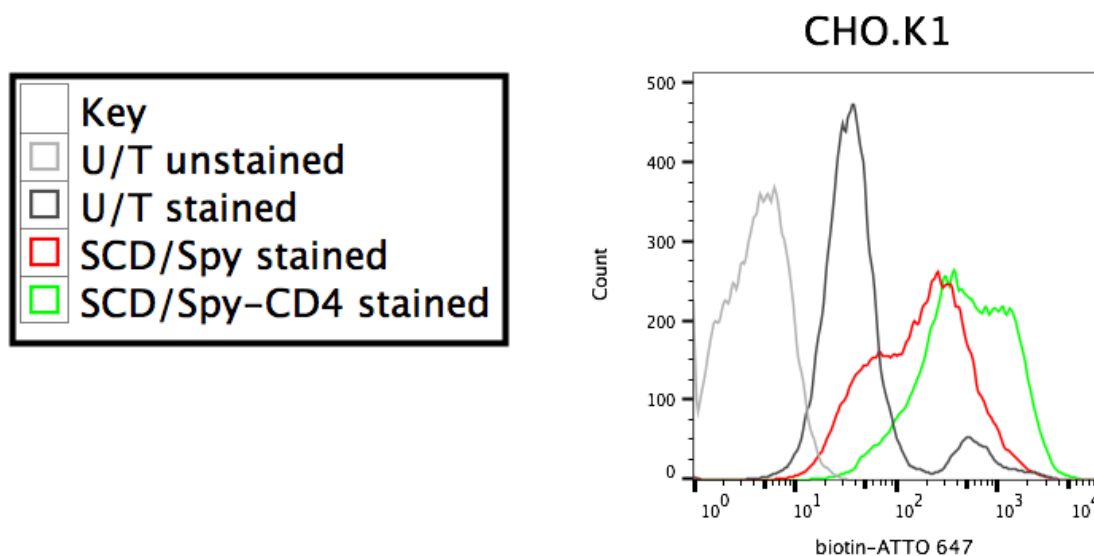


Figure 4.20: Analysis of monovalent coupling levels for the SCD-based ligand cells used for stimulation of the Jurkat receptor cells, showing flow cytometry results of U/T CHO.K1 cells (dark grey), CHO.K1 SCD/Spy ligand cells (red) and CHO.K1 SCD/Spy-CD4 ligand cells (green) coupled with 200 nM of monovalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647. Unstained U/T CHO.K1 cells are shown in light grey. Results are representative of seven independent experiments.

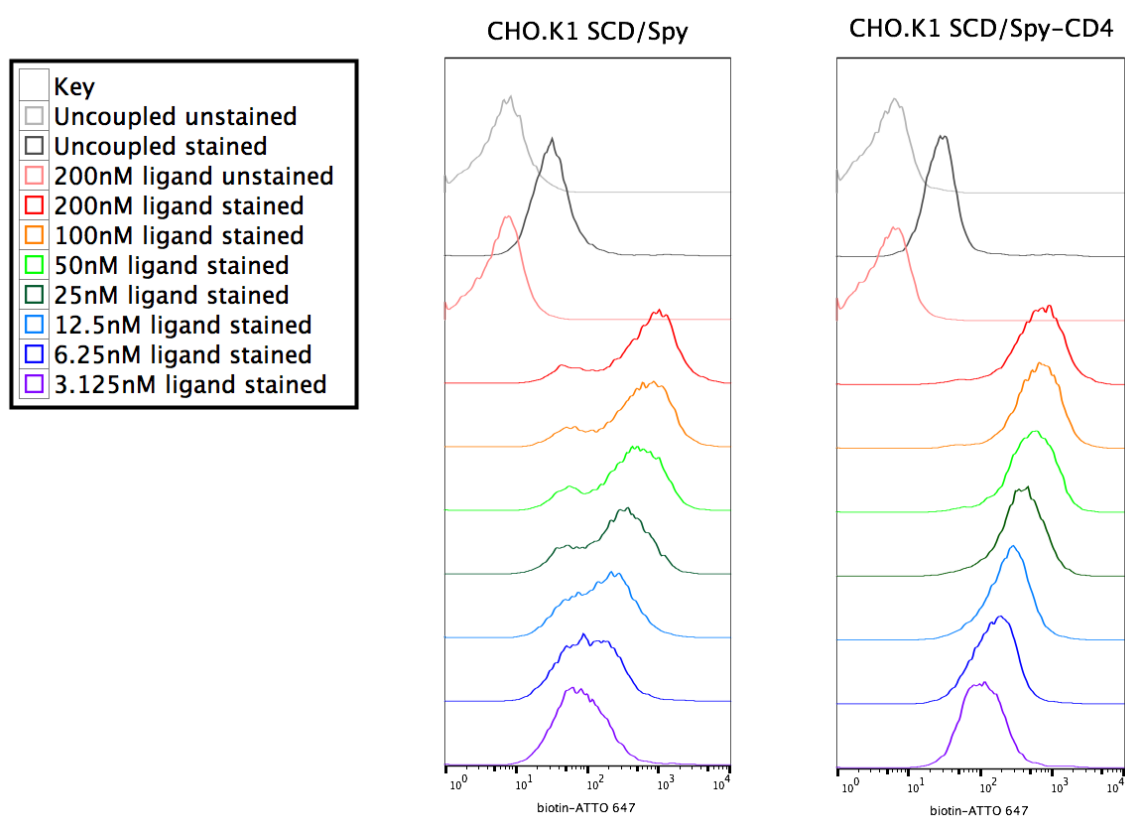


Figure 4.21: Analysis of tetravalent coupling levels for the SCD-based ligand cells used for stimulation of the Jurkat receptor cells, showing flow cytometry results of CHO.K1 SCD/Spy ligand cells and CHO.K1 SCD/Spy-CD4 ligand cells. Ligand cells were coupled with the indicated concentration of tetravalent StrepTactin-SpyCatcher and stained with $3 \mu\text{M}$ of a 3:1 mixture of D-biotin:biotin-ATTO 647. Controls include unstained uncoupled cells (light grey), stained uncoupled cells (dark grey), and unstained coupled cells (pink). Results are representative of seven independent experiments.

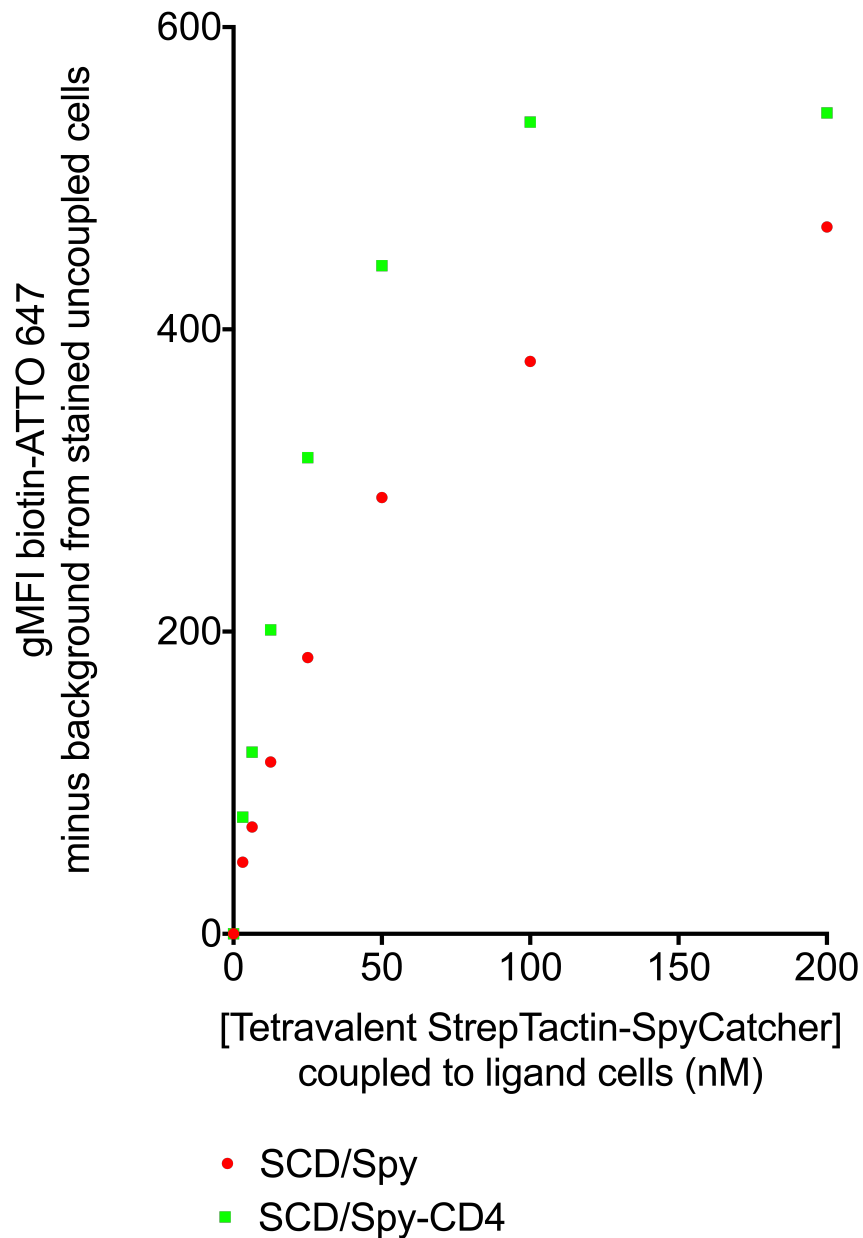


Figure 4.22: Quantification of flow cytometry results from Figure 4.21, showing the gMFI of CHO.K1 SCD/Spy (red) and SCD/Spy-CD4 (green) ligand cells coupled with tetravalent StrepTactin-SpyCatcher and stained with 3 μ M of a 3:1 mixture of D-biotin:biotin-ATTO 647.

4.3.3.1 10 nM peptide stimulation of Jurkat receptor cells

We proceeded to test the CHO.K1 SCD-based ligand cells in the two-ligand system to determine whether they were capable of stimulating the Jurkat NF- κ B-eGFP/1G4 TCR receptor cells and whether any PD-1-mediated inhibition could be observed upon stimulation with ligand cells coupled with the monovalent or tetravalent StrepTactin-SpyCatcher. We started by loading the SCD-based ligand cells with 10 nM NY-ESO-1 C9V peptide as this should give a moderate amount of 1G4 TCR-mediated activation from which we can measure PD-1-mediated inhibition (Appendix A.5).

Results from three independent experiments were combined by normalising NF- κ B-eGFP gMFI to levels from stimulation with uncoupled ligand cells (Figures 4.23 and 4.24).

Stimulation with CHO.K1 SCD/Spy cells

Stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 cells using uncoupled CHO.K1 SCD/Spy ligand cells loaded with 10 nM peptide resulted in an increase in NF- κ B-eGFP gMFI well above the background measured from culturing with untransfected CHO.K1 cells (Figure 4.23). As seen in Figure 4.23 A, coupling of 200 nM monovalent StrepTactin-SpyCatcher did not result in inhibition ($P = 0.5755$); however, coupling of 200 nM tetravalent StrepTactin-SpyCatcher resulted in a reproducible and statistically significant inhibition of NF- κ B-eGFP gMFI ($P = 0.0074$). This inhibition was not observed from stimulation of the PD-1 control cells (panel B, Figure 4.23; $P = 0.9886$ and $P = 0.2581$ for monovalent and tetravalent ligand respectively), so is specific to engagement of the PD-1 Strep-tag II construct via the Strep-tag II tag.

Stimulation with CHO.K1 SCD/Spy-CD4 cells

Stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells using uncoupled CHO.K1 SCD/Spy-CD4 ligand cells loaded with 10 nM peptide resulted in stimulation levels above background but considerably lower compared to stimulation with the CHO.K1 SCD/Spy cells (Appendix B.5). The reason for the lower NF- κ B-eGFP gMFI upon activation with these ligand cells compared to stimulation with the CHO.K1 SCD/Spy ligand cells is currently unknown but was considered upon interpretation of the results of these experiments.

Neither the monovalent or tetravalent StrepTactin-SpyCatcher coupled to the CHO.K1 SCD/Spy-CD4 cells resulted in statistically significant inhibition of NF-

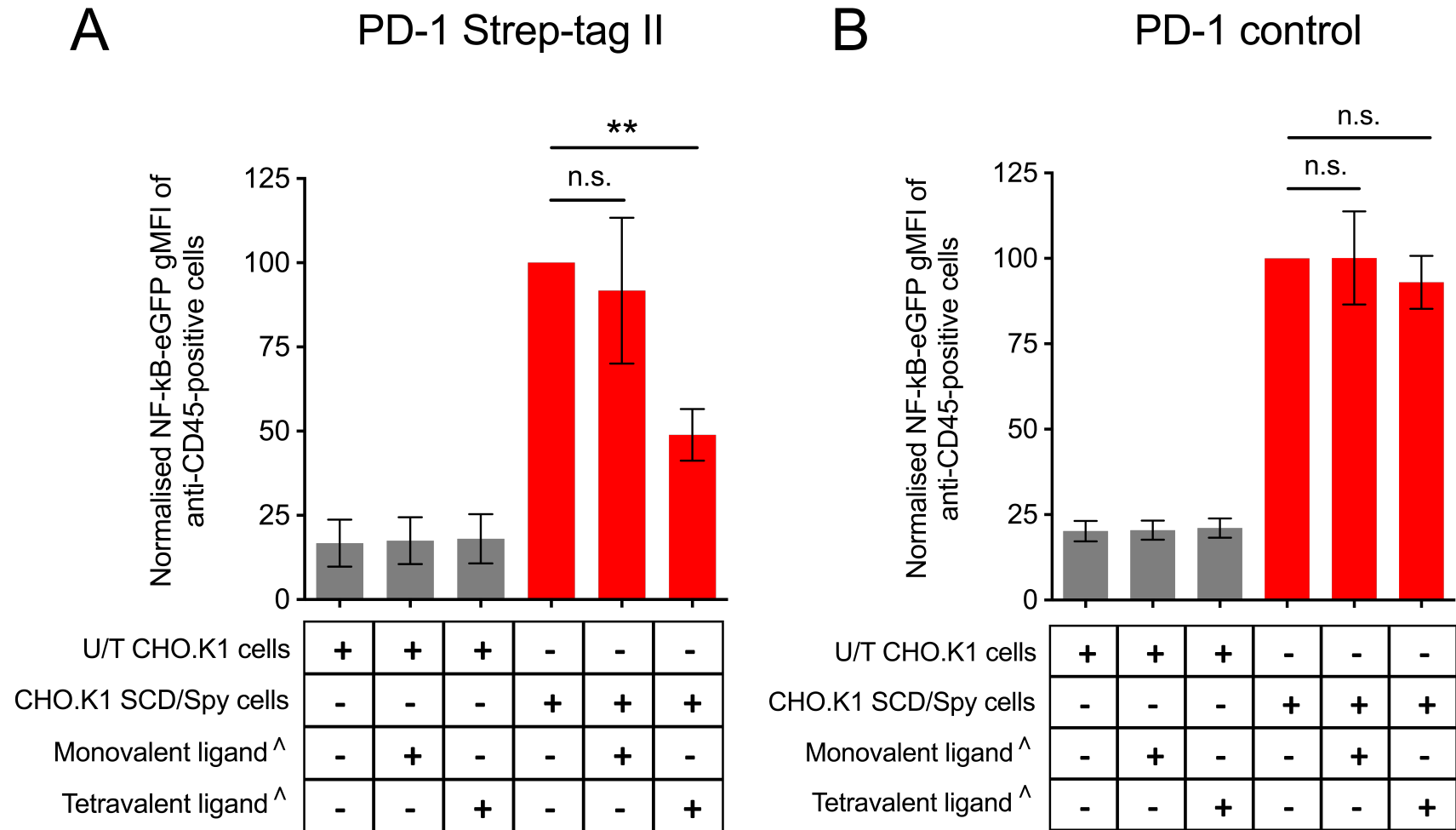


Figure 4.23: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with CHO.K1 SCD/Spy ligand cells loaded with 10 nM peptide. Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 Strep-tag II (A) or PD-1 control (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells, either uncoupled or coupled with 200 nM monovalent or tetravalent StrepTactin-SpyCatcher as indicated. NF- κ B-eGFP gMFI was measured by flow cytometry and normalised to stimulation with uncoupled CHO.K1 SCD/Spy cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; ** = $P \leq 0.01$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

κ B-eGFP gMFI in the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells ($P = 0.0834$ and $P = 0.3299$ respectively, panel A, Figure 4.24), but whether this was due to a lack of signal integration or the low activation levels mentioned above was unclear (Appendix B.5). To investigate this result, we repeated these stimulation experiments using a higher concentration of C9V peptide to increase 1G4 TCR-mediated activation levels.

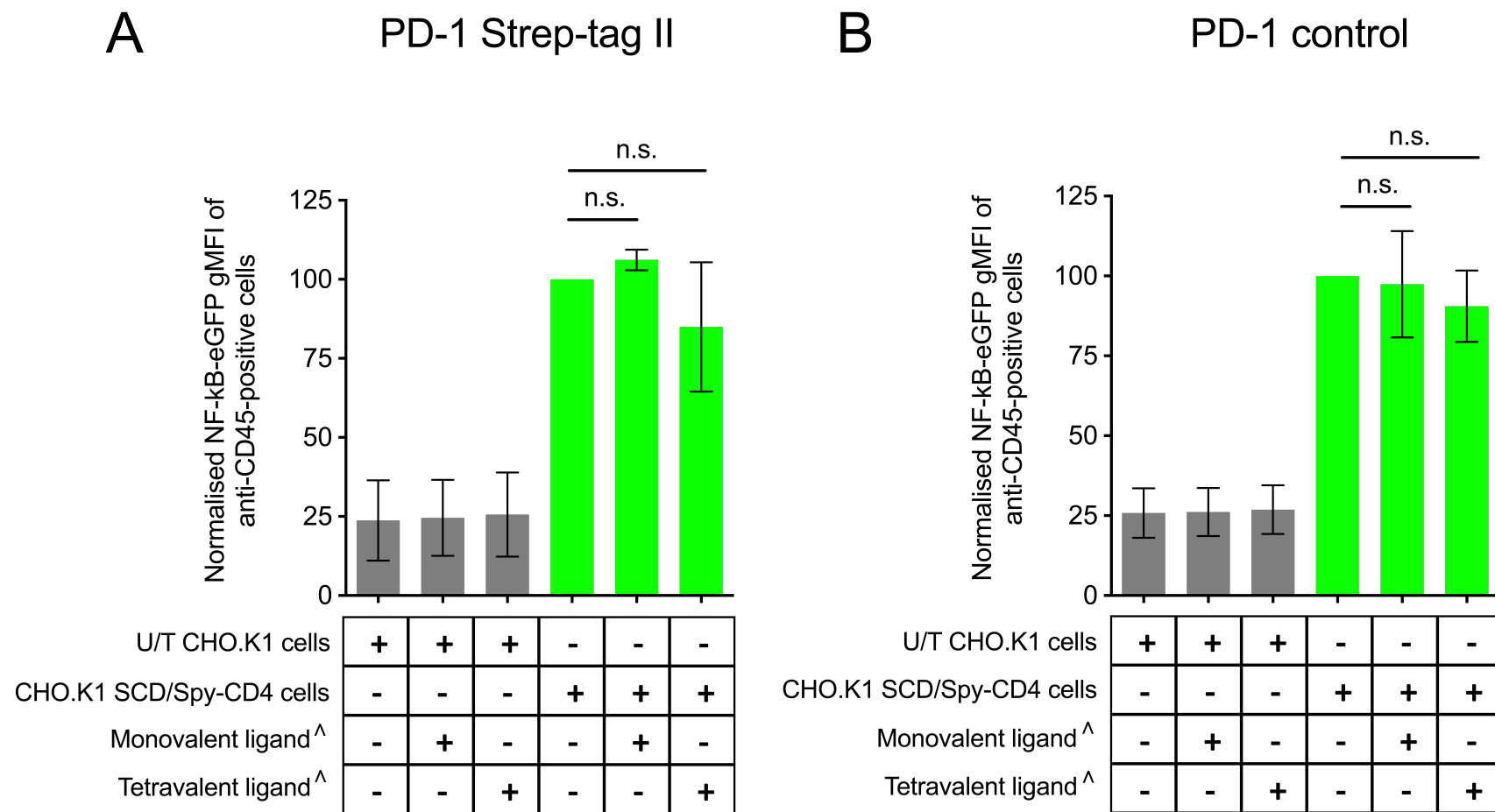


Figure 4.24: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with CHO.K1 SCD/Spy-CD4 ligand cells loaded with 10 nM peptide. Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 Strep-tag II (A) or PD-1 control (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy-CD4 ligand cells, either uncoupled or coupled with 200 nM monovalent or tetravalent StrepTactin-SpyCatcher as indicated. NF- κ B-eGFP gMFI was measured by flow cytometry and normalised to stimulation with uncoupled CHO.K1 SCD/Spy-CD4 cells, showing mean plus standard deviation from three independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

4.3.3.2 100 nM peptide stimulation of Jurkat receptor cells

Stimulation with CHO.K1 SCD/Spy cells

The results from stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells using uncoupled CHO.K1 SCD/Spy ligand cells loaded with 10 nM peptide (panel A, Figure 4.23) were reinforced by repeating the experiment with 100 nM peptide; coupling of the monovalent StrepTactin-SpyCatcher did not show inhibition ($P = 0.9122$), but coupling of tetravalent StrepTactin-SpyCatcher to the CHO.K1 SCD/Spy ligand cells resulted in a reproducible and statistically significant inhibition of NF- κ B-eGFP gMFI ($P = 0.0008$; panel A, Figure 4.25). This inhibition was once again absent from stimulation of the PD-1 control cells ($P = 0.0957$ and $P = 0.0821$ for monovalent and tetravalent ligand respectively; panel B, Figure 4.25), so is specific to PD-1 Strep-tag II being engaged via the Strep-tag II tag.

Stimulation with CHO.K1 SCD/Spy-CD4 cells

In contrast to the stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells using uncoupled CHO.K1 SCD/Spy-CD4 ligand cells loaded with only 10 nM peptide (Figure 4.23), the same stimulation assay using 100 nM peptide showed statistically significant inhibition of NF- κ B-eGFP gMFI upon coupling of tetravalent StrepTactin-SpyCatcher ($P = 0.0040$; panel A, Figure 4.26). However, inhibition was also observed with stimulation of the PD-1 control cells ($P = 0.0352$ and 0.0356 for monovalent and tetravalent ligand respectively; panel B, Figure 4.26), casting doubt on the specificity of the inhibition observed with the PD-1 Strep-tag II cells.

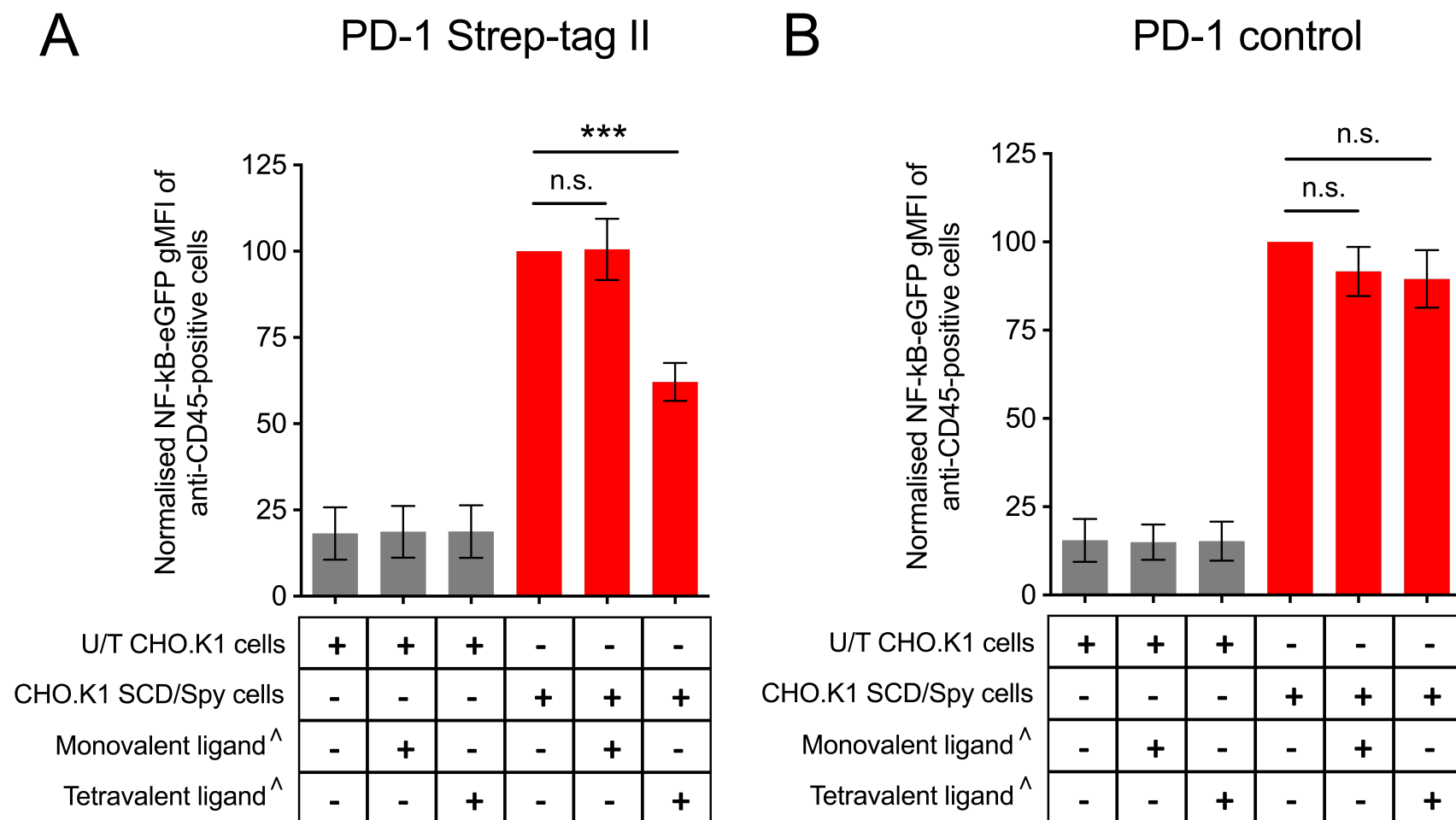


Figure 4.25: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with CHO.K1 SCD/Spy ligand cells loaded with 100 nM peptide. Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 Strep-tag II (A) or PD-1 control (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells, either uncoupled or coupled with 200 nM monovalent or tetravalent StrepTactin-SpyCatcher as indicated. NF- κ B-eGFP gMFI was measured by flow cytometry and normalised to stimulation with uncoupled CHO.K1 SCD/Spy cells, showing mean plus standard deviation from four independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; *** = $P \leq 0.001$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

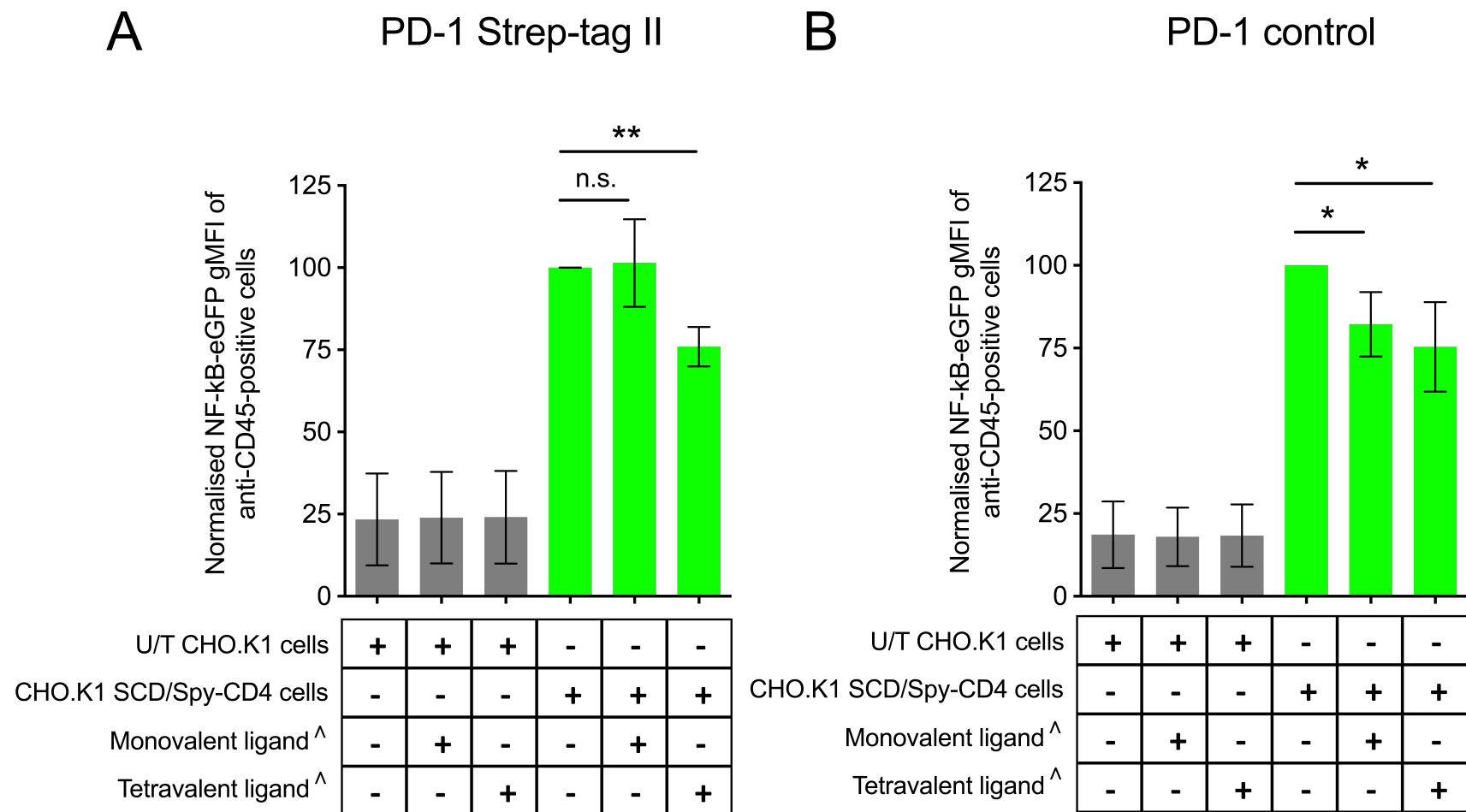


Figure 4.26: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells with CHO.K1 SCD/Spy-CD4 ligand cells loaded with 100 nM peptide. Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 Strep-tag II (A) or PD-1 control (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy-CD4 ligand cells, either uncoupled or coupled with 200 nM monovalent or tetravalent StrepTactin-SpyCatcher as indicated. NF- κ B-eGFP gMFI was measured by flow cytometry and normalised to stimulation with uncoupled CHO.K1 SCD/Spy-CD4 cells, showing mean plus standard deviation from four independent experiments performed with biological replicates. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

4.3.3.3 Stimulation of Jurkat receptor cells with a titration of StrepTactin-SpyCatcher

For analysis of the stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II receptor cells with SCD-based ligand cells loaded with 100 nM peptide and coupled with a titration of tetravalent StrepTactin-SpyCatcher, NF- κ B-eGFP gMFI was plotted against relative coupling levels to control for the relative amount of Strep-tag II binding sites available between the ligand cell lines and between experiments. This was possible due to the reasonably similar and consistent levels of coupling obtained for these cells (Figures 4.20 - 4.22).

As seen in Figure 4.27, there was a statistically significant trend of inhibition of NF- κ B-eGFP gMFI with increased relative coupling of tetravalent StrepTactin-SpyCatcher for stimulation with both the CHO.K1 SCD/Spy cells (non-zero slope of -0.1939 with P-value < 0.0001) and CHO.K1 SCD/Spy-CD4 cells (non-zero slope of -0.0932 with P-value < 0.0001), using a null hypothesis of a slope of zero. As well as being negative, the slopes were different to each other (comparison of fits using a null hypothesis of the same slope for both data sets P-value = 0.0001), suggesting that PD-1 Strep-tag II-mediated inhibition was impaired upon stimulation with the CHO.K1 SCD/Spy-CD4 cells compared to stimulation with the CHO.K1 SCD/Spy cells.

4.4 Chapter Discussion

This chapter has explored the use of CHO.K1 ligand cells expressing ligands of different lengths to test the importance of receptor-ligand dimensions for PD-1 signal integration in both THP-1 and Jurkat receptor cells in the two-ligand system.

4.4.1 Ligand cell lines with matched and mismatched receptor-ligand dimensions

A set of CHO.K1 ligand cells was generated for stimulation of the THP-1 and Jurkat receptor cells with matched or mismatched receptor-ligand dimensions (Table 4.1). The expression levels of the CD48-based and HLA-A2 SCD activating ligands were well-matched between the different ligand cell lines (Figures 4.3 and 4.5). However, expression levels of the Spy-based constructs were not well-matched and this proved to be one of the biggest challenges faced throughout the THP-1 receptor cell stimulation experiments. Despite continuous co-selection of the ligands using MSX and G418 as well as several rounds of FACS, expression levels of the different Spy-based constructs

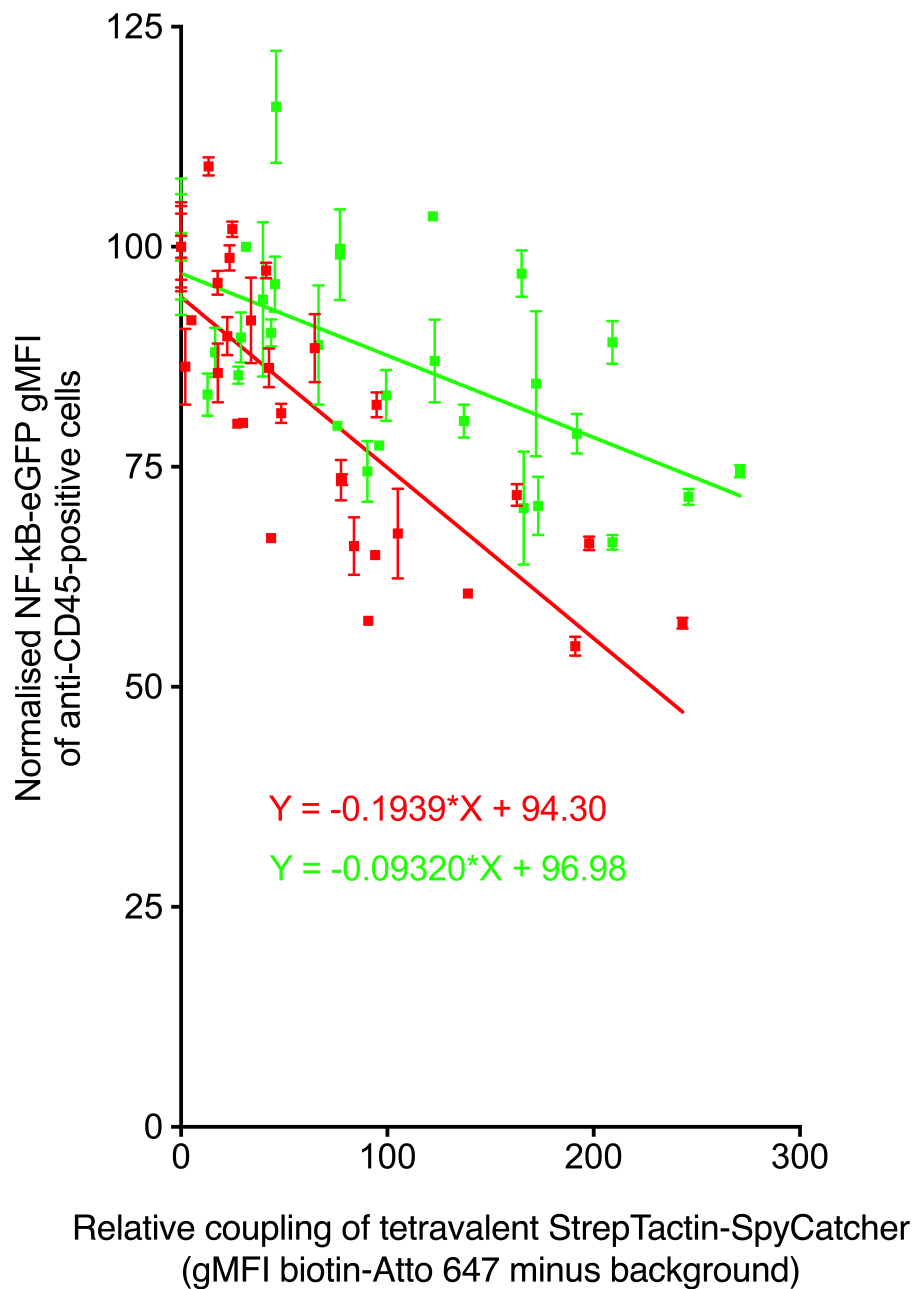


Figure 4.27: Quantification of NF-κB-eGFP gMFI from cell-cell stimulation of Jurkat NF-κB-eGFP/1G4 TCR receptor cells with CHO.K1 SCD/Spy (red) or CHO.K1 SCD/Spy-CD4 (green) ligand cells loaded with 100 nM peptide. The Jurkat receptor cells were stimulated with CHO.K1 SCD-based ligand cells either uncoupled or coupled with a titration of tetravalent StrepTactin-SpyCatcher. NF-κB-eGFP gMFI was measured by flow cytometry and normalised to stimulation with uncoupled ligand cells plotted against relative coupling of tetravalent StrepTactin-SpyCatcher as measured from biotin-ATTO 647 gMFI. Results show mean plus range for biological replicates from four independent experiments. Equations for line of best fits are shown inset for the SCD/Spy (red) and SCD/Spy-CD4 (green) cells.

were variable. The Spy expression levels were particularly low for both the CHO.K1 CD48/Spy-CD2-CD2 cells and the CHO.K1 CD48-CD4/Spy-CD2-CD2 cells (Figure 4.2). This was reflected in the poor coupling of tetravalent StrepTactin-SpyCatcher to these ligand cells (Figures 4.6 - 4.11).

4.4.2 THP-1 stimulation results

Due to the poor and variable coupling of the StrepTactin-SpyCatcher to the CD48-based ligand cells, the results from stimulation of the THP-1 receptor cells with the different ligand cells are inconclusive; variation in coupling means that the stimulation results from the different ligand cells cannot be compared to one another. However, it appears from the results with the CHO.K1 CD48/Spy ligand cells coupled with a titration of tetravalent StrepTactin-SpyCatcher that PD-1 Strep-tag II can be specifically engaged via the Strep-tag II tag, with a modest trend of inhibition of IL-8 production by the THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with increased coupling (Figure 4.13).

Stimulation with the CHO.K1 CD48/Spy-CD2-CD2 ligand cells coupled with a titration of tetravalent StrepTactin-SpyCatcher showed a possible trend of IL-8 inhibition from low to intermediate coupling levels but no further inhibition at higher levels of coupling (Figure 4.15). However, more efficient and comparable levels of coupling of the StrepTactin-SpyCatcher need to be achieved before the strength and kinetics of the PD-1 Strep-tag II-mediated inhibition with matched and mismatched receptor-ligand dimensions can be further investigated in this system. Moreover, full dose response curves are needed in order to perform meaningful statistical analyses comparing stimulation with the different ligand cell lines.

For the CHO.K1 CD48-CD4/Spy and CHO.K1 CD48-CD4/CD2-CD2 ligand cells, there were lower IL-8 levels from stimulation of the THP-1 receptor cells with these uncoupled ligand cells compared to stimulation with ligand cells expressing normal length CD48 (Appendices B.2 - B.4). This did not appear to be due to variation in expression levels of the CD48-based constructs (Figure 4.3); therefore we hypothesise that the lower level of IL-8 production from stimulation with the CD48-CD4 construct is due to a decrease in segregation of the large phosphatases CD45 and CD148 from areas of triggering through the activating 2B4-CD64 receptor. This hypothesis could be tested using microscopy to analyse the co-localisation of ligated receptor and phosphatases in areas of close contact between cells, as has been done in previous studies of NTRs (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009); Cordoba *et al.* (2013); Lim *et al.* (2015)). This approach could also be used to determine the degree

of co-localisation of receptor-ligand complexes with matched or mismatched dimensions, as has been done for activating and co-inhibitory NTRs in NK cells (Brzostek *et al.* (2010); Köhler *et al.* (2010)).

There was no clear trend of inhibition (or otherwise) from stimulation of the THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with either the CHO.K1 CD48-CD4/Spy or CHO.K1 CD48-CD4/CD2-CD2 ligand cells coupled with tetravalent StrepTactin-SpyCatcher (Figures 4.16 - 4.19). However, these results are inconclusive as they may be due to poor coupling of the StrepTactin-SpyCatcher to these ligand cells (Figures 4.6 - 4.11).

4.4.3 Jurkat stimulation results

For experiments using the Jurkat receptor cells, both the Spy construct expression levels and the coupling of StrepTactin-SpyCatcher were substantially better for the SCD-based ligand cells than for the CD48-based ligand cells (Figures 4.4 and 4.20 - 4.22). The well-matched levels of coupling of StrepTactin-SpyCatcher to the SCD-based ligand cells allows for comparison of results from stimulation of the Jurkat receptor cells with these different ligand cells.

Stimulation of the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II receptor cells with ligand cells loaded with 10 nM C9V peptide resulted in specific and statistically significant inhibition upon coupling of tetravalent StrepTactin-SpyCatcher to the CHO.K1 SCD/Spy ligand cells ($P = 0.0074$; panel A, Figure 4.23). This inhibition was not detected from stimulation of the same Jurkat receptor cells with the CHO.K1 SCD/Spy-CD4 cells coupled with tetravalent ligand ($P = 0.3299$; panel A, Figure 4.24). Nonetheless, it was unclear whether the lack of statistically significant inhibition from stimulation with the CHO.K1 SCD/Spy-CD4 cells was due to abrogated signal integration or lower overall activation from stimulation with these cells (Appendix B.5).

This issue was addressed by repeating the Jurkat stimulation experiments with a higher concentration of C9V peptide to increase activation of the Jurkat receptor cells. Upon loading ligand cells with 100 nM peptide, there was statistically significant inhibition of NF- κ B-eGFP gMFI when stimulating with either the CHO.K1 SCD/Spy ($P = 0.0008$; panel A, Figure 4.25) or the CHO.K1 SCD/Spy-CD4 ($P = 0.0040$; panel A, Figure 4.26) ligand cells coupled with tetravalent StrepTactin-SpyCatcher.

However, it is important to note that the specificity of the inhibition observed from stimulation with the CHO.K1 SCD/Spy-CD4 ligand cells is unclear as there

was also a statistically significant inhibition of NF- κ B-eGFP gMFI in the Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 control cells stimulated with the same CHO.K1 SCD/Spy-CD4 cells loaded with 100 nM C9V peptide and coupled with monovalent ($P = 0.0352$) or tetravalent ($P = 0.0356$) StrepTactin-SpyCatcher (panel B, Figure 4.26). The cause of this non-specific inhibition is currently unknown.

Statistical analysis of the NF- κ B-eGFP gMFI of Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II cells stimulated with CHO.K1 SCD/Spy or CHO.K1 SCD/Spy-CD4 ligand cells coupled with a titration of tetravalent StrepTactin-SpyCatcher shows a difference between the slopes of the line-of-best-fits for stimulations with the two SCD-based ligand cell lines (Figure 4.27). A comparison of fits with slopes of -0.1939 and -0.0932 from stimulation with the CHO.K1 SCD/Spy and CHO.K1 SCD/Spy-CD4 ligand cells respectively shows a statistically significant difference between the slopes (P -value = 0.0001). This difference highlights a partial abrogation of PD-1 Strep-tag II-mediated inhibition from stimulation with the CHO.K1 SCD/Spy-CD4 ligand cells compared to the CHO.K1 SCD/Spy cells, supporting “hypothesis 1” that well-matched NTR-ligand dimensions are required for optimal signal integration (Figure 1.3). As with the THP-1 system, this could be further investigated using microscopy to analyse the co-localisation of receptor-ligand complexes with each other and with phosphatases at the cell-cell interface.

4.4.4 Areas for further development

An alternative approach is needed to overcome the challenge of variable Spy construct expression levels and coupling of the StrepTactin-SpyCatcher, including differences both between experiments and between ligand cell lines, in particular for the CD48-based ligand cells. One possibility is incorporation of the elongation of receptor-ligand dimensions into the soluble component of the generic ligand. This would allow us to use the same ligand cells expressing the Spy construct coupled with soluble ligand components of different lengths e.g. CHO.K1 CD48/Spy ligand cells coupled with normal length or elongated StrepTactin-SpyCatcher.

In addition to improving ligand coupling, another area for development of the two-ligand system is increasing the strength of the PD-1-mediated inhibition. The inhibition of IL8 production observed from stimulation of the THP-1 receptor cells was relatively weak and not statistically significant, even with the strongest inhibition seen with the CHO.K1 CD48/Spy ligand cells ($P = 0.0685$; panel A, Figure 4.12), and the inhibition of NF- κ B-eGFP gMFI of Jurkat receptor cells was still only modest even with efficient coupling of the StrepTactin-SpyCatcher to the SCD-based ligand

cells (up to approximately 50% inhibition compared to stimulation with uncoupled ligand cells; panel A, Figure 4.23). Therefore, options for increasing the strength of the PD-1-mediated inhibition should be explored, as discussed in detail in Section 6.4.1.

Another aspect of this system that requires further development is generation of ligand cells with elongated activating ligand for stimulation of the Jurkat receptor cells. Since insertion of Ig domains into the HLA-A2 SCD renders it non-functional (elongated SCD is not surface expressed; data not shown), we were only able to test stimulation of the Jurkat receptor cells with CHO.K1 SCD/Spy and CHO.K1 SCD/Spy-CD4 ligand cells. Ideally we would also want to test stimulation with the complementary ligand cells expressing elongated activating ligand.

A possible approach for this would be to generate a single chain trimer (SCT) version of HLA-A2 that also encodes the desired peptide (Choudhuri *et al.* (2005)). Generation of these additional ligand cells with an elongated ligand for the 1G4 TCR would allow us to investigate whether elongation of both activating and inhibitory receptor-ligand dimensions can restore signal integration compared to mismatched dimensions, as has been found for some NK cell NTRs (Brzostek *et al.* (2010); Köhler *et al.* (2010)). If elongated activating NTR-ligand signalling can be effectively inhibited by similarly elongated inhibitory receptor-ligand interactions, this would provide strong support for the requirement for matched receptor-ligand dimensions for optimal signal integration. This is because such an experiment would control for the possibility that ligand elongation somehow results in abrogation of inhibitory signalling, such as through disrupting receptor-ligand interactions or the inhibitory signalling itself.

Investigating the importance of intracellular tail length for the function of the inhibitory NTR, programmed cell death protein 1 (PD-1)

5.1 Chapter Introduction

5.1.1 NTR signalling through immunoreceptor tyrosine-based motifs

As indicated by their name, NTRs have no intrinsic catalytic activity, instead signalling via motifs in their intracellular tails or associated adaptors (reviewed in Biladeau & Leibson (2002); Verbrugge & Meyaard (2005)). These motifs include immunoreceptor tyrosine-based activating, inhibitory, and switch motifs (ITAMs, ITIMs and ITSMs respectively), which contain conserved tyrosine residues. Downstream signalling depends on the phosphorylation state of the tyrosine residues and is mediated through recruitment of cytoplasmic proteins. Phosphorylation of these motifs is regulated by the opposing function of SFKs such as Lck and RPTPs such as CD45 (Figure 1.1).

Signalling by ITAMs

ITAMs are made up of tandem tyrosine residues in the conserved amino acid consensus

sequence Yxx(L/I)_{x6-8}Yxx(L/I), where x is any amino acid (Reth (1989); Cambier (1995)). Phosphorylation of ITAMs by SFKs creates docking sites for recruitment of tandem-SH2-containing signalling molecules including members of the Syk family of tyrosine kinases such as Syk and ZAP70 (Chan *et al.* (1992); Wange *et al.* (1993); Kihara & Siraganian (1994); Narula *et al.* (1995)). For instance, the CD3 ζ chains associated with the T cell receptor contain 3 ITAMs each, which when phosphorylated recruit ZAP70 to initiate downstream signalling and T cell activation (Chan *et al.* (1992); as reviewed by Humphrey *et al.* (2005); Love & Hayes (2010)).

Similarly, the activating receptor CD64 (Fc γ RI) associates with the ITAM-containing adaptor molecule referred to as the FcR γ chain (Küster *et al.* (1990); Orloff *et al.* (1990)). Phosphorylation of the ITAM of the FcR γ chain results in downstream signalling through recruitment of Syk and is required for CD64-mediated activation (Agarwal *et al.* (1993); Crowley *et al.* (1997); Van Vugt *et al.* (1998)).

Signalling by ITIMs

The ITAM-mediated signalling described above can be opposed by the inhibitory function of ITIM-containing receptors, which have the conserved amino acid sequence (S/I/V/L)xYxx(I/V/L) (Amigorena *et al.* (1992); Muta *et al.* (1994)). Unlike ITAMs, phosphorylation of the tyrosine residue in ITIMs results in recruitment of phosphatases such as the inositol phosphatase SHIP-1 or protein tyrosine phosphatases SHP-1 or SHP-2 (Amigorena *et al.* (1992); Muta *et al.* (1994); D'Ambrosio *et al.* (1995); Ono *et al.* (1996); Vély *et al.* (1996); Kuroiwa *et al.* (1998)). These phosphatases can then dephosphorylate nearby molecules to regulate downstream signalling. Examples of ITIM-containing NTRs include LAIR-1 (Meyaard *et al.* (1997)), the inhibitory members of the KIR family (reviewed by Long (2008)), and most Siglecs (reviewed by Crocker & Varki (2001)).

Signalling by ITSMs

The third main type of immunoreceptor tyrosine-based motif is the ITSM or “switch” motif. The ITSM was first described by Shlapatska and colleagues who studied its role in the NTR CD150 (Shlapatska *et al.* (2001)). The action of ITSMs is dependent on the presence of members of the SLAM-associated protein (SAP) family of adaptors such as SAP (Sayos *et al.* (1998)) and Ewing sarcoma-associated transcript (EAT)-2 (Cruz-Munoz *et al.* (2009)). In the absence of SAP family adaptors, inhibitory molecules such as SHP-1/SHP-2 are directly recruited to the ITSM, whereas in the

presence of SAP/EAT-2, activating molecules such as Fyn and PLC- γ are recruited via SAP and EAT-2 respectively (reviewed by Wu & Veillette (2016)). The consensus sequence of the ITSM, TxYxx(I/V), contains a conserved threonine residue that distinguishes it from the ITIM (Shlapatska *et al.* (2001)). Examples of ITSM-containing NTRs include members of the SLAM family of receptors (reviewed by Detre *et al.* (2010)).

5.1.2 The PD-1 intracellular tail

The focus of this study is the co-inhibitory NTR PD-1, which contains both an ITIM and an ITSM within its intracellular tail. Interestingly, evidence suggests that neither of these motifs function in a characteristic way in this receptor. Firstly, it has been shown that the ITIM of PD-1 is not essential for its inhibitory function (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012)). Instead, it is the ITSM that is most important for the inhibitory function of PD-1. Okazaki *et al.* used a murine B cell tumour line to show that the ITSM was required for inhibition of BCR-mediated calcium flux and downstream signalling (Okazaki *et al.* (2001)). Similarly, the ITSM was found to be most important for inhibition of T cell activation by murine PD-1 (Yokosuka *et al.* (2012)) and by a murine CD28-human PD-1 chimeric receptor (Chemnitz *et al.* (2004); Parry *et al.* (2005)).

The second aspect of the PD-1 signalling motifs that is uncharacteristic is the lack of binding of the small adaptor SAP to the ITSM. This is somewhat unusual since the ITSM was originally defined according to its binding of SAP (Shlapatska *et al.* (2001)). Both Okazaki and colleagues as well as Chemnitz and colleagues found no evidence of binding of SAP to PD-1 in B or T cells (Okazaki *et al.* (2001); Chemnitz *et al.* (2004)). In contrast, several studies have demonstrated the ability of PD-1 to recruit the phosphatase SHP-2 (Latchman *et al.* (2001); Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Yokosuka *et al.* (2012)), resulting in inhibition of Akt, Ras and MEK signalling (Parry *et al.* (2005); Patsoukis *et al.* (2012)). These studies showed that SHP-2 recruitment was required for PD-1 inhibitory function, making SHP-2 a candidate key effector of PD-1-mediated inhibition. Recent preliminary findings from Hui and colleagues have also highlighted specific binding of SHP-2 to PD-1 (Hui *et al.* (2016)), further strengthening the proposal that SHP-2 is a key effector of PD-1-mediated inhibitory function.

Importantly, Chemnitz and colleagues showed that although SHP-2 recruitment was required for PD-1 signalling, it was not sufficient in the absence of PD-1 receptor engagement (Chemnitz *et al.* (2004)). This requirement for engagement of PD-1

for its inhibitory function suggests that PD-1 may need to be co-localised with its signalling target in order to affect its inhibitory function. This is supported by the finding that PD-1 is able to inhibit T cell proliferation when anti-CD3 and PD-L1 Ig are presented on the same microspheres but not when they are presented on separate microspheres (Bennett *et al.* (2003)). Results from Yokosuka *et al.* provide further evidence for the requirement of close proximity for PD-1 signalling, with elongation of the PD-1 ectodomain resulting in prevention of co-localisation with TCR microclusters and abrogation of the inhibitory function of the receptor (Yokosuka *et al.* (2012)). They also showed that the abrogation of inhibitory function of the elongated PD-1 variants could not be rescued by fusion of the phosphatases SHP-1 or SHP-2 to their intracellular tails, confirming that the loss of inhibitory function was not due to lack of recruitment of these phosphatases.

5.1.3 PD-1 intracellular tail length: important or not?

As detailed above, several studies have investigated the role of the ITIM and ITSM in PD-1 signalling. However, the importance of the positioning of these motifs within the intracellular tail has not been analysed. These motifs recruit cytoplasmic molecules such as phosphatases to regulate downstream signalling by acting on proximal targets such as the TCR CD3 chains, the associated kinase ZAP70, and/or the co-stimulatory receptor CD28 (Sheppard *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012); Zinselmeyer *et al.* (2013)). It has been suggested that the intracellular tails of NTRs are mostly unstructured (Dushek *et al.* (2012)) and we expect the position of the motifs within the tails of NTRs to affect the reach of the intracellular tail and any recruited molecules bound to it (Figure 1.4).

Interestingly, it has been pointed out that not only the sequence but also the position of these motifs is shared between related receptors (Riley (2009)), suggesting a functional relevance of motif positioning. However, to our knowledge, this has not been further investigated. It has also been observed that the conserved tyrosine residues in ITIM- and ITSM-containing NTRs are on average more than 20 amino acids further from the plasma membrane than those in ITAMs (Dr Jesse Goyette, unpublished). This indicates that there may be a functional relevance of long intracellular tails in these receptors.

We propose that, in addition to the receptor-ligand dimensions investigated in Chapter 4, NTR signalling may also be dependent on the dimensions on the other side of the membrane: the intracellular tail. Specifically, we hypothesise that optimal signal integration by inhibitory NTRs is dependent on the length of their intracellular

tail, with longer tails enabling inhibition throughout a greater volume (“hypothesis 2”; Section 1.7.2; Figure 1.4).

There are relatively few studies that systematically investigate the importance of cytoplasmic dimensions of transmembrane proteins (Ohuchi *et al.* (1998); Chan *et al.* (2004)), and to our knowledge there are none that investigate the intracellular dimensions of NTRs. Most studies of NTRs either completely remove the intracellular tail or use targeted amino acid mutations to specifically knock out the function of signalling motifs (Engelhardt *et al.* (1991); Delgado *et al.* (2000); Hueber *et al.* (2006)). Romeo and colleagues tested signalling by truncated versions of CD3 ζ (Romeo *et al.* (1992)), but no studies have systematically tested NTRs with a series of elongated and truncated cytoplasmic tails.

In light of this, we set out to test the co-inhibitory NTR PD-1 to determine if altering the length of its intracellular tail would impact its inhibitory function.

5.2 Chapter Aim

The aim of this chapter is to use the two-ligand system to investigate the importance of the length of the intracellular tail of PD-1 for its inhibitory function.

5.3 Results

5.3.1 Generation of PD-1 intracellular tail mutants of different lengths

A series of Strep-tag II-tagged human PD-1 constructs with intracellular tails of different lengths were generated by either truncation or elongation of the intracellular tail using the primers set out in Section 2.4. For the truncated mutants, amino acid residues Q207 - P243 were removed, including the entire ITIM. For the elongated mutants, either one copy (elongated x1) or two copies (elongated x2) of the PD-1 ITIM/ITSM mutant intracellular tail were inserted at amino acid position 207.

Variants of each length with mutations to the ITIM (Y223F; ITIM mutant), ITSM (Y248F; ITSM mutant) or both (Y223/Y248F; ITIM/ITSM mutant) were generated to allow investigation of the role of these motifs in PD-1 constructs of different lengths. These PD-1 constructs are detailed in Table 5.1, with the full set of PD-1 variants, including PD-1 control and PD-1 Strep-tag II, represented in Figure 5.1.

Receptor construct	Phosphorylatable motif	Number of amino acids from membrane to phosphorylatable tyrosine
PD-1 truncated	ITSM	20
PD-1 truncated ITSM mutant	None	N/A
PD-1 ITSM mutant	ITIM	32
PD-1 ITIM mutant	ITSM	57
PD-1 ITIM/ITSM mutant	None	N/A
PD-1 ITSM mutant elongated x1	ITIM	114
PD-1 ITIM mutant elongated x1	ITSM	139
PD-1 ITIM/ITSM mutant elongated x1	None	N/A
PD-1 ITSM mutant elongated x2	ITIM	196
PD-1 ITIM mutant elongated x2	ITSM	221
PD-1 ITIM/ITSM mutant elongated x2	None	N/A

Table 5.1: Details of Strep-tag II-tagged PD-1 variants cloned with different length intracellular tails and mutations to the ITIM and/or ITSM. For the elongated mutants, either one copy (elongated x1) or two copies (elongated x2) of the PD-1 ITIM/ITSM mutant intracellular tail were inserted at amino acid position 207. Mutation of the ITIM = Y223F; mutation of the ITSM = Y248F.

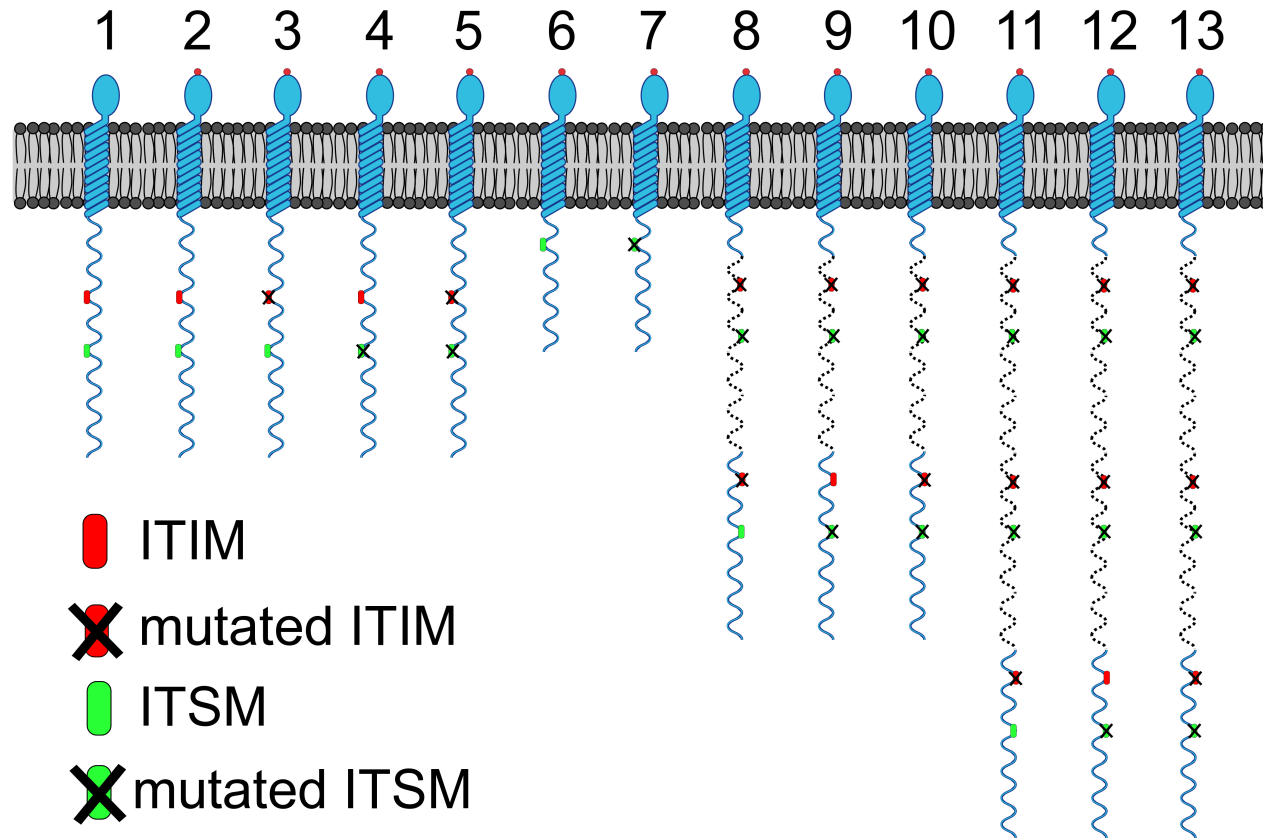


Figure 5.1: Schematic representation of the full set of PD-1 variants cloned with different length intracellular tails and mutations to the ITIM and/or ITSM. Variants include 1. PD-1 control, 2. PD-1 Strep-tag II, 3. PD-1 ITIM mutant, 4. PD-1 ITSM mutant, 5. PD-1 ITIM/ITSM mutant, 6. PD-1 truncated, 7. PD-1 truncated ITSM mutant, 8. PD-1 ITIM mutant elongated $x1$, 9. PD-1 ITSM mutant elongated $x1$, 10. PD-1 ITIM/ITSM mutant elongated $x1$, 11. PD-1 ITIM mutant elongated $x2$, 12. PD-1 ITSM mutant elongated $x2$, 13. PD-1 ITIM/ITSM mutant elongated $x2$. All variants except for the PD-1 control have an extracellular (N-terminal) Strep-tag II tag (small red circle). Inserted copies of the PD-1 ITIM/ITSM mutant intracellular tail in the elongated variants are represented by dashed lines. Mutated ITIM = Y223F mutant; mutated ITSM = Y248F mutant.

5.3.2 Testing PD-1 ITIM, ITSM, and ITIM/ITSM mutants in THP-1 receptor cells

To start with, the PD-1 variants of normal length (PD-1 control, PD-1 Strep-tag II, PD-1 ITIM mutant, PD-1 ITSM mutant and PD-1 ITIM/ITSM mutant) were tested to determine if they have inhibitory function in the THP-1 receptor cells.

Lentiviral transduction of THP-1 cells with the PD-1 constructs followed by sorting of positive cells by anti-Strep-tag II-magnetic bead sorting and/or FACS resulted in THP-1 receptor cells with well-matched expression levels of the different PD-1 constructs (Figure 5.2). Levels of CD64 were also well-matched (Figure 5.3).

The receptor cells were vitamin D-differentiated then stimulated with platebound antibodies including 2.5 $\mu\text{g}/\text{ml}$ anti-CD64 plus increasing concentrations of anti-Strep-tag II. The total amount of platebound antibody was kept constant using an IgG1 isotype control.

The PD-1 Strep-tag II and PD-1 ITIM mutant, which both have a functional ITSM, showed strong inhibition of IL-8 production with increasing anti-Strep-tag II (Figure 5.4). This inhibition was not observed with the THP-1 receptor cells expressing PD-1 control, indicating that the inhibition is specific to engagement of the Strep-tag II-tagged PD-1 constructs. No inhibition was measured from stimulation of THP-1 cells expressing the PD-1 ITSM mutant or the PD-1 ITIM/ITSM mutant (Figure 5.4).

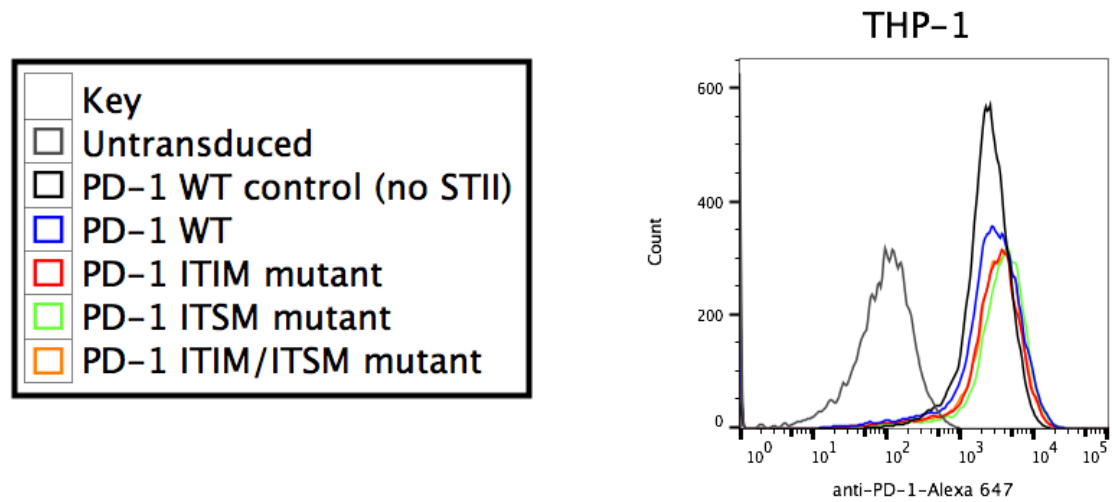


Figure 5.2: Flow cytometry results showing untransduced THP-1 cells (grey) and THP-1 receptor cells expressing the indicated PD-1 constructs stained with anti-PD-1-Alexa 647, including PD-1 control (black), PD-1 Strep-tag II (blue), PD-1 ITIM mutant (red), PD-1 ITSM mutant (green), and PD-1 ITIM/ITSM mutant (orange).

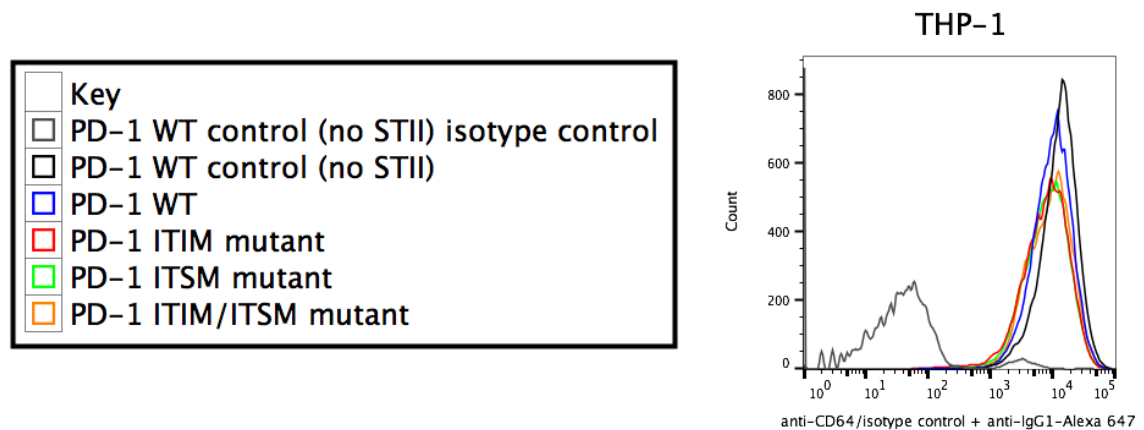


Figure 5.3: Flow cytometry results showing CD64 expression levels on THP-1 receptor cells expressing the indicated PD-1 constructs stained with anti-CD64 plus anti-mouse IgG1-Alexa 647, including PD-1 control (black), PD-1 Strep-tag II (blue), PD-1 ITIM mutant (red), PD-1 ITSM mutant (green), and PD-1 ITIM/ITSM mutant (orange). THP-1 PD-1 control cells stained with an isotype control antibody plus anti-mouse IgG1-Alexa 647 are shown in grey.

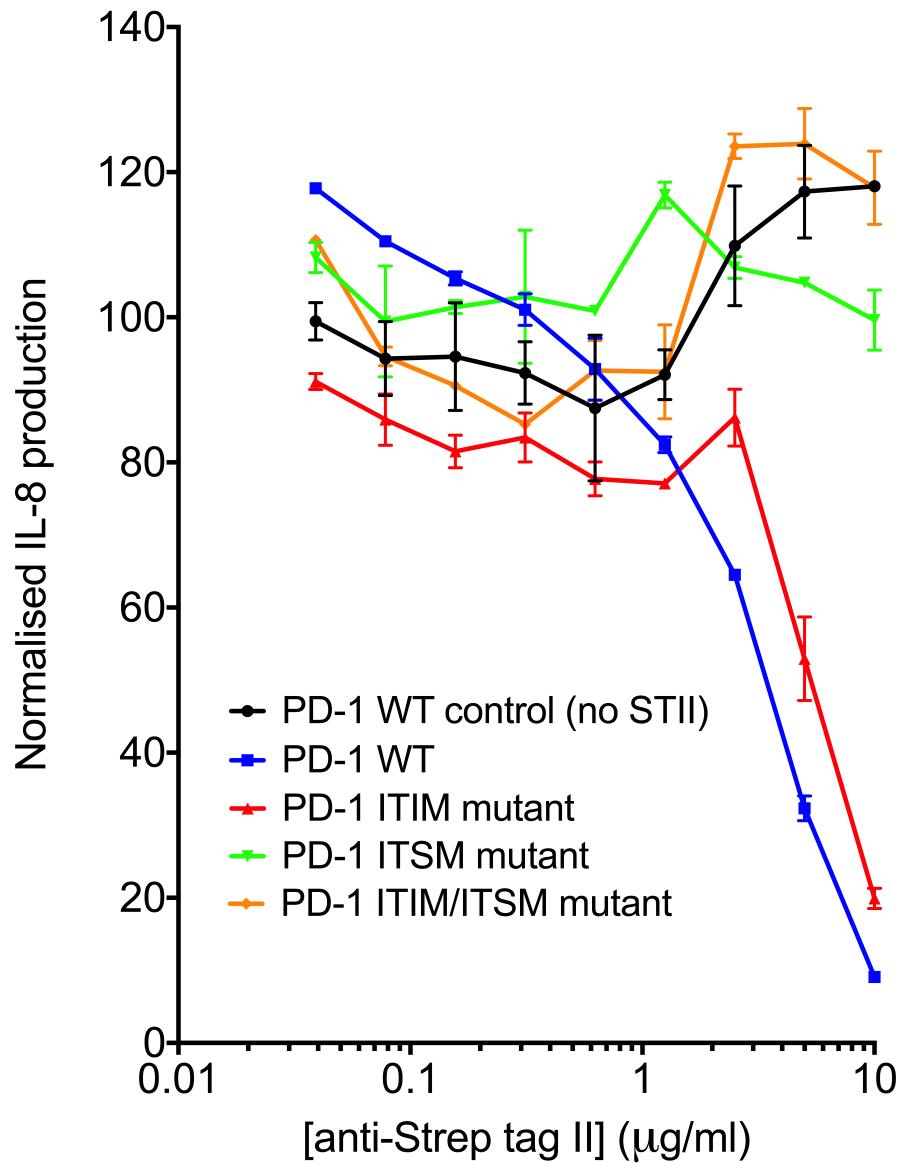


Figure 5.4: Normalised IL-8 production from platebound antibody stimulation of vitamin D-differentiated THP-1 receptor cells expressing the indicated forms of PD-1, including PD-1 control (black), PD-1 Strep-tag II (blue), PD-1 ITIM mutant (red), PD-1 ITSM mutant (green), and PD-1 ITIM/ITSM mutant (orange). The THP-1 cells were stimulated with 2.5 µg/ml platebound anti-CD64 plus a titration of anti-Strep-tag II. Total IgG levels were kept constant using an IgG1 isotype control antibody. IL-8 levels in supernatant were measured by ELISA and normalised to levels from anti-CD64 stimulation without anti-Strep-tag II. Results show mean and range of biological replicates and are representative of three independent experiments.

5.3.3 Testing PD-1 intracellular tail mutants in Jurkat receptor cells

To further investigate the PD-1 intracellular tail we moved onto testing PD-1-mediated inhibition of T cell receptor signalling, which may have implications for several immunotherapies currently under development. For these experiments the full set of PD-1 variants of different lengths and with different mutations (Figure 5.1) were tested in Jurkat NF κ -B-eGFP/1G4 TCR receptor cells.

Lentiviral transduction of Jurkat NF κ -B-eGFP/1G4 TCR cells with the PD-1 constructs followed by sorting of positive cells by FACS resulted in Jurkat receptor cells with reasonably well-matched expression levels of the different PD-1 constructs (Figure 5.6). The expression levels of the 1G4 TCR remained well-matched after the transduction and sorting process (Figure 5.6).

5.3.3.1 Platebound antibody stimulation of Jurkat receptor cells

Preliminary platebound antibody stimulations of the Jurkat NF κ -B-eGFP/1G4 TCR cells expressing the PD-1 ITIM/ITSM mutant showed higher (approximately double) NF κ -B-eGFP gMFI upon stimulation with anti-CD3 plus anti-Strep-tag II compared to stimulation with anti-CD3 plus an IgG1 isotype control ($P = 0.0131$; Figure 5.5). We hypothesised that this was due to engagement of a signalling incompetent form of PD-1 using an immobilised platebound antibody (in this case the PD-1 ITIM/ITSM mutant being engaged by platebound anti-Strep-tag II), which could result in an increase in the number and/or area of close contacts formed, promoting CD3-mediated signalling. If this is the case then increased activation should also be induced by engagement of an irrelevant cell surface molecule such as transferrin receptor 1 (TfR1). Therefore we investigated this hypothesis by performing a platebound antibody stimulation of four of the Jurkat receptor cell lines (PD-1 control, PD-1 Strep-tag II, PD-1 ITIM mutant, and PD-1 ITIM/ITSM mutant) with anti-CD3 plus an IgG1 isotype control, anti-TfR1, or anti-Strep-tag II.

As seen from the results in Figure 5.7, all four receptor cell lines showed a trend of increase in NF κ -B-eGFP gMFI when stimulated with a combination of anti-CD3 plus anti-TfR1 compared to stimulation with anti-CD3 plus IgG1 isotype control, with a statistically significant increase for the PD-1 ITIM mutant cells ($P = 0.0122$). This supports our hypothesis that engagement of cell surface molecules with immobilised platebound antibody can increase NF κ -B upregulation in the Jurkat receptor cells.

The increase in NF κ -B-eGFP gMFI was statistically significant upon stimulation of the Jurkat receptor cells expressing the PD-1 ITIM/ITSM mutant with a combination of anti-CD3 plus anti-Strep-tag II (panel D, Figure 5.7; $P = 0.0286$). There was no increase in NF κ -B-eGFP gMFI measured from stimulation of the PD-1 Strep-tag II cells with anti-CD3 plus anti-Strep-tag II (panel B, Figure 5.7), most likely because of opposing signalling from PD-1-mediated inhibition via engagement of the Strep-tag II tag.

Overall these results indicate that the anti-Strep-tag II-mediated inhibitory function of PD-1 is being masked by a non-specific increase in activation from platebound antibody binding to cell surface molecules, in this case the Strep-tag II-tagged PD-1 variants. This is problematic for studying signal integration in the Jurkat receptor cells and provides further impetus for developing a cell-cell stimulation approach such as the two-ligand system.

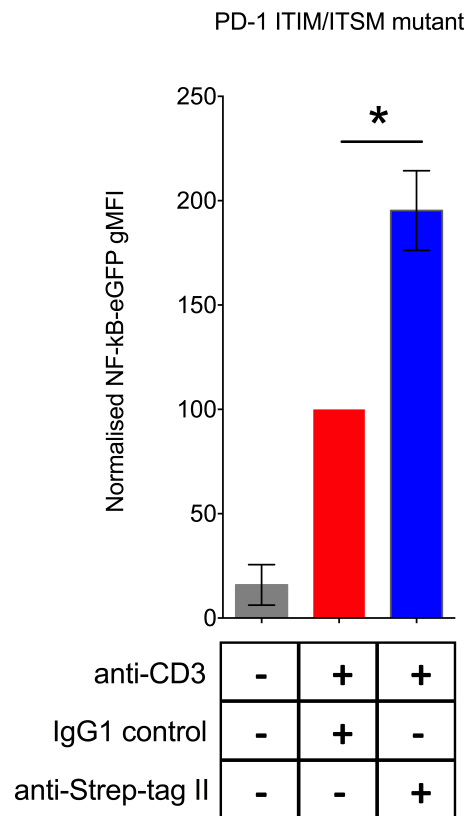


Figure 5.5: Normalised $\text{NF-}\kappa\text{B-eGFP}$ gMFI of Jurkat $\text{NF-}\kappa\text{B-eGFP}/1\text{G}4$ TCR receptor cells expressing PD-1 ITIM/ITSM mutant stimulated with platebound antibodies. The cells were unstimulated (PBS + 1% BSA), or stimulated with $0.5\text{ }\mu\text{g/ml}$ anti-CD3 plus either $10\text{ }\mu\text{g/ml}$ IgG1 isotype control or $10\text{ }\mu\text{g/ml}$ anti-Strep-tag II as indicated. Results are from three independent experiments performed with biological replicates showing mean plus standard deviation normalised to stimulation with anti-CD3 plus isotype control. * = $P \leq 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

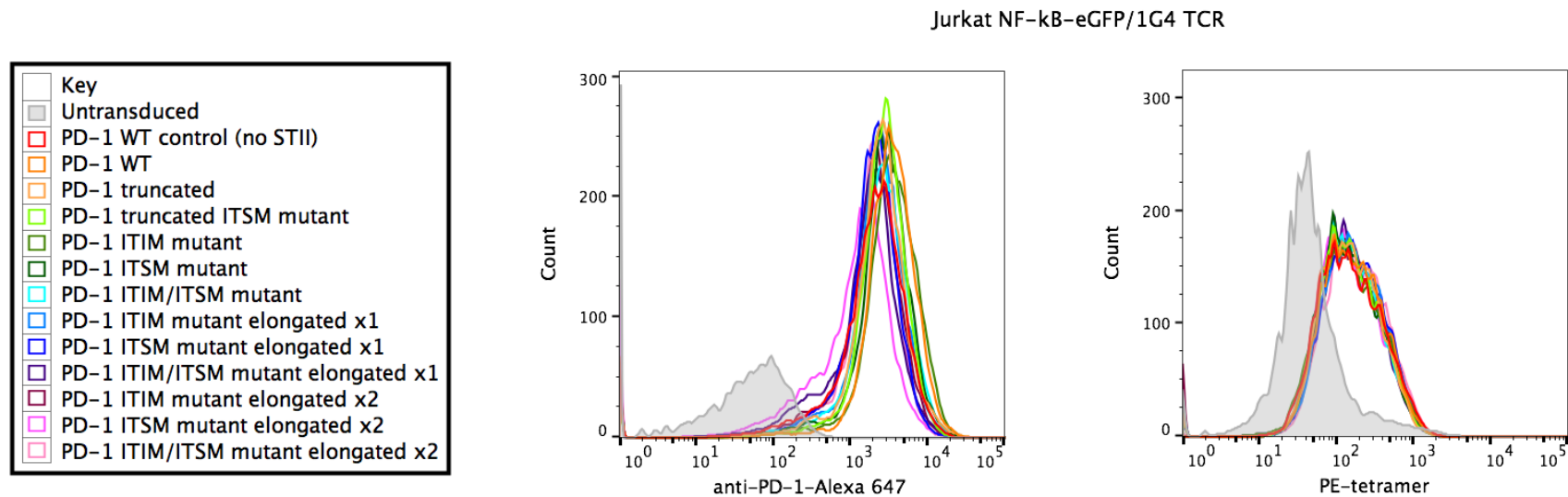


Figure 5.6: Flow cytometry results showing Jurkat NF κ -B-eGFP/1G4 TCR cells either untransduced (filled grey histogram) or lentivirally transduced with PD-1 constructs with various intracellular tails as detailed in the figure key. The cells were stained with anti-PD-1-Alexa 647 and PE-tetramer (HLA-A2-C9V; binds to the 1G4 TCR).

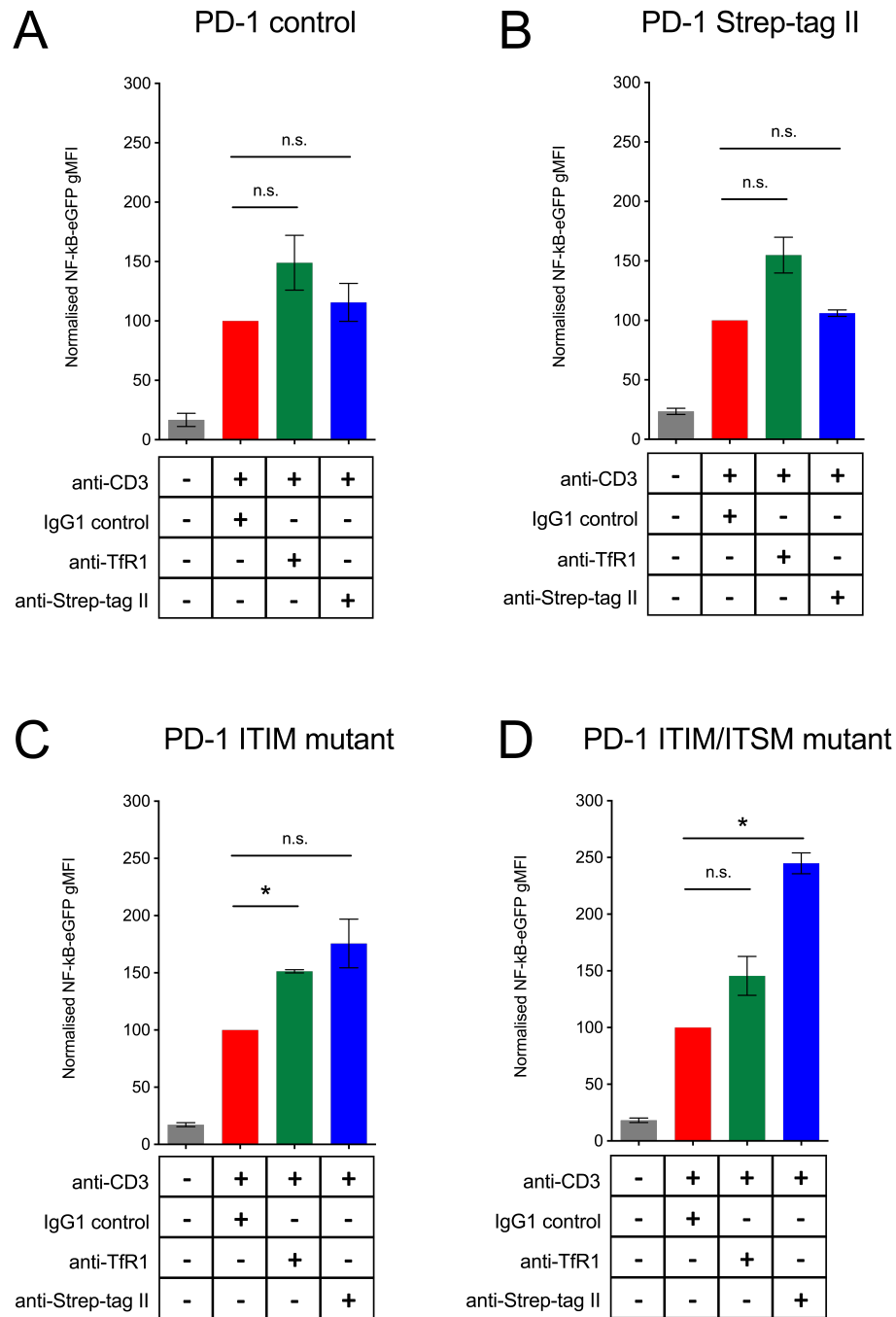


Figure 5.7: Normalised NF- κ B-eGFP gMFI of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 control (A), PD-1 Strep-tag II (B), PD-1 ITIM mutant (C) or PD-1 ITIM/ITSM mutant (D). The cells were unstimulated (PBS + 1% BSA), or stimulated with 0.5 μ g/ml anti-CD3 plus 10 μ g/ml IgG1 isotype control, 10 μ g/ml anti-TfR1, or 10 μ g/ml anti-Strep-tag II platebound antibody as indicated. Results are from two independent experiments performed with biological replicates showing mean plus standard deviation normalised to stimulation with anti-CD3 plus isotype control. n.s. = $P > 0.05$; * = $P \leq 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

5.3.3.2 Cell-cell stimulation of Jurkat receptor cells using the two-ligand system

Cell-cell stimulation experiments were performed using the two-ligand system. The Jurkat receptor cell lines expressing the full set of PD-1 intracellular tail variants (Figure 5.1) were stimulated with untransfected CHO.K1 cells or CHO.K1 SCD/Spy ligand cells loaded with 100 nM C9V peptide and either uncoupled or coupled with 200 nM tetravalent StrepTactin-SpyCatcher as demonstrated in Figures 4.21 and 4.22. Results from three independent experiments are shown in Figures 5.8 - 5.11.

Truncated PD-1 variants

Stimulation with uncoupled or coupled CHO.K1 SCD/Spy ligand cells resulted in no statistically significant difference in NF- κ B-eGFP gMFI for the Jurkat receptor cells expressing either the PD-1 truncated or PD-1 truncated ITSM mutant (Figure 5.8; $P = 0.4038$ and $P = 0.2237$ for PD-1 truncated and PD-1 truncated ITSM mutant respectively). The implications of this result are discussed in Section 5.4.4, in particular in relation to the lack of inhibition observed for the PD-1 truncated variant, which contains an ITSM.

PD-1 ITIM mutant variants

As seen in Figure 5.9 A, there was a statistically significant inhibition of the NF- κ B-eGFP gMFI of Jurkat receptor cells expressing the PD-1 ITIM mutant upon stimulation with CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher. The NF- κ B-eGFP gMFI was inhibited by approximately 25% compared to stimulation with uncoupled CHO.K1 SCD/Spy ligand cells ($P = 0.0161$; panel A, Figure 5.9).

There was also a statistically significant inhibition of NF- κ B-eGFP gMFI for both the Jurkat receptor cells expressing the PD-1 ITIM mutant elongated x1 ($P = 0.0017$; panel B, Figure 5.9) or the PD-1 ITIM mutant elongated x2 ($P = 0.0221$; panel C, Figure 5.9) when stimulating with CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher. Both of these receptor cell lines showed approximately 20% inhibition of NF- κ B-eGFP gMFI compared to stimulation with uncoupled CHO.K1 SCD/Spy ligand cells.

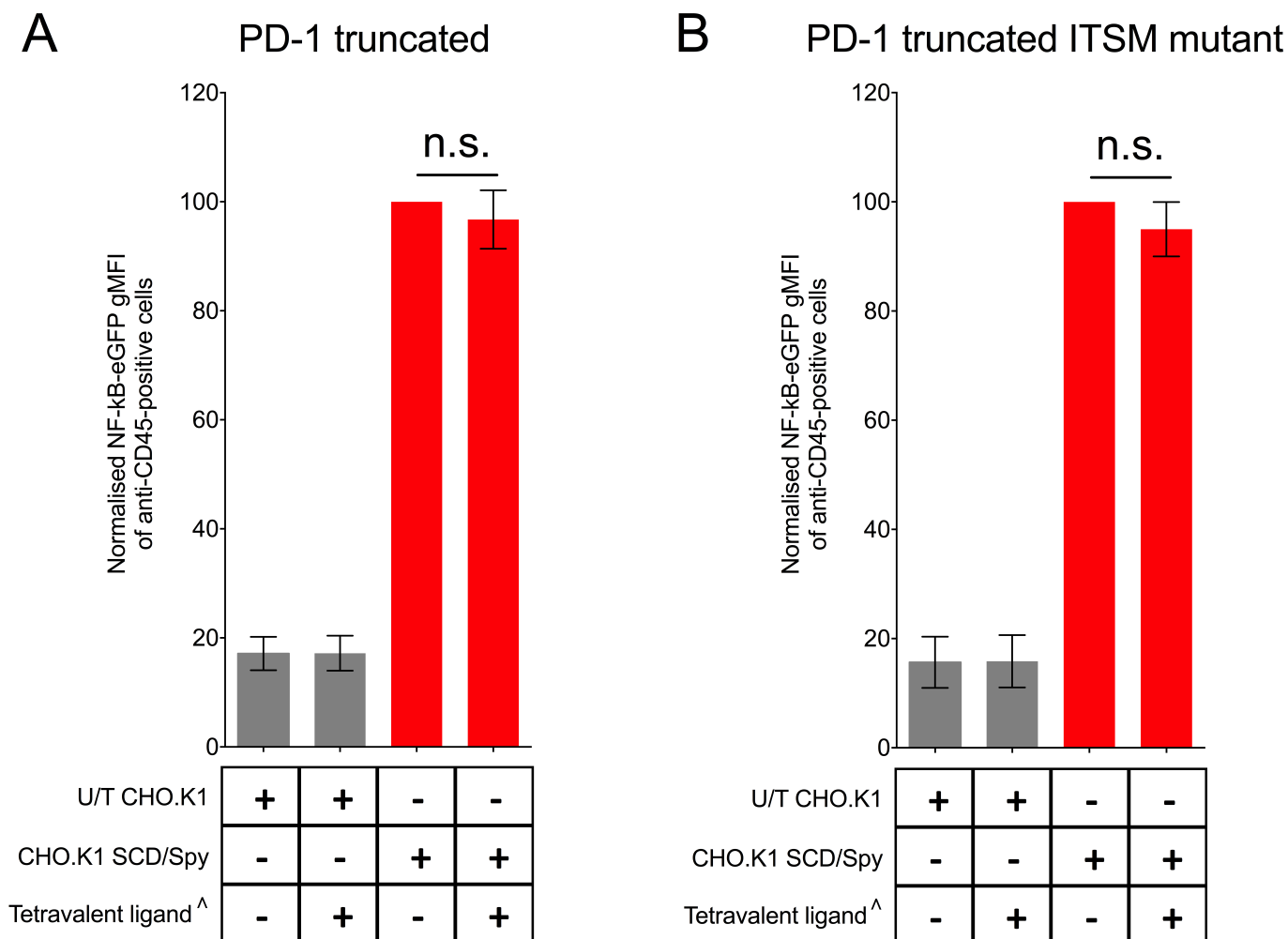


Figure 5.8: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing truncated PD-1 intracellular tail constructs including PD-1 truncated (A) and PD-1 truncated ITSM mutant (B). The cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells loaded with 100 nM NY-ESO-1 C9V peptide and either uncoupled or coupled with 200 nM tetravalent StrepTactin-SpyCatcher as indicated. Results are from three independent experiments performed with biological replicates showing mean plus standard deviation. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

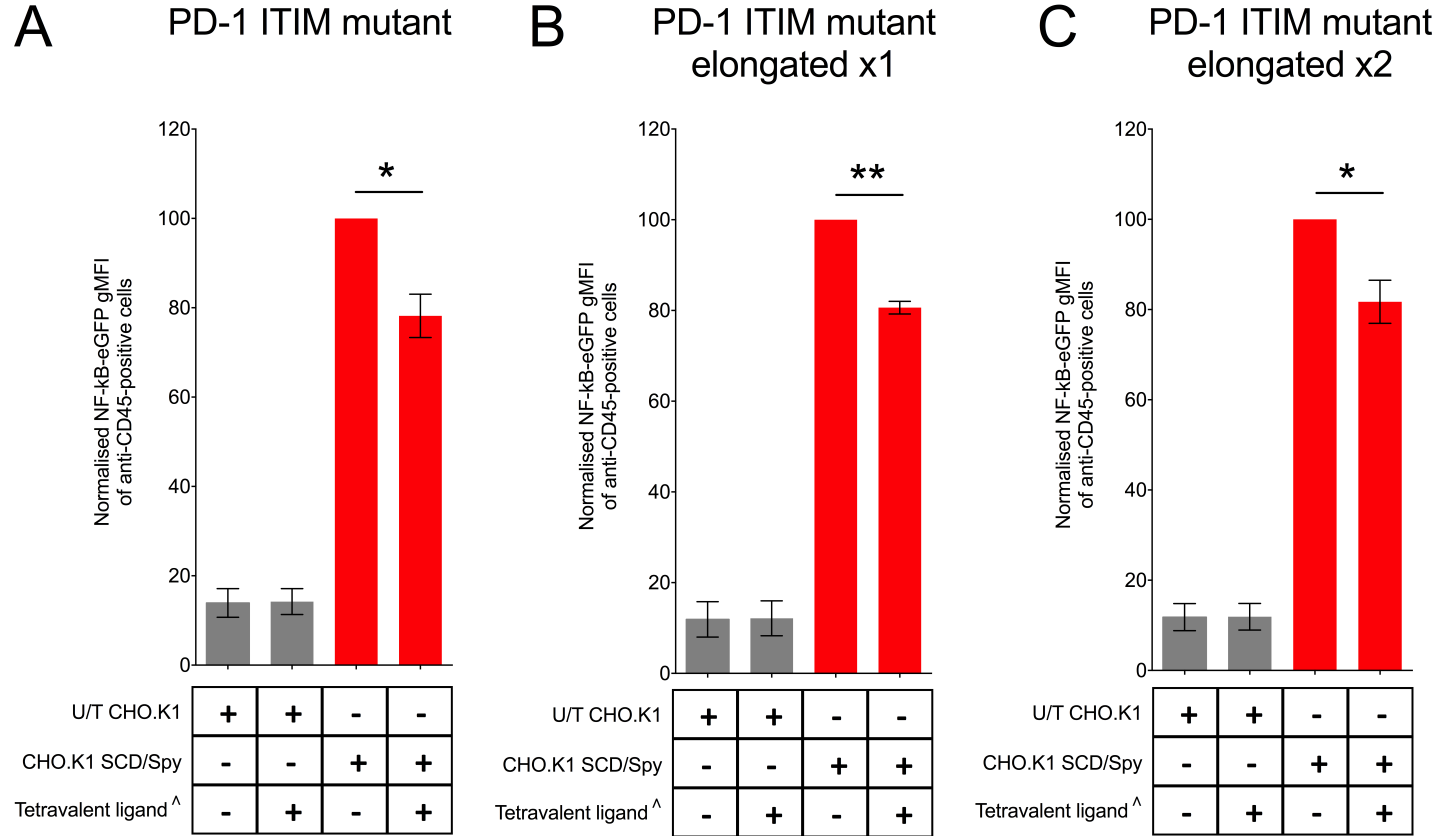


Figure 5.9: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 intracellular tail constructs of different lengths with mutations to the ITIM, including PD-1 ITIM mutant (A), PD-1 ITIM mutant elongated x1 (B), and PD-1 ITIM mutant elongated x2 (C). The Jurkat cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells loaded with 100 nM NY-ESO-1 C9V peptide and either uncoupled or coupled with 200 nM tetravalent StrepTactin-SpyCatcher as indicated. Results are from three independent experiments performed with biological replicates showing mean plus standard deviation. [^]Ligand = StrepTactin-SpyCatcher. * = $P \leq 0.05$; ** = $P \leq 0.01$; statistical significance determined by performing a one-sample *t* test comparing the experimental mean to a specified value of 100.

PD-1 ITSM mutant variants

In contrast to the PD-1 ITIM mutant variants, there was no statistically significant inhibition of NF- κ B-eGFP gMFI upon stimulation of the Jurkat receptor cells expressing the PD-1 ITSM mutant variants with CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher (Figure 5.10). This was irrelevant of intracellular tail length, with no statistically significant inhibition measured for the PD-1 ITSM mutant ($P = 0.4796$), PD-1 ITSM mutant elongated x1 ($P = 0.1046$), or PD-1 ITSM mutant elongated x2 ($P = 0.1215$; Figure 5.10).

PD-1 ITIM/ITSM mutant variants

For the Jurkat receptor cells expressing the PD-1 ITIM/ITSM mutant, there was an approximately 5% inhibition of NF- κ B-eGFP gMFI upon stimulation with coupled compared to uncoupled CHO.K1 SCD/Spy cells ($P = 0.0066$; panel A, Figure 5.5). Stimulation with uncoupled or coupled CHO.K1 SCD/Spy ligand cells showed no difference in NF- κ B-eGFP gMFI for the Jurkat receptor cells expressing PD-1 ITIM/ITSM mutant elongated x1 ($P = 0.0618$) or PD-1 ITIM/ITSM mutant elongated x2 ($P = 0.2003$; panels B and C respectively, Figure 5.5).

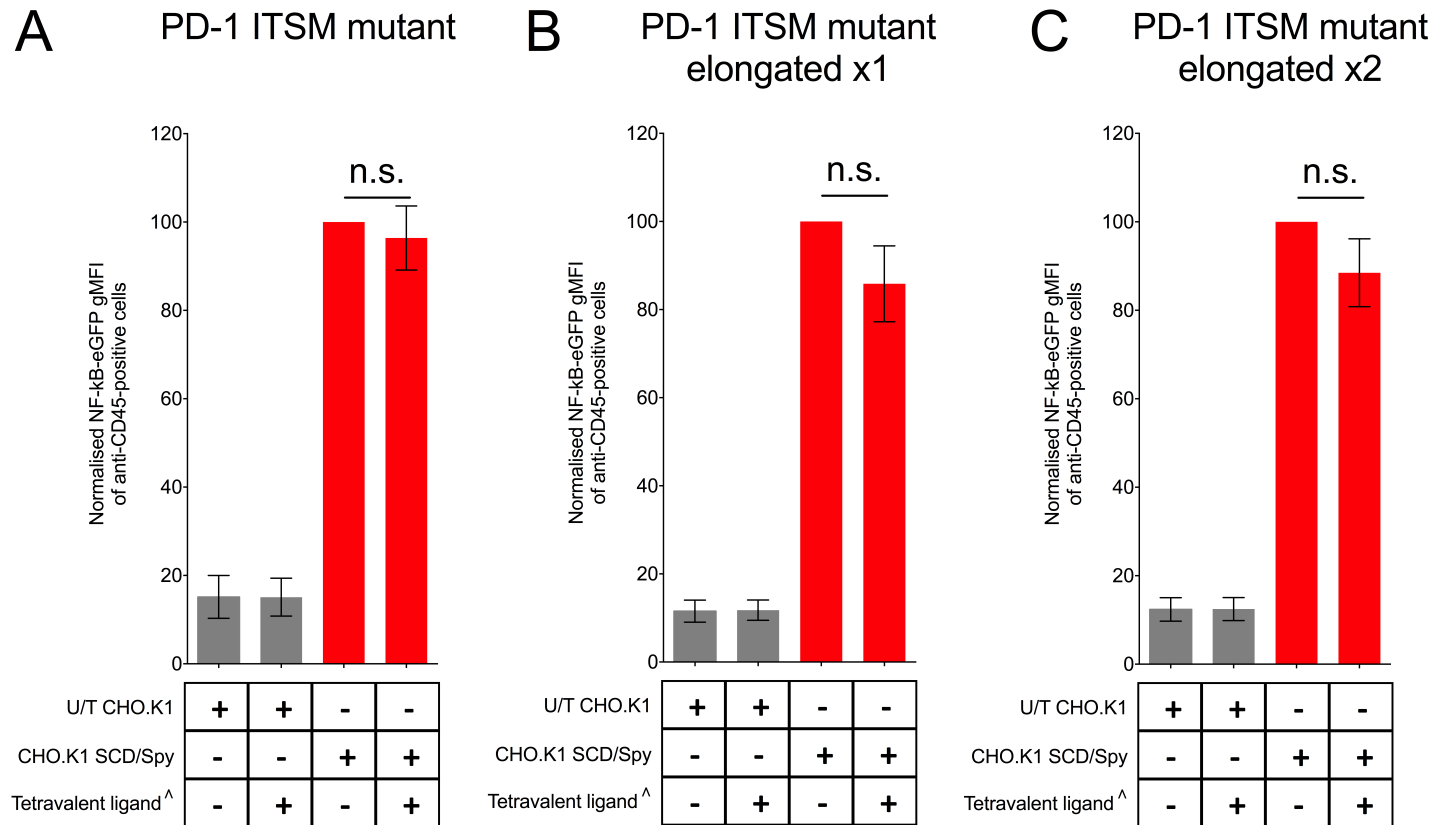


Figure 5.10: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 intracellular tail constructs of different lengths with mutations to the ITSM, including PD-1 ITSM mutant (A), PD-1 ITSM mutant elongated x1 (B), and PD-1 ITSM mutant elongated x2 (C). The Jurkat cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells loaded with 100 nM NY-ESO-1 C9V peptide and either uncoupled or coupled with 200 nM tetravalent StrepTactin-SpyCatcher as indicated. Results are from three independent experiments performed with biological replicates showing mean plus standard deviation. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

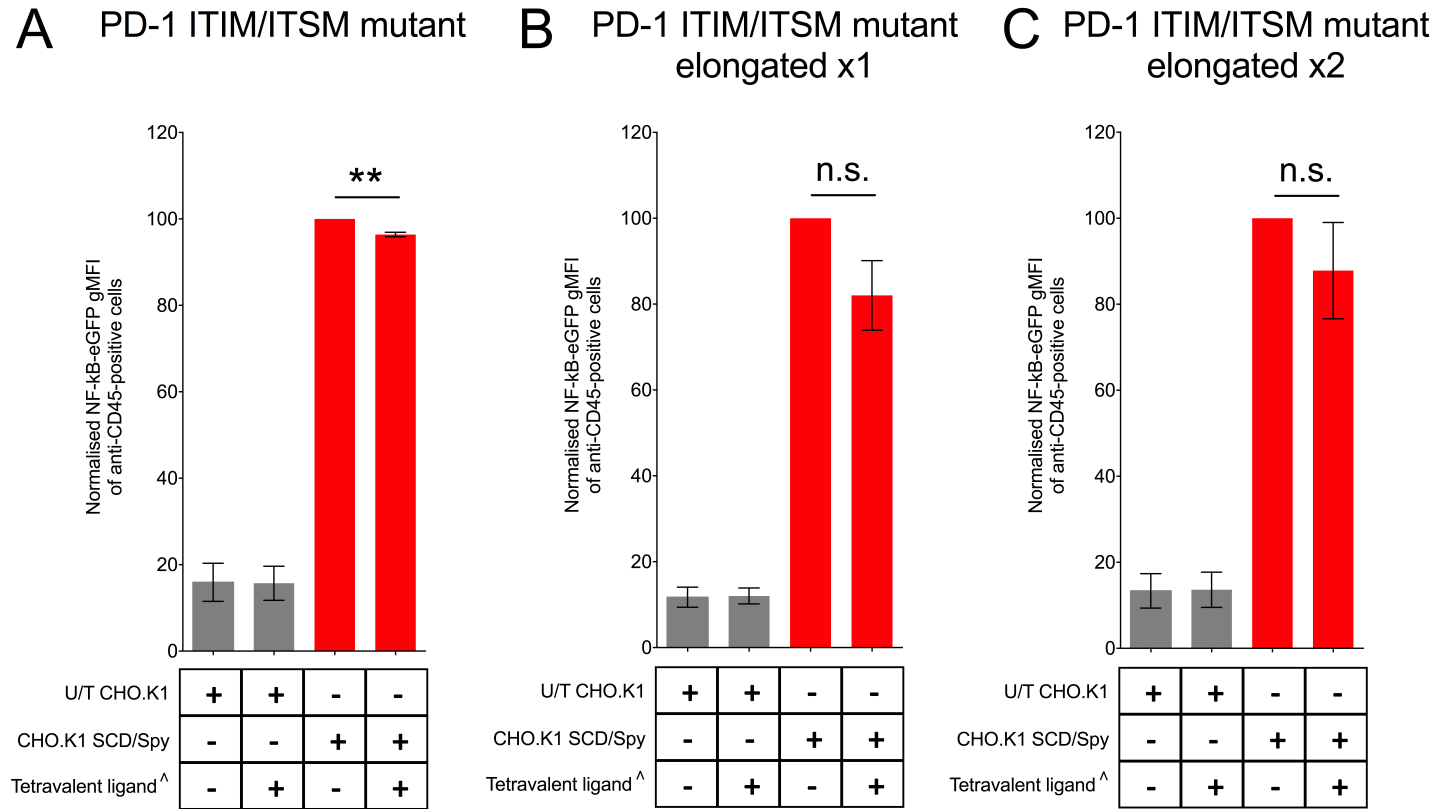


Figure 5.11: Quantification of NF- κ B-eGFP gMFI from cell-cell stimulation of Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing PD-1 intracellular tail constructs of different lengths with mutations to the ITIM and ITSM, including PD-1 ITIM/ITSM mutant (A), PD-1 ITIM/ITSM mutant elongated x1 (B), and PD-1 ITIM/ITSM mutant elongated x2 (C). The Jurkat cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD/Spy ligand cells loaded with 100 nM NY-ESO-1 C9V peptide and either uncoupled or coupled with 200 nM tetravalent StrepTactin-SpyCatcher as indicated. Results are from three independent experiments performed with biological replicates showing mean plus standard deviation. [^]Ligand = StrepTactin-SpyCatcher. n.s. = $P > 0.05$; ** = $P \leq 0.01$; statistical significance determined by performing a one-sample t test comparing the experimental mean to a specified value of 100.

5.4 Chapter Discussion

This chapter has begun exploring the role of intracellular tail length for the function of the inhibitory NTR PD-1. A series of PD-1 variants were tested in both THP-1 and Jurkat receptor cells, with the two-ligand system being utilised for analysis of the full set of truncated and elongated intracellular tails variants, represented in Figure 5.1, in the Jurkat receptor cells.

5.4.1 Generation of receptor cell lines expressing PD-1 intracellular tail variants

Lentiviral transduction was used to generate THP-1 and Jurkat receptor cell lines expressing PD-1 intracellular tail variants. Cell sorting by antibody-coated bead-sorting and/or FACS resulted in well-matched expression levels of the PD-1 variants in both the THP-1 and Jurkat receptor cells (Figures 5.2 and 5.6 respectively). The expression levels of CD64 in the THP-1 receptor cell lines and the 1G4 TCR in the Jurkat receptor cell lines were also well-matched (Figure 5.3 and 5.6 respectively).

5.4.2 Stimulation of THP-1 receptor cells with platebound antibody

Stimulation of THP-1 receptor cells expressing the normal length PD-1 variants (PD-1 control, PD-1 Strep-tag II, PD-1 ITIM mutant, PD-1 ITSM mutant and PD-1 ITIM/ITSM mutant) resulted in inhibition of CD64-mediated IL-8 production from stimulation of the THP-1 receptor cells expressing PD-1 Strep-tag II and PD-1 ITIM mutant with increasing levels of anti-Strep-tag II (Figure 5.4). There was no trend of inhibition from stimulation of the THP-1 receptor cells expressing PD-1 control, indicating that this inhibition is specific to engagement of the Strep-tag II-tagged PD-1.

In contrast to the ITSM-containing PD-1 variants, the two PD-1 variants lacking a phosphorylatable ITSM (PD-1 ITSM mutant and PD-1 ITIM/ITSM mutant) showed no inhibition of IL-8 production with increasing anti-Strep-tag II (Figure 5.4). This is consistent with the results of previous studies, which have shown that the ITSM is most critical for PD-1-mediated inhibition (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012)).

5.4.3 Stimulation of Jurkat receptor cells with platebound antibody

Further experiments were carried out with Jurkat NF- κ B-eGFP/1G4 TCR receptor cells expressing the PD-1 intracellular tail variants. Preliminary platebound antibody experiments showed that stimulation with anti-CD3 plus anti-Strep-tag II (compared to anti-CD3 plus an IgG1 control) resulted in a statistically significant increase in NF- κ B-eGFP gMFI with the Jurkat receptor cells expressing the PD-1 ITIM/ITSM mutant ($P = 0.0131$; Figure 5.5).

Further investigation of this observation uncovered a trend of increased NF- κ B-eGFP gMFI in several of the Jurkat receptor cell lines upon addition of an antibody against the unrelated cell surface molecule Tfr1 (Figure 5.7). These results suggest that engagement of a cell surface molecule with platebound antibody can result in increased activation of the Jurkat receptor cells as measured by NF- κ B upregulation. We propose that this is due to an increase in the number and/or area of close contacts formed, which could promote increased engagement of CD3 and/or increased signalling through engaged CD3. We also expect that binding of cell surface molecules by immobilised antibody would hold the apposing membranes close together, increasing the probability of binding/rebinding of other nearby activating receptors.

This increased activation was not observed from similar platebound antibody stimulation of the THP-1 receptor cells (Figure 5.4). However, a constant level of 10 μ g/ml IgG1 was used to control for Fc receptor-mediated signalling during platebound antibody stimulation of the THP-1 receptor cells. This level of Fc receptor-mediated signalling may mask any effect of increased engagement of signalling incompetent PD-1 variants such as was observed with the Jurkat receptor cells expressing the PD-1 ITIM/ITSM mutant (Figures 5.5 and 5.7). As Jurkat cells do not express Fc receptors, this may explain the difference observed between platebound antibody stimulation of the Jurkat and THP-1 receptor cells.

Overall, the results from platebound antibody stimulation of the Jurkat receptor cells suggest that alternative approaches such as a cell-cell system should be pursued for studying signal integration between these cell surface receptors.

5.4.4 Stimulation of Jurkat receptor cells with the two-ligand system

In light of these results, all further experiments used cell-cell stimulation of the Jurkat receptor cells expressing the full set of PD-1 intracellular tail variants (Figure 5.1).

For these experiments the two-ligand system was utilised to determine whether stimulation of Jurkat receptor cells via the 1G4 TCR could be inhibited by engagement of the different PD-1 variants.

These experiments showed no statistically significant inhibition of NF- κ B-eGFP gMFI in either the Jurkat receptor cells expressing the PD-1 truncated or PD-1 truncated ITSM mutant variants upon stimulation with the CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher (Figure 5.8). This indicates that no PD-1-mediated inhibition of TCR signalling can be measured under these conditions for these PD-1 variants. This is somewhat unsurprising for the PD-1 truncated ITSM mutant, which contains no phosphorylatable signalling motifs. In the case of the PD-1 truncated variant, which contains an ITSM, there are a number of reasons why there may be no inhibition evident. For example, truncation of the tail may prevent or reduce 1) phosphorylation of the ITSM, 2) binding of phosphatases such as SHP-2, and/or 3) the ability to dephosphorylate nearby molecules. These possible explanations are not mutually exclusive and could contribute to varying degrees to abrogate the inhibitory function of PD-1. This will be investigated in future experiments by comparing stimulation-induced phosphorylation and recruitment of SHP-2 to the truncated PD-1 variants compared to normal length PD-1.

When assessing the PD-1 ITIM mutant variants of different lengths, statistically significant inhibition of NF- κ B-eGFP gMFI was measured upon engagement of the PD-1 ITIM mutant, PD-1 ITIM mutant elongated x1, and PD-1 ITIM mutant elongated x2 using the CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher (Figure 5.9). For each of these Jurkat receptor cell lines, inhibition of approximately 20 - 25% was measured compared to approximately 40% inhibition measured from stimulation of the PD-1 Strep-tag II cells with coupled CHO.K1 SCD/Spy ligand cells (Figure 4.25).

In contrast to the Jurkat cells expressing the PD-1 ITIM mutant variants, none of the Jurkat receptor cells expressing PD-1 ITSM mutant variants showed statistically significant inhibition of NF- κ B-eGFP gMFI, regardless of intracellular tail length (Figure 5.10).

Together these results support the critical role of the ITSM for the inhibitory function of PD-1, which has previously been demonstrated in a number of studies (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012)). The difference between the strength of inhibition measured with the PD-1 Strep-tag II ($\approx$ 40% inhibition; panel A, Figure 4.25) and the PD-1 ITIM mutant ($\approx$ 25% inhibition; panel A, Figure 5.9) provides some evidence that the ITIM does

contribute to the strength of this PD-1-mediated inhibition, however this needs to be investigated further once the system has been optimised.

Importantly, these results do not provide evidence to support our hypothesis that elongating the intracellular tail increases the inhibitory function of PD-1 as there does not appear to be increasing inhibition with increased length of the PD-1 ITIM mutants (Figure 5.9). Since we have only performed this experiment with one concentration of StrepTactin-SpyCatcher so far, we plan to further investigate this hypothesis by comparing the inhibition via these PD-1 constructs with various concentrations of StrepTactin-SpyCatcher to determine if altering ligand density reveals any differences between the PD-1 variants of different lengths. For instance, at lower ligand densities some PD-1 variants may be more effective than others at inhibiting TCR-mediated activation. As discussed in relation to the PD-1 truncated variant, such a result could indicate a difference in ITSM phosphorylation, recruitment of effector molecules such as SHP-1/SHP-2, and/or a difference in “reach” of the intracellular tail of the PD-1 variants as represented in Figure 1.4.

Before proceeding with these experiments we aim to further optimise the two-ligand system. For example, we will trial use of twin Strep-tag II to increase the avidity of the StrepTactin-SpyCatcher binding to the Strep-tag II-tagged PD-1, as discussed further in Section 6.4.1. This should help to strengthen PD-1-mediated inhibition and as such improve the signal to noise of the assay.

Surprisingly, a weak but still statistically significant inhibition of NF- κ B-eGFP gMFI of approximately 5% was measured from stimulation of the Jurkat receptor cells expressing the PD-1 ITIM/ITSM mutant stimulated with coupled compared to uncoupled CHO.K1 SCD/Spy ligand cells (panel A, Figure 5.11). The cause and physiological relevance of this level of inhibition from the PD-1 ITIM/ITSM mutant are unknown and should be investigated as the two-ligand system is further developed.

General Discussion

6.1 Overview

As the field of immunotherapy comes of age, the push to understand more about the receptors and signalling pathways behind many of these emerging treatments provides not only a great challenge but also countless opportunities.

The founding targets of checkpoint blockade, CTLA-4 and PD-1, belong to a group of more than 120 immune cell-surface receptors classified as NTRs (Dushek *et al.* (2012)). This group of receptors includes activating, co-stimulatory, and co-inhibitory receptors with many potential targets for a variety of immune-related diseases. Although some NTRs and their ligands have been studied in detail, a number of NTRs have no known ligands and we have little understanding about the signalling mechanism of many NTRs.

With more than 120 NTRs identified, the task of studying these receptors one by one with their native ligands would be both time and resource intensive. This task is further compounded when attempting to study signal integration between multiple activating and co-inhibitory or co-stimulatory NTRs. Therefore we set out to develop a system with a generic ligand that can bind to NTRs cloned with a short peptide tag.

The system, referred to as a two-ligand system, includes CHO.K1 ligand cells expressing both an activating ligand to provide activating signals to receptor cells and a generic ligand to engage tagged co-stimulatory or co-inhibitory NTRs for investigation of signal integration. The activating ligands used in this study were CD48 to engage a 2B4-CD64 activating receptor in THP-1 receptor cells and an HLA-A2

single chain dimer loaded with cognate peptide to bind to the 1G4 T cell receptor in Jurkat NF κ -B-eGFP receptor cells.

The generic ligand utilised in the system contains a soluble StrepTactin-based component, StrepTactin-SpyCatcher, which was titrated onto the ligand cells. The surface-coupled StrepTactin can then be used to engage Strep-tag II-tagged NTRs expressed by Jurkat or THP-1 receptor cells.

During the current study, this two-ligand system was used to investigate signal integration by PD-1, an inhibitory NTR from the CD28 family of receptors. After developing the system, we utilised it to test the importance of both extracellular receptor-ligand dimensions and intracellular tail length for PD-1-mediated inhibition in THP-1 and Jurkat receptor cells. The results from these experiments, areas for further development of this system, and future directions of this research are discussed below.

6.2 Results on the importance of receptor-ligand dimensions

6.2.1 Signal integration between PD-1 and a CD64-based receptor

For analysis of signal integration in a monocyte-like cell line, CHO.K1 ligand cells expressing CD48 or CD48-CD4 were used to activate monocyte-like THP-1 receptor cells expressing a 2B4-CD64 fusion protein. The CHO.K1 cells also expressed Spy or Spy-CD2-CD2 constructs to allow coupling of the soluble StrepTactin-SpyCatcher generic ligand for engagement of Strep-tag II-tagged PD-1 constructs on the THP-1 receptor cells. These ligand and receptor cells were used to analyse signal integration between PD-1 and the CD64-based activating receptor with matched or mismatched receptor-ligand dimensions by measuring IL-8 production by the THP-1 cells.

Throughout these experiments, low and variable levels of coupling of the StrepTactin-SpyCatcher to the CD48-based ligand cells (Figures 4.6 - 4.11) prevented comparison of THP-1 receptor cells stimulated with CHO.K1 ligand cells expressing ligands with matched and mismatched receptor-ligand dimensions. Therefore, this aspect of the system needs to be improved in order to test stimulation of the THP-1 receptor cells with the four CD48-based ligand cell lines. Some approaches for addressing this are discussed below in Section 6.4.

However, despite the low and variable coupling levels, the results from these experiments did show a modest trend of inhibition of IL-8 production from stimulation of the THP-1 receptor cells with the coupling of tetravalent StrepTactin-SpyCatcher to the CHO.K1 CD48/Spy cells (Figure 4.13). There was also a possible trend of IL-8 inhibition from low to intermediate coupling levels but no further inhibition at higher levels of coupling with the CHO.K1 CD48/Spy-CD2-CD2 ligand cells (Figure 4.15).

Due to the weak levels of PD-1-mediated inhibition measured and the lack of full dose response curves from these stimulation experiments, meaningful statistical analyses of these results are not possible. Therefore, whether the observed inhibition is statistically significant and/or whether there is a statistically significant difference between stimulation with the different ligand cell lines should be further investigated once the system has been optimised so that StrepTactin-SpyCatcher coupling levels are comparable between the different ligand cell lines.

Overall these results indicate that the two-ligand system can be used to study signal integration between CD64 and PD-1 in the THP-1 receptor cells. Once the two-ligand system has been further optimised using PD-1, it would be interesting to utilise it to study signal integration by monocytic NTRs, such as Siglec-9, in the THP-1 receptor cells, which are themselves a monocytic cell line. As detailed in Section 1.6.5, Siglec-9 is highly expressed on monocytes and is of clinical interest as a potential target for both tumour- and autoimmune-related therapies (Beatson *et al.* (2016); Matsumoto *et al.* (2016)).

6.2.2 Signal integration between PD-1 and the 1G4 TCR

For analysis of signal integration in a T cell line, CHO.K1 ligand cells expressing HLA-A2 SCD were used to activate Jurkat NF κ -B-eGFP receptor cells expressing the 1G4 TCR. The CHO.K1 cells also expressed Spy or Spy-CD4 constructs to allow coupling of the soluble StrepTactin-SpyCatcher generic ligand for engagement of Strep-tag II-tagged PD-1 constructs on the Jurkat receptor cells. These ligand and receptor cells were used to analyse signal integration between PD-1 and the 1G4 T cell receptor with matched or mismatched receptor-ligand dimensions by measuring NF κ -B-eGFP gMFI of the Jurkat receptor cells.

In contrast to the poor coupling of the StrepTactin-SpyCatcher to the CD48-based ligand cells, coupling was more effective with the SCD-based ligand cells, which were used for stimulation of the Jurkat receptor cells. The SCD/Spy and SCD/Spy-CD4 ligand cells showed similar and reproducible levels of coupling of the tetravalent StrepTactin-SpyCatcher (Figures 4.21 and 4.22) and higher levels of coupling of the

monovalent StrepTactin-SpyCatcher to the SCD/Spy-CD4 cells (Figure 4.20). This allowed comparison of results from stimulation with these two ligand cell lines as any decrease in PD-1-mediated inhibitory function from the mismatched receptor-ligand dimensions of the SCD/Spy-CD4 cells would not be due to lower levels of coupling of the StrepTactin-based ligand to those cells.

Statistically significant inhibition of NF κ -B-eGFP gMFI was measured from stimulation of the Jurkat NF κ -B-eGFP/1G4 TCR/PD-1 Strep-tag II receptor cells with the CHO.K1 SCD/Spy but not the CHO.K1 SCD/Spy-CD4 ligand cells, loaded with 10 nM C9V peptide and coupled with tetravalent StrepTactin-SpyCatcher ($P = 0.0074$, Figure 4.23 and $P = 0.3299$, Figure 4.24 respectively).

The same experiment repeated with a concentration of 100 nM C9V peptide showed statistically significant inhibition of NF κ -B-eGFP gMFI with both the CHO.K1 SCD/Spy and the CHO.K1 SCD/Spy-CD4 ligand cells coupled with tetravalent StrepTactin-SpyCatcher ($P = 0.0008$, Figure 4.25 and $P = 0.0040$, Figure 4.26 respectively). However, inhibition was also measured from stimulation of the PD-1 control cells with the CHO.K1 SCD/Spy-CD4 cells ($P = 0.0352$ and 0.0356 for monovalent and tetravalent ligand respectively), so this needs to be further investigated.

Nonetheless, stimulation with the CHO.K1 SCD/Spy ligand cells showed a trend of stronger inhibition than the CHO.K1 SCD/Spy-CD4 cells (Figure 4.27). This is in line with our hypothesis that matched receptor-ligand dimensions are required for optimal NTR signal integration (“hypothesis 1”; Section 1.7 and Figure 1.3). However, one factor that may contribute to abrogation of PD-1-mediated inhibition upon mismatching of receptor-ligand dimensions is decreased net phosphorylation of receptors with elongated receptor-ligand dimensions. Differences in phosphorylation levels could be controlled for by fusing SHP-1 or SHP-2 to the intracellular tails of the PD-1 variants, such as performed by Yokosuka and colleagues (Yokosuka *et al.* (2012)). This approach could be used provide evidence as to whether mismatched receptor-ligand dimensions affect NTR signal integration independent of phosphorylation and phosphatase recruitment. Alternatively, if a suitable elongated ligand for the 1G4 TCR could be generated, as discussed in Section 6.4.4, this could be used to provide evidence that matched dimensions are important for signal integration in a similar manner to the experiments performed by Köhler and colleagues for the human NK cell receptors NKG2D and KIR2DL1, in which elongation of both the activating and inhibitory ligands partially rescued co-localisation of and signal integration between these receptors (Köhler *et al.* (2010)).

It is interesting to note that a previous study of murine PD-1 found that a mismatching of receptor-ligand dimensions by four Ig domains resulted in almost complete abrogation of not only PD-1-mediated inhibition of TCR signalling, but also of SHP-2 recruitment and co-localisation with TCR microclusters (Yokosuka *et al.* (2012)). As such, it would be useful to further investigate the differences in signalling from stimulation of the Jurkat receptor cells with the CHO.K1 SCD/Spy and CHO.K1 SCD/Spy-CD4 ligand cells to determine if SHP-2 recruitment and co-localisation with TCR are similarly affected by mismatched dimensions in this system. For example, confocal microscopy of these ligand and receptor cell lines could be used to determine the degree of co-localisation of the activating 1G4 TCR-C9V-SCD complex (≈ 12 nm) with the normal length (≈ 15 nm) and elongated (≈ 27 nm) PD-1-ligand complexes at the cell-cell interface (estimated dimensions as described in Table 4.1). This would shed light on the level of segregation of ligated PD-1 and TCR complexes caused by the mismatched receptor-ligand dimensions.

6.3 Results on the importance of NTR intracellular tail length

After investigating the importance of receptor-ligand dimensions in Chapter 4, we went on to test the importance of the PD-1 intracellular tail length in Chapter 5. For these experiments we generated a series of truncated and elongated PD-1 variants with mutations to the ITIM and/or ITSM (Table 5.1 and Figure 5.1) and lentivirally transduced them into THP-1 and Jurkat NF κ -B-eGFP receptor cells (Figures 5.2, 5.3 and 5.6).

6.3.1 Signal integration between PD-1 and a CD64-based receptor

The THP-1 receptor cells were analysed using platebound antibody stimulation with anti-CD64 for activation and anti-Strep-tag II to engage the Strep-tag II-tagged PD-1 variants. Increasing anti-Strep-tag II resulted in clear inhibition of IL-8 production by the THP-1 receptor cells expressing the PD-1 Strep-tag II and PD-1 ITIM mutant, but not the PD-1 ITSM mutant or PD-1 ITIM/ITSM mutant (Figure 5.4). This confirms the critical role of the ITSM in PD-1-mediated inhibition, which has been reported previously in B and T cells (Okazaki *et al.* (2001); Chemnitz *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012)). It also demonstrates that the expected

PD-1 variants (i.e. those containing a functional ITSM) are able to exhibit inhibitory function in the THP-1 receptor cells stimulated through CD64 engagement.

6.3.2 Signal integration between PD-1 and the 1G4 TCR

The results from the THP-1 receptor cells were extended using the two-ligand system to analyse the Jurkat NF κ -B-eGFP receptor cells expressing the 1G4 TCR. Statistically significant inhibition of NF κ -B-eGFP gMFI was measured in several of the Jurkat receptor cells lines expressing PD-1 variants containing an ITSM (Figure 5.9). This included the Jurkat receptor cells expressing the PD-1 ITIM mutant, PD-1 ITIM mutant elongated x1, and PD-1 ITIM mutant elongated x2, all of which showed approximately 20 - 25% inhibition of NF κ -B-eGFP gMFI when stimulated with CHO.K1 SCD/Spy ligand cells coupled with tetravalent StrepTactin-SpyCatcher compared to stimulation with uncoupled CHO.K1 SCD/Spy ligand cells.

These results once again reinforce the importance of the ITSM for PD-1-mediated inhibition. They also show no indication of a difference in PD-1 inhibitory function with increasing intracellular tail length under these experimental conditions (Figure 5.9). However, it is possible that differences between these PD-1 constructs will manifest with a lower density of StrepTactin-SpyCatcher or with mismatched receptor-ligand dimensions; conditions which remain to be tested. This may be the case because if longer intracellular tails improve inhibitory efficiency by increasing the “reach” of the intracellular tail and any recruited cytoplasmic molecules, as represented in Figure 1.4, then it would be expected that this effect would be most prominent in conditions upon which the average distance between the activating and inhibitory receptors is increased - such as at lower ligand density and/or with mismatched receptor-ligand dimensions. Longer intracellular tails may then be able to compensate for this increased distance between receptor-ligand complexes by increasing the reach of the inhibitory receptor to enable signal integration, as proposed by “hypothesis 2” (Section 1.7.2; Figure 1.4).

6.4 Further optimisation of the two-ligand system

A number of aspects of the two-ligand system have been optimised throughout the current study, including assays for stimulation of both the THP-1 and Jurkat receptor cells (Section 3.3.4), concentrations of StrepTactin-SpyCatcher for coupling to the ligand cells (Appendices A.2 and A.1), and use of a 3:1 mixture of D-biotin:biotin-ATTO 647 for measuring the relative number of available binding sites for Strep-tag

II (Figure 3.13). Nonetheless, there are several areas of the two-ligand system that would benefit from further optimisation, as discussed below.

6.4.1 Increasing the affinity of the StrepTactin-Strep-tag II interaction

PD-1-mediated inhibition in the two-ligand system was not detectable using the monovalent StrepTactin-SpyCatcher (Figures 3.16 and 3.18), despite successful coupling of the ligand as confirmed by flow cytometry (Appendix A.3). Use of tetravalent StrepTactin-SpyCatcher was able to induce PD-1-mediated inhibition in both the THP-1 and Jurkat receptor cells in the two-ligand system, however the measured inhibition was relatively weak even with PD-1 Strep-tag II ($\approx 30\%$ and $\approx 55\%$ inhibition for THP-1 and Jurkat receptor cells respectively, relative to stimulation with uncoupled ligand cells; Figures 4.12 and 4.23). Most variants of PD-1 are expected to have weaker inhibitory function than PD-1 Strep-tag II, therefore this does not provide a very large dynamic range to use as a starting point for investigation of the inhibitory function of PD-1 variants.

The modest level of PD-1-mediated inhibition measured in the two-ligand system may be due to the low affinity of the interaction between StrepTactin and Strep-tag II compared to the physiological interaction between PD-1 and PD-L1 or PD-L2. This is supported by the fact that inhibition was observed upon coupling of tetravalent StrepTactin-SpyCatcher, but not monovalent StrepTactin-SpyCatcher (Figures 3.16 and Figure 3.18).

It has been suggested by Voss and colleagues that Strep-tag II has a physiologically relevant affinity of $1\ \mu\text{M}$ for tetravalent StrepTactin (Voss & Skerra (1997)). This result has been further explored in our laboratory, with recent investigation of the affinity of Strep-tag II for monovalent StrepTactin indicating an affinity in the order of tens of μM (Dr Ben de Wet, unpublished). This affinity is approximately an order of magnitude weaker than the physiological affinities of PD-1 for its native ligands, which have been measured to be $K_d \approx 8\ \mu\text{M}$ and $K_d \approx 2\ \mu\text{M}$ for PD-1 binding to PD-L1 and PD-L2 respectively (Cheng *et al.* (2013)). Therefore the strength of PD-1-mediated inhibition in the two-ligand system may be improved by increasing the affinity of the generic ligand-receptor interaction between StrepTactin and Strep-tag II-tagged NTRs.

One option for achieving this would be to tag NTRs with twin Strep-tag II instead of the single Strep-tag II used here. Twin-Strep-tag II, previously referred to as “Strep-tag III” and “One-STrEP-tag”, contains sequential Strep-tag II sequences joined by

a short linker (Junttila *et al.* (2005)). Several variations of the twin-Strep-tag II have been used, with the predominantly used version having the amino acid sequence SAWSHPQFEKGGGSGGGSGGSAWSHPQFEK (Strep-tag II sequence underlined; Schmidt *et al.* (2013)).

The presence of two copies of the Strep-tag II tag has been shown to increase the affinity of binding of fusion proteins to StrepTactin by at least an order of magnitude, from a K_d in the 100 nM range to the 10 nM range in the case of a GFP fusion protein, and from a K_d in the 10 μ M range to the 100 nM range for a cytochrome b_{562} fusion protein (Schmidt *et al.* (2013)). This increased affinity with the twin Strep-tag II was due to a substantial reduction of the off-rate compared to the single Strep-tag II.

Therefore, by tagging NTRs with twin-Strep-tag II we hope to increase the affinity of binding to StrepTactin to a range that gives stronger signalling. This would improve the signal to noise for stimulation assays and may uncover underlying differences between the signalling efficiencies of different variants or experimental conditions, such as with the different ligand cells and PD-1 variants investigated here. Additionally, our group has generated mutants of StrepTactin with intermediate affinities for Strep-tag II (Tudor Ilca and Dr Jesse Goyette, unpublished), so there is the potential to use the twin Strep-tag II with generic ligands with a range of affinities.

6.4.2 Incorporation of co-stimulatory signalling

As discussed in Section 1.6.7, PD-1-mediated inhibition has been reported to inhibit signalling through the CD3 chains of the TCR, the associated kinase ZAP70, and/or the co-stimulatory receptor CD28 (Sheppard *et al.* (2004); Parry *et al.* (2005); Yokosuka *et al.* (2012); Zinselmeyer *et al.* (2013)). However, recent preliminary results from Vale and colleagues indicate that CD28 may be the most sensitive target of PD-1-mediated inhibition (Hui *et al.* (2016)). Considering these results, more effective inhibition by PD-1 may be obtained upon incorporation of CD28 co-stimulation in the current system.

Indeed, expression of a ligand for CD28, namely CD80, has been shown to substantially increase Jurkat reporter cell activation in the TSC and eAPC systems described in Section 3.1.2 (Leitner *et al.* (2010); Roskopf *et al.* (2016)), and statistically significant inhibition was measured upon engagement of PD-1 by either PD-L1 or PD-L2 in the eAPC system (Roskopf *et al.* (2016)). This PD-1-mediated inhibition was only statistically significant in the presence of co-stimulation via CD28, indicating that inclusion of CD28 co-stimulatory signalling may be more effective for investigating signal integration by PD-1 than TCR stimulation alone.

6.4.3 Obtaining comparable levels of coupling of StrepTactin-SpyCatcher to different ligand cell lines

Coupling of StrepTactin-SpyCatcher, the soluble component of the generic ligand, was found to vary between experiments and between the ligand cell lines used in the two-ligand system, in particular the CD48-based ligand cell lines (Figures 4.6 - 4.11). This aspect of the system must be addressed in order to be able to compare stimulation with different ligand cell lines.

One approach for obtaining well-matched levels of coupling of StrepTactin- SpyCatcher to the ligand cells would be to generate a single ligand cell line expressing the SpyTag cell-surface component. Instead of ligand cell lines expressing SpyTag constructs of different lengths, as was done in the current work, the difference in receptor-ligand dimensions could be incorporated into the soluble StrepTactin-SpyCatcher component of the system. For instance, titin immunoglobulin-like domain 27 (I27; Improta *et al.* (1996)) could be included in the soluble ligand component to generate elongated versions of the ligand.

After selecting/sorting ligand cells with high levels of expression of the SpyTag construct and confirming effective coupling of the StrepTactin-SpyCatcher soluble components, this cell line could be used as a “parent” cell line for all subsequent ligand cell lines. Other ligands e.g. CD48 or SCD could be transfected into the parent cell line and sorted for high and well-matched expression levels, as was done here. Since such ligand cell lines would have come from the same original Spy ligand cell line, coupling them with either short or elongated StrepTactin-SpyCatcher should result in well-matched coupling levels.

6.4.4 Elongated ligand for the 1G4 TCR

The use of HLA-A2 SCD loaded with cognate peptide as the activating ligand for stimulation of the Jurkat receptor cells was limited by the lack of surface expression of elongated SCD (data not shown). Since insertion of Ig domains renders the SCD non-functional, most likely because of an impairment of peptide loading (Dr Keith Gould, personal communication), we were only able to compare stimulation of the Jurkat receptor cells with CHO.K1 ligand cells expressing SCD and either Spy or Spy-CD4. We were therefore unable to determine whether elongation of both receptor-ligand dimensions (e.g. SCD-CD4/Spy-CD4) can restore signal integration compared to mismatched dimensions. Using an approach similar to that of Köhler *et al.* and Brzostek and colleagues (Brzostek *et al.* (2010); Köhler *et al.* (2010)), ligand cell

lines expressing an elongated ligand for the 1G4 TCR would allow us to test whether elongation impacts signal integration for reasons other than mismatched receptor-ligand dimensions. For instance, elongation may abrogate binding to and/or signaling through the receptor.

Such ligand cells expressing elongated ligand for activation of the Jurkat receptor cells could be generated by replacing the SCD in the system with SCT that also encodes cognate peptide, thus avoiding the requirement for peptide loading. This approach would eliminate the ability to load peptides of different affinity for the TCR, but it should be feasible since it has been used previously to express elongated TCR ligand (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009)).

Designing a functional SCT for investigation of PD-1 signal integration is not a trivial matter as the peptide must be encoded in a way that allows proper folding. Approaches for improving SCT folding are under development and could be considered for the design of an appropriate SCT for use in the two-ligand system (Honda *et al.* (2015); Li *et al.* (2010); reviewed by Hansen *et al.* (2010)). Another challenge for this approach is that quite low expression levels of the SCT may be needed in order to measure PD-1-mediated inhibition and to determine any differences between stimulation with matched or mismatched receptor-ligand dimensions. It may therefore be prudent to first use the SCD-based ligand cells to explore the effect of affinity on 1G4-mediated activation of the Jurkat NF κ -B-eGFP cells in the two-ligand system before selecting a suitable peptide or peptides for development of an SCT-based version.

6.4.5 Adding fluorescence to ligand cells

The experiments presented here using the Jurkat NF κ -B-eGFP receptor cells involved post-stimulation staining of the mixture of ligand and receptor cells with anti-human CD45-Alexa 647, which was then used to gate on the Jurkat receptor cells. This assay could be simplified by expression of a fluorescent protein on the ligand cells so that they can be gated out without any additional staining.

It is important to consider the spectral properties of such a fluorophore due to the use of a fluorescent reporter in this system. For example, a protein with fluorescence emission in the red or far-red region should be compatible with the NF κ -B-eGFP reporter used here.

6.5 Future experiments

Several experiments may help to shed further light on the results described here. A number of suggested experiments are introduced below.

6.5.1 Teasing out the role of SHP-1/SHP-2

Interestingly, the Jurkat receptor cells expressing PD-1 truncated, which contains an ITSM, showed no inhibitory function (Figure 5.8). It is unclear what role phosphatases such as SHP-1/SHP-2 play in this lack of inhibitory function, as it could be due to a reduction in 1) phosphorylation of the ITSM, 2) binding of phosphatases such as SHP-1/SHP-2 to the phosphorylated ITSM, and/or 3) the ability of recruited phosphatases to dephosphorylate nearby molecules. The first step to clarify the cause/s of the lack of inhibition by the PD-1 truncated variant would be to compare phosphotyrosine levels and recruitment of phosphatases upon stimulation of receptor cells expressing PD-1 Strep-tag II or PD-1 truncated. This approach could be used to investigate the full set of PD-1 variants.

If such analyses uncover differences between the PD-1 variants, SHP-1 or SHP-2 could be fused to their intracellular tails such as performed by Yokosuka and colleagues (Yokosuka *et al.* (2012)). This would account for differences in phosphorylation of the ITSM and/or phosphatase recruitment to determine if “reach” affects the inhibitory function of the PD-1 intracellular tail variants. As mentioned in Section 6.2.2, this approach could also be used for comparing stimulation with matched or mismatched receptor-ligand dimensions.

6.5.2 Combining manipulation of extra- and intra-cellular dimensions

The current study separately investigated the effects of manipulating extracellular (i.e. receptor-ligand) and intracellular dimensions on NTR signal integration. The techniques and cell lines developed for this work could be combined to test the impact of simultaneous manipulation of extra- and intra-cellular dimensions. For example, if mismatched receptor-ligand dimensions result in decreased PD-1-mediated inhibitory efficiency then elongation of the PD-1 intracellular tail may be able to compensate for this (Figure 6.1).

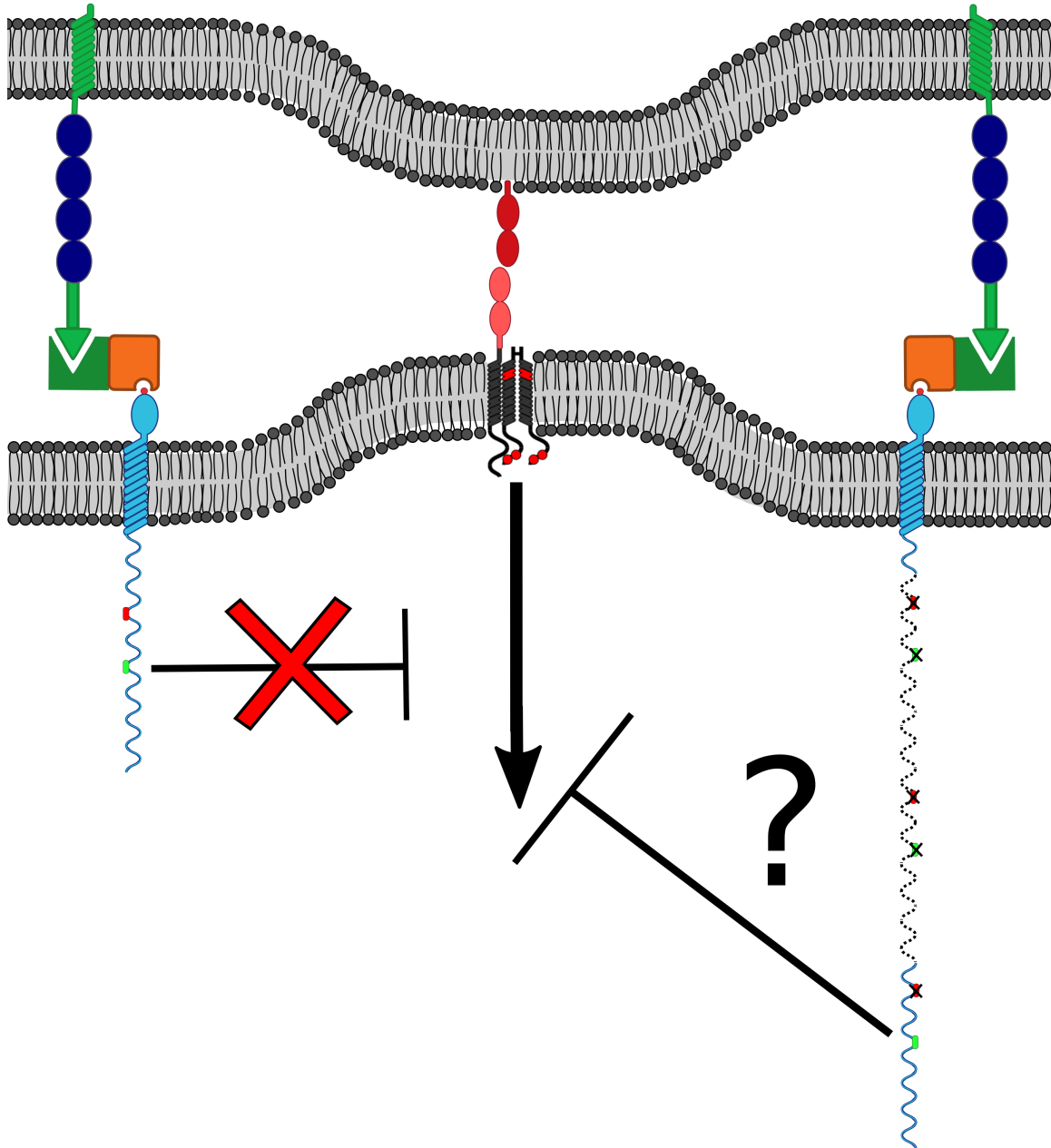


Figure 6.1: Schematic representation showing combined manipulation of extra- and intra-cellular dimensions in the two-ligand system. Mismatched receptor-ligand dimensions may prevent an inhibitory NTR with a normal-length intracellular tail (left) from inhibiting signalling by an engaged activating receptor, but elongation of the intracellular tail of the inhibitory NTR (right) may compensate for the mismatched receptor-ligand dimensions and restore inhibitory signalling.

6.5.3 Confirmation of results in primary cells

Cell lines such as the Jurkat and THP-1 cells used here are invaluable tools for investigation of a wide range of immunological questions (reviewed by Abraham & Weiss (2004); Chanput *et al.* (2014)). Advantages of using cell lines include that they are easily accessible, widely used, well-studied and relatively easy to work with. These features make cell lines useful for setting up and testing biological systems such as the two-ligand system described here.

Nonetheless, cell lines are known to have defects in certain cellular pathways so do not necessarily provide an accurate model of signalling. For instance, the Jurkat T cell line is deficient in phosphatase and tensin homolog (PTEN), making it hyperresponsive to TCR-mediated stimulation (Shan *et al.* (2000)). Similarly, it has been found that variations in THP-1 differentiation protocols result in differences in activation as measured by TNF- α production (Lund *et al.* (2016)). It would therefore be useful to strengthen the physiological relevance of key results using primary cells once the system has been optimised.

6.5.4 Use of native ligands

Another approach for assessing the physiological relevance of important results is to use the native ligand. This could be in the form of the complete native ligand expressed on ligand cells, or it could be incorporated into the two-ligand system through use of native ligand-SpyCatcher fusion proteins, which could be coupled to the ligand cells in the same way as the StrepTactin-SpyCatcher used here. For example, soluble PD-L1-SpyCatcher fusion protein could be coupled to the surface of ligand cells and used to engage PD-1 variants on receptor cells.

This would allow us to determine if similar results are obtained from engagement of NTRs by the generic StrepTactin-based ligand and by their native ligand. However, use of a native ligand prevents specific engagement of the introduced receptors because the native ligand will also bind to endogenous receptors. Therefore endogenous receptors need to be considered when designing experiments using native ligand. One possibility for overcoming the complication of endogenous receptors would be to use receptor cells lacking expression of the native receptor, such as PD-1-negative Jurkat cells.

6.5.5 Spatial organisation of receptor-ligand complexes

An area of investigation that holds extensive opportunities for the two-ligand system is analysis of spatial localisation of receptor-ligand complexes and the SFKs and RPTPs that act upon them. A wide range of studies have used a variety of microscopy techniques to analyse clustering of cell-surface proteins (reviewed by Garcia-Parajo *et al.* (2014); Goyette & Gaus (2016)), co-localisation of NTR-ligand complexes (Brzostek *et al.* (2010); Köhler *et al.* (2010)), and segregation of the phosphatases CD45 and CD148 (Choudhuri *et al.* (2005); Choudhuri *et al.* (2009); Cordoba *et al.* (2013); Lim *et al.* (2015)). Similar approaches could be applied here to determine the degree of phosphatase segregation and co-localisation of receptor-ligand complexes with matched or mismatched dimensions.

It would also be interesting to determine if changing the length of the PD-1 intracellular tail affects clustering of the receptor, as has been found for other cell surface proteins (Wade *et al.* (1989); Capps *et al.* (2004); Umemura *et al.* (2008); Lavi *et al.* (2012); Torreno-Pina *et al.* (2016)). For instance, it has been shown that for the antigen-presenting molecule CD1d that the intracellular tail controls the density and size of CD1d nanoclusters (Torreno-Pina *et al.* (2016)). Removal of the CD1d intracellular tail resulted in formation of larger nanoclusters, as did the use of cytochalasin D to disrupt the actin cytoskeleton (Torreno-Pina *et al.* (2016)). These results indicate that interactions between the CD1d intracellular tail and the actin cytoskeleton are important for controlling clustering of this molecule at the cell surface.

In contrast, for MHC I and MHC II molecules disruption of the actin cytoskeleton or deletion of the intracellular tail has been shown to increase mobility and reduce the lifetime of MHC I and MHC II nanoclusters (Wade *et al.* (1989); Capps *et al.* (2004); Umemura *et al.* (2008); Lavi *et al.* (2012)). Therefore, interactions between the actin cytoskeleton and the intracellular tails of transmembrane proteins can differ and should be investigated separately for each protein of interest.

6.6 Implications for adoptive cell transfer (ACT) approaches

A better understanding of the function of the intracellular tail of inhibitory NTRs may help to guide the development of a range of ACT-based techniques, including CAR therapies. These approaches commonly include targeting two or more NTRs or using split receptors to increase specificity and functionality (reviewed by Jensen

& Riddell (2014)). Signal integration between the receptors then directs the immune response towards the relevant target such as tumour cells.

Most CARs currently under development include components of activating and/or co-stimulatory receptors such as CD3 ζ , CD28, 4-1BB and OX40 (reviewed by Sadelain *et al.* (2013); Srivastava & Riddell (2015)). This approach has shown great promise; however, it can result in immune-related adverse effects (irAEs), such as fatal acute respiratory distress syndrome (Morgan *et al.* (2010)) and B cell aplasia (Brentjens *et al.* (2011); Kalos *et al.* (2011); Brentjens *et al.* (2013)). Current options for managing these irAEs include high-dose corticosteroid therapy and suicide genes, but these approaches are not specific and shut down desirable as well as undesirable immune responses.

An alternative approach is the use of inhibitory CARs (iCARs), which utilise inhibitory receptor components to prevent unwanted T cell responses in a specific manner (Fedorov *et al.* (2013); reviewed by Sadelain (2016)). Proof-of-principle for iCARs has been demonstrated using a single-chain variable fragment (scFv) specific for human prostate-specific membrane antigen along with the cytoplasmic tail of CTLA-4 or PD-1 (Fedorov *et al.* (2013)). Initial results from these iCARs have been promising, with antigen-specific inhibition of unwanted T cell cytotoxicity whilst maintaining on-target killing (Fedorov *et al.* (2013)).

Despite increasing interest in CARs as a treatment option for immune-related pathologies, there has been little investigation into the importance of the dimensions of intracellular tails and the positioning of signalling motifs within them, in particular for inhibitory receptors such as PD-1. Therefore further investigation is required to develop a better understanding of the intracellular tail of these receptors in order to maximise the effectiveness and safety of CAR-based therapies.

For instance, initial investigations into the PD-1 intracellular tail presented in Chapter 5 demonstrate a lack of inhibitory effectiveness upon truncation of the PD-1 intracellular tail (Figure 5.8), indicating that positioning of the ITSM too close to the plasma membrane abrogates PD-1 inhibitory function. This result should be considered for the development of iCARs, as positioning of the PD-1 ITSM may impact their efficacy.

Overall, continued investigation of the intracellular tails of PD-1 and other NTRs should provide additional insights into strategies for utilising these receptors and their signalling components in ACT-based therapies.

6.7 NTRs: future targets for immunotherapy

Alongside the emergence of ACT-based therapies, another exciting development in the field of immunotherapy is the use of antibodies against NTRs. Of note are the recent successes of CTLA-4 and PD-1 checkpoint blockade (Wolchok *et al.* (2013); Schadendorf *et al.* (2015)), which have paved the way for antibody-based therapies against other immune receptors. For example, there is particular interest in targeting other co-inhibitory receptors such as TIM-3 and LAG-3 to fight tumours (reviewed by Anderson (2014); Anderson *et al.* (2016); Liu *et al.* (2016a); Romero (2016)).

Building on this progress with tumour immunotherapies, there is also a push for a similar approach for the treatment of autoimmune diseases such as rheumatoid arthritis, psoriasis, and SLE (reviewed by Ceeraz *et al.* (2014); van der Vlist *et al.* (2016); Lee *et al.* (2016)). In contrast to the emerging immunotherapies targeting tumours, which have mainly used blocking antibodies against inhibitory receptors, immunotherapies for autoimmune diseases could involve agonistic antibodies or artificial ligands against inhibitory receptors to dampen unwanted immune responses (reviewed by van der Vlist *et al.* (2016)).

As described in Section 1.6, there are numerous co-inhibitory NTRs of interest that are potential targets for antibody-based immunotherapies, such as inhibitory NK cell receptors, TIGIT, TACTILE, Siglec-2, Siglec-9, and BTLA. These NTRs could be further investigated through analysing signal integration using the two-ligand system described here. In addition to these NTRs, there is a wide range of NTRs about which even less is currently known. Developing a better understanding of many more of these receptors and their mechanisms of signal integration may help to develop safer and more effective immunotherapies.

6.8 Concluding remark

The current study describes the development of a novel two-ligand system including ligand cells expressing a generic and titratable ligand for stimulation of receptor cells. This system was set up to analyse signal integration by an important group of immune cell surface receptors known as NTRs. The current experiments have focused on the co-inhibitory receptor PD-1, with analyses in both THP-1 and Jurkat receptor cells. Initial results demonstrate PD-1-mediated inhibition in the two-ligand system and provide a starting point for further investigation of the importance of extra- and intra-cellular dimensions for signal integration by a variety of NTRs.

Many of these NTRs are potential targets for emerging immunotherapies such as the ACT- and antibody-based approaches discussed above. With so many promising immunological targets, one of the biggest challenges for the field of immunotherapy will be to determine which treatment combinations are the most effective in different diseases and indeed in individual patients as well as at different stages of disease. Therefore we must develop a better understanding of target molecules and how they direct the immune response temporally, spatially (on both macro and micro scales), in different cell types, different patients, and in a pathological context. This will require continued collaborations across disciplines and from different stages of research, from fundamental research all the way to the clinic. It also calls for development of innovative techniques to accelerate the investigation of NTR signalling and signal integration. We hope that the two-ligand system presented here will be one of many new approaches for pursuing this goal.

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Appendices

Appendix

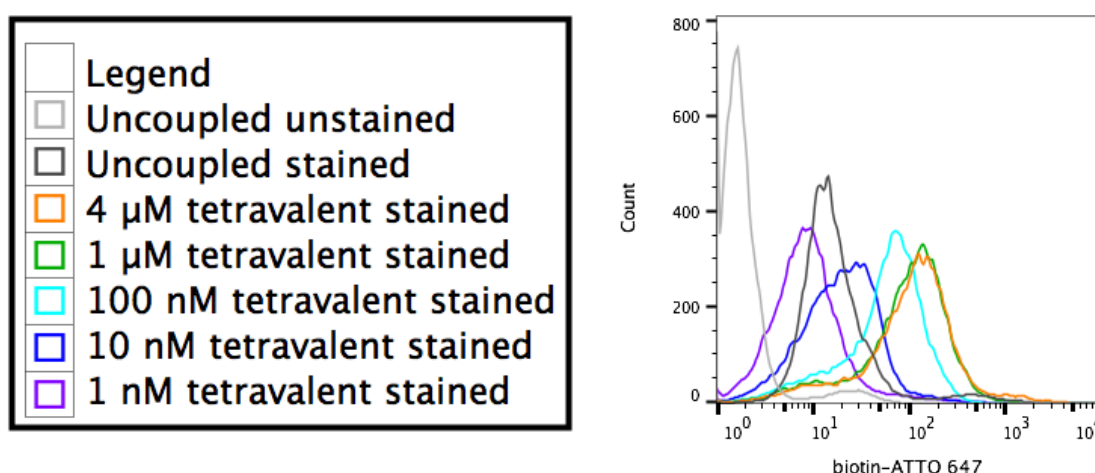


Figure A.1: Flow cytometry results showing CHO.K1 CD48/Spy ligand cells coupled with a titration of tetravalent StrepTactin-SpyCatcher from 4 μM down to 1 nM as indicated. Cells were stained with 3 μM of a 3:1 mixture of D-biotin:biotin-ATTO 647. Uncoupled CHO.K1 CD48/Spy ligand cells either unstained or stained are shown in light grey and dark grey respectively.

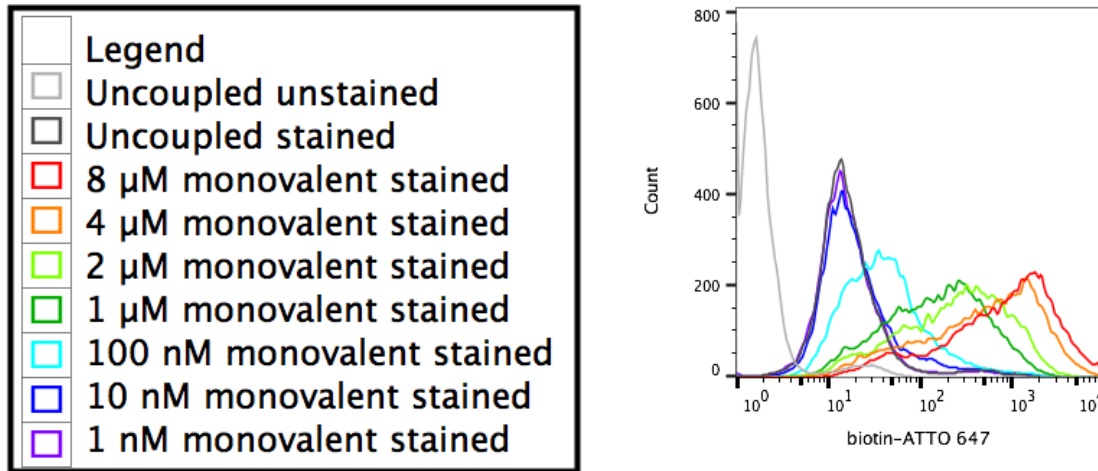


Figure A.2: Flow cytometry results showing CHO.K1 CD48/Spy ligand cells coupled with a titration of monovalent StrepTactin-SpyCatcher from 8 μM down to 1 nM as indicated. Cells were stained with 3 μM of a 3:1 mixture of D-biotin:biotin-ATTO 647. Uncoupled CHO.K1 CD48/Spy ligand cells either unstained or stained are shown in light grey and dark grey respectively.

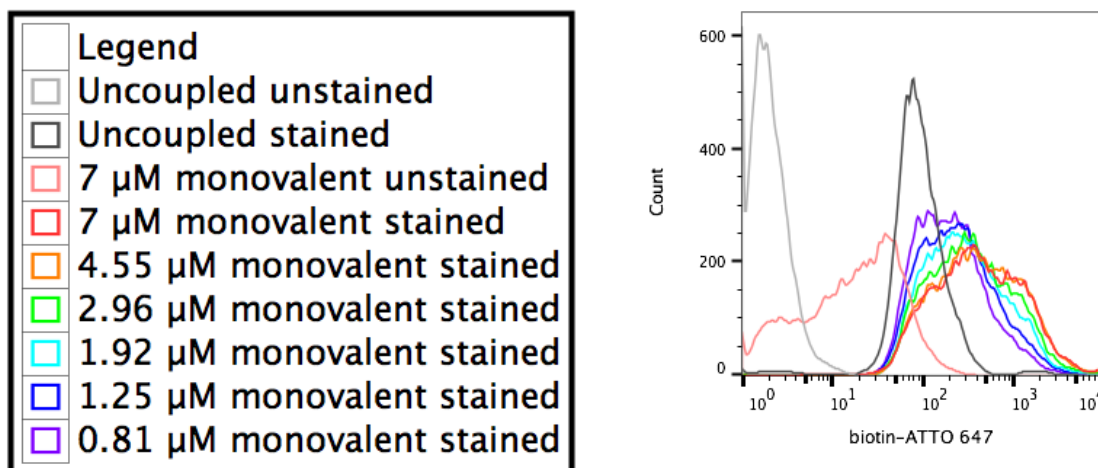


Figure A.3: Flow cytometry results showing CHO.K1 CD48/Spy ligand cells coupled with a titration of monovalent StrepTactin-SpyCatcher from 7 μM down to 0.81 μM as indicated. Cells were stained with 0.5 μM biotin-ATTO 647. Unstained uncoupled (light grey), stained uncoupled (dark grey) and unstained coupled (pink) CHO.K1 CD48/Spy cells are shown as controls.

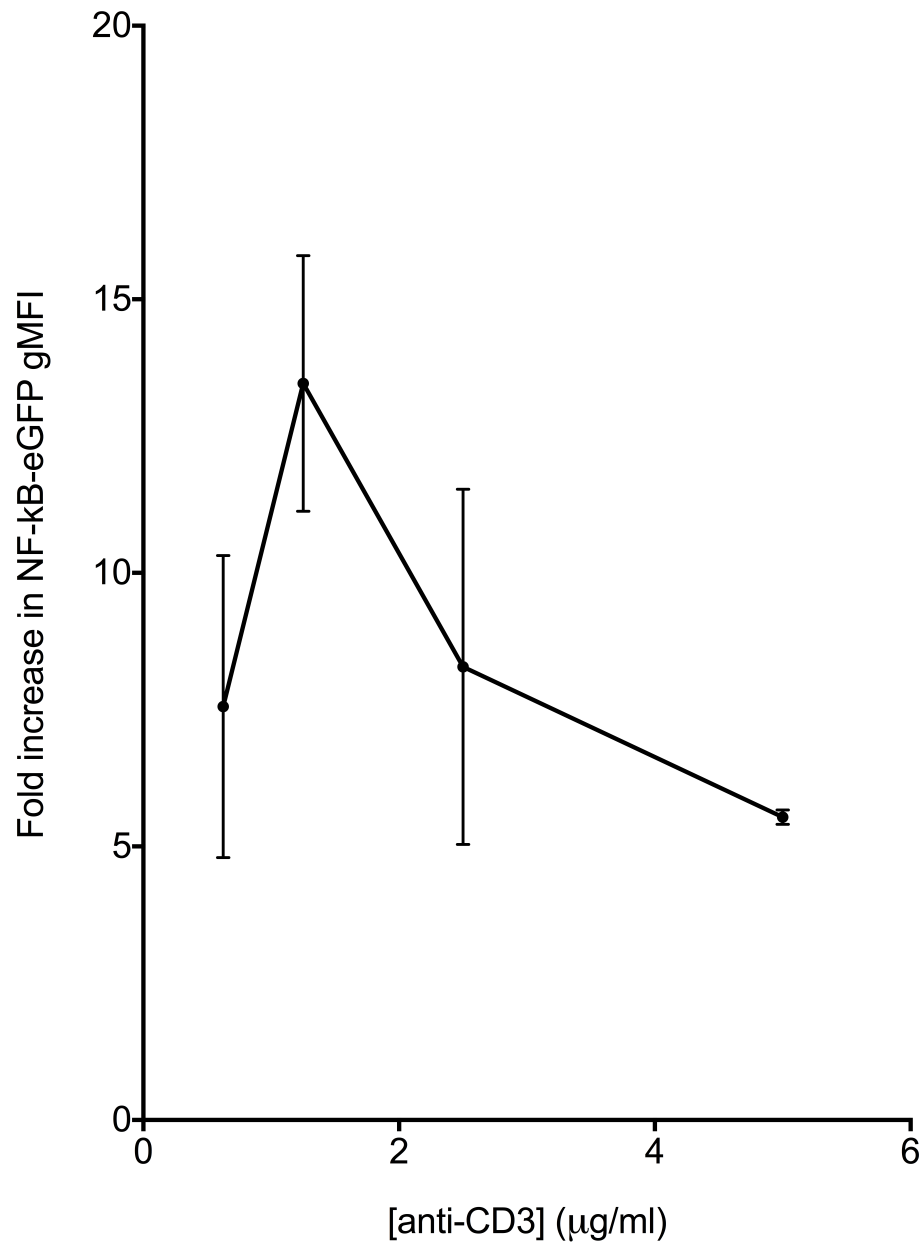


Figure A.4: Flow cytometry to assess fold increase in NF- κ B-eGFP gMFI of Jurkat NF- κ B-eGFP/1G4 TCR reporter cells stimulated with a titration of platebound anti-CD3 (UCHT1). The cells were unstimulated (5 μ g/mL platebound IgG1 isotype control) or stimulated with a 1/2 serial dilution from 5 μ g/mL down to 0.625 μ g/mL of platebound anti-CD3, with total IgG1 controlled for using an IgG1 isotype control. NF- κ B-eGFP gMFI is expressed as a fold increase relative to stimulation with 5 μ g/mL IgG1 isotype control alone. Mean plus standard deviation from triplicates are shown for each experimental condition.

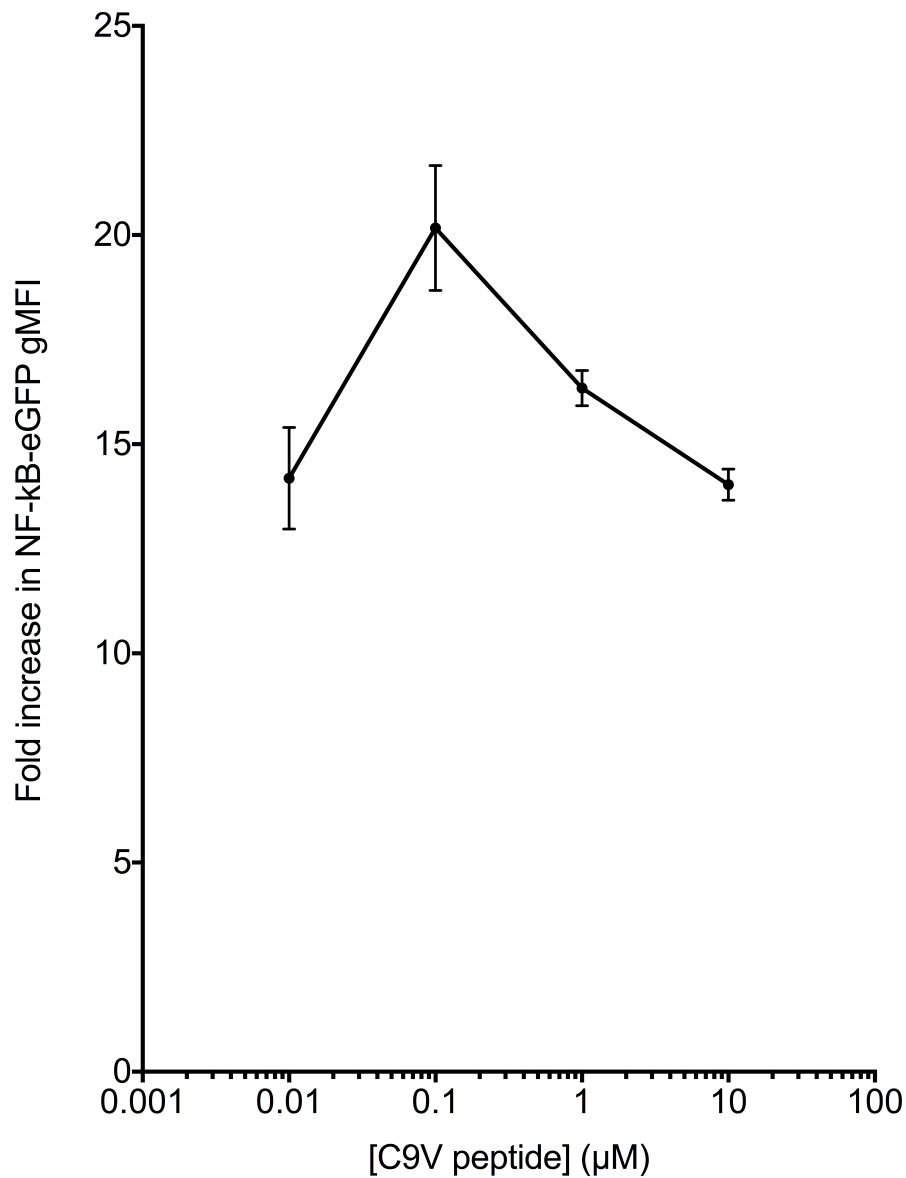


Figure A.5: Flow cytometry to assess fold increase in NF- κ B-eGFP gMFI of Jurkat NF- κ B-eGFP/1G4 TCR reporter cells stimulated with CHO.K1 HLA-A2 single-chain dimer ligand cells loaded with a titration of cognate NY-ESO-1 C9V peptide. NF- κ B-eGFP gMFI is expressed as a fold increase relative to stimulation with unloaded CHO.K1 ligand cells (no peptide). Mean plus standard deviation from triplicates are shown for each experimental condition.

Appendix

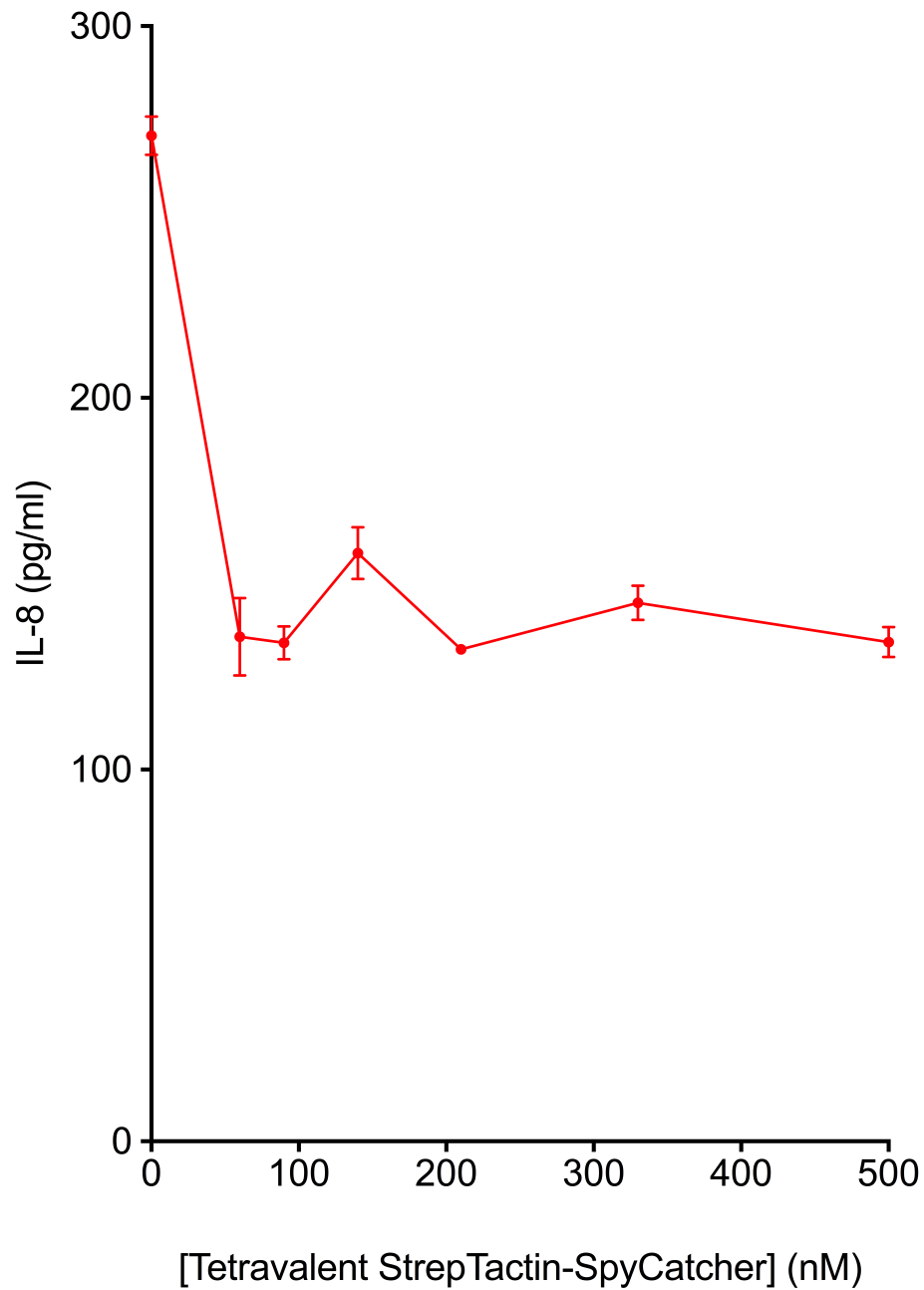


Figure B.1: *IL-8 production from a pilot cell-cell stimulation of vitamin D-differentiated THP-1 2B4-CD64/PD-1 Strep-tag II receptor cells with CHO.K1 CD48/Spy cells. The THP-1 receptor cells were stimulated with CHO.K1 CD48/Spy ligand cells either uncoupled or coupled with a titration of tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in the supernatant were measured by ELISA, showing mean plus range from a single experiment.*

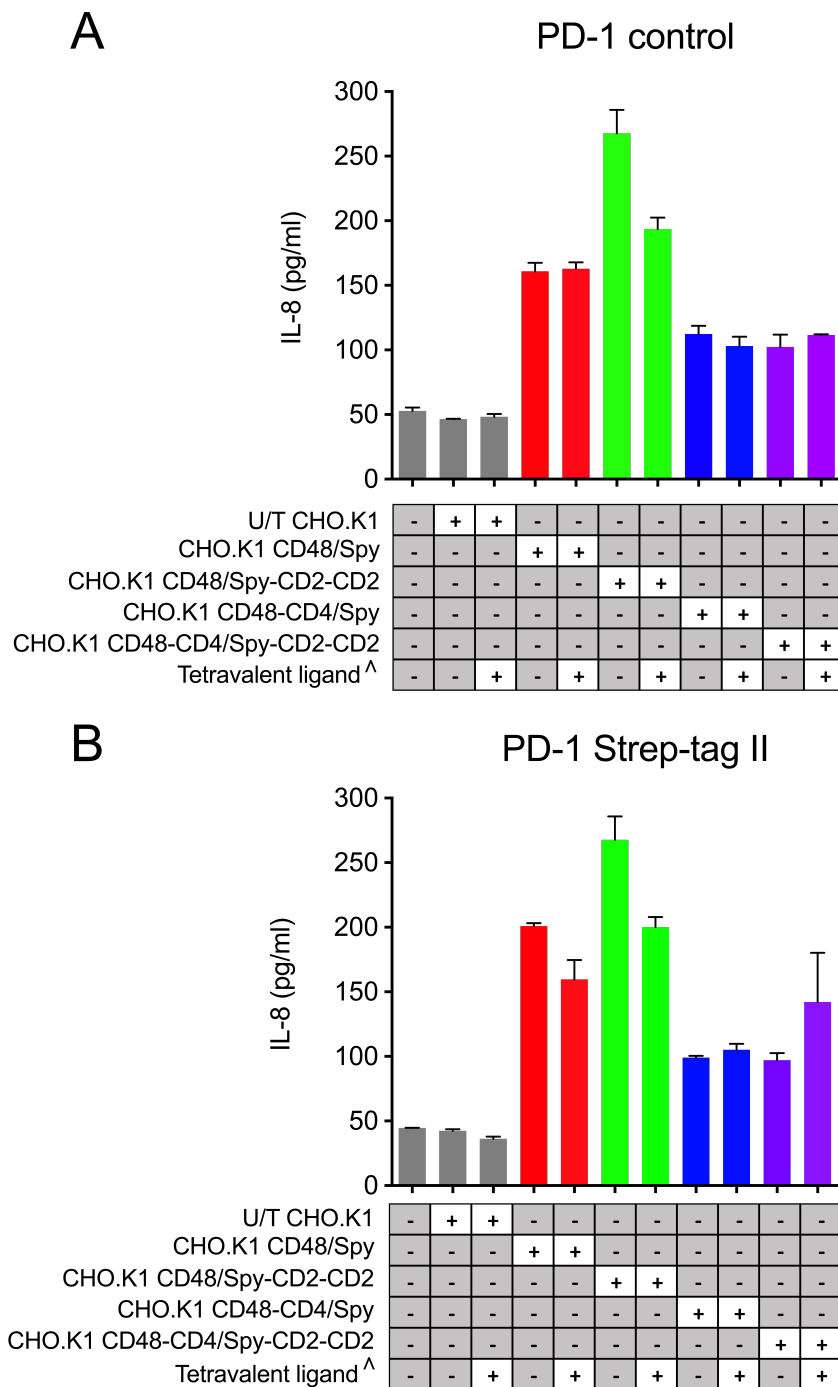


Figure B.2: Raw IL-8 production from the first stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48-based ligand cells. THP-1 2B4-CD64 receptor cells expressing PD-1 control (A) or PD-1 Strep-tag II (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48-based ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in the supernatant were measured by ELISA, showing mean plus range from duplicate samples. [^]Ligand = StrepTactin-SpyCatcher.

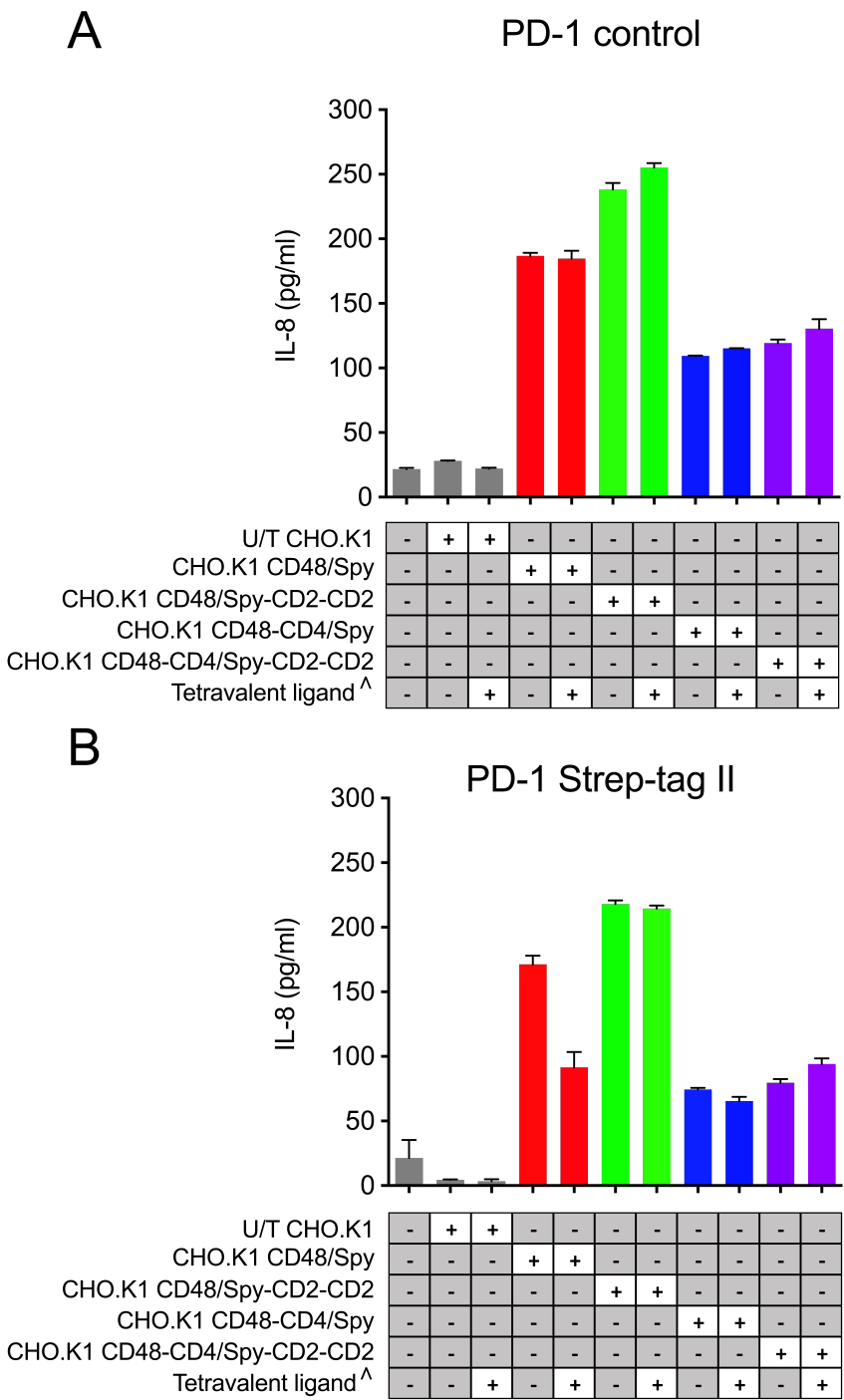


Figure B.3: Raw IL-8 production from the second stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48-based ligand cells. THP-1 2B4-CD64 receptor cells expressing PD-1 control (A) or PD-1 Strep-tag II (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48-based ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in the supernatant were measured by ELISA, showing mean plus range from duplicate samples. [^]Ligand = StrepTactin-SpyCatcher.

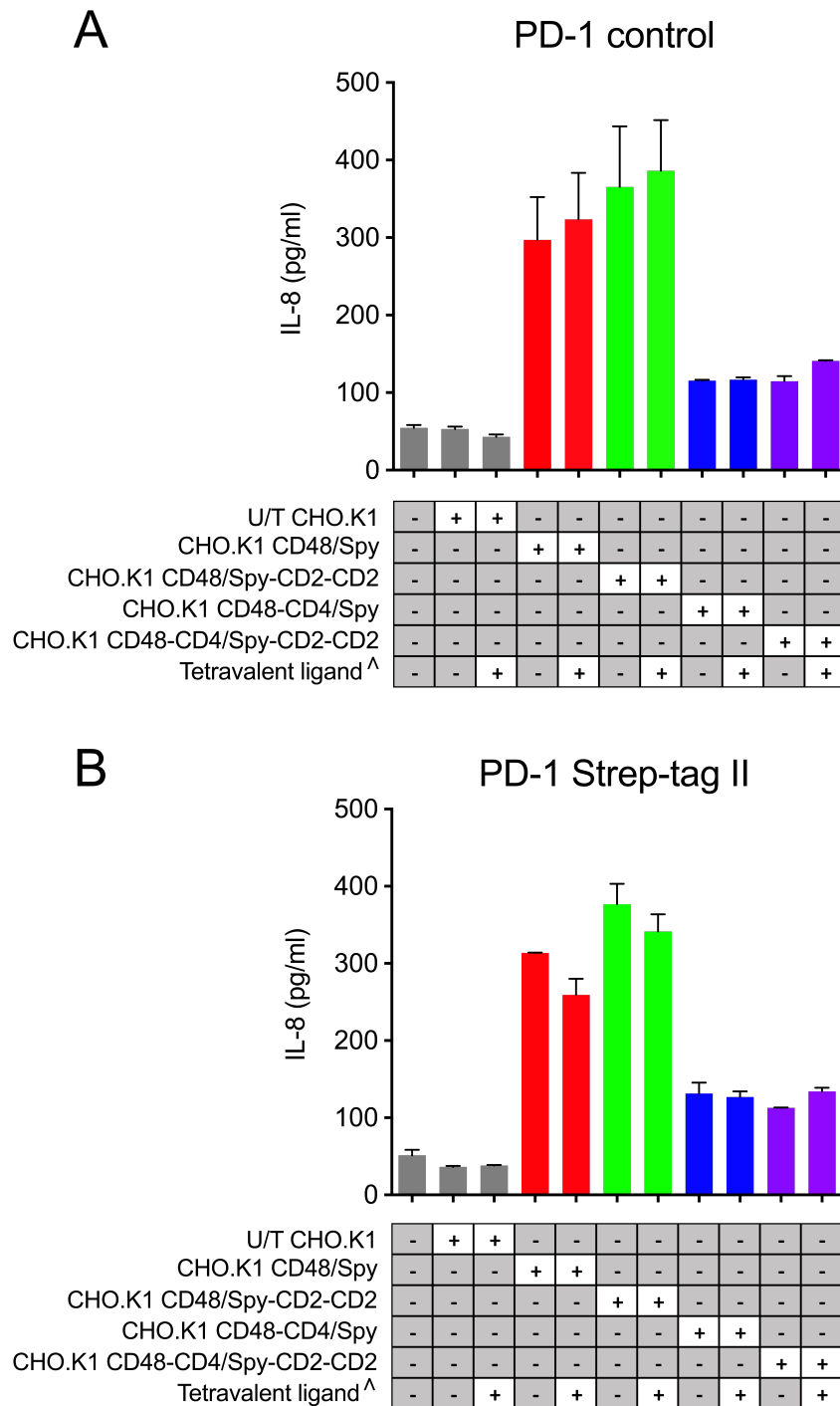


Figure B.4: Raw IL-8 production from the third stimulation of vitamin D-differentiated THP-1 receptor cells with CHO.K1 CD48-based ligand cells. THP-1 2B4-CD64 receptor cells expressing PD-1 control (A) or PD-1 Strep-tag II (B) were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 CD48-based ligand cells, either uncoupled or coupled with 60 nM tetravalent StrepTactin-SpyCatcher as indicated. IL-8 levels in the supernatant were measured by ELISA, showing mean plus range from duplicate samples. [^]Ligand = StrepTactin-SpyCatcher.

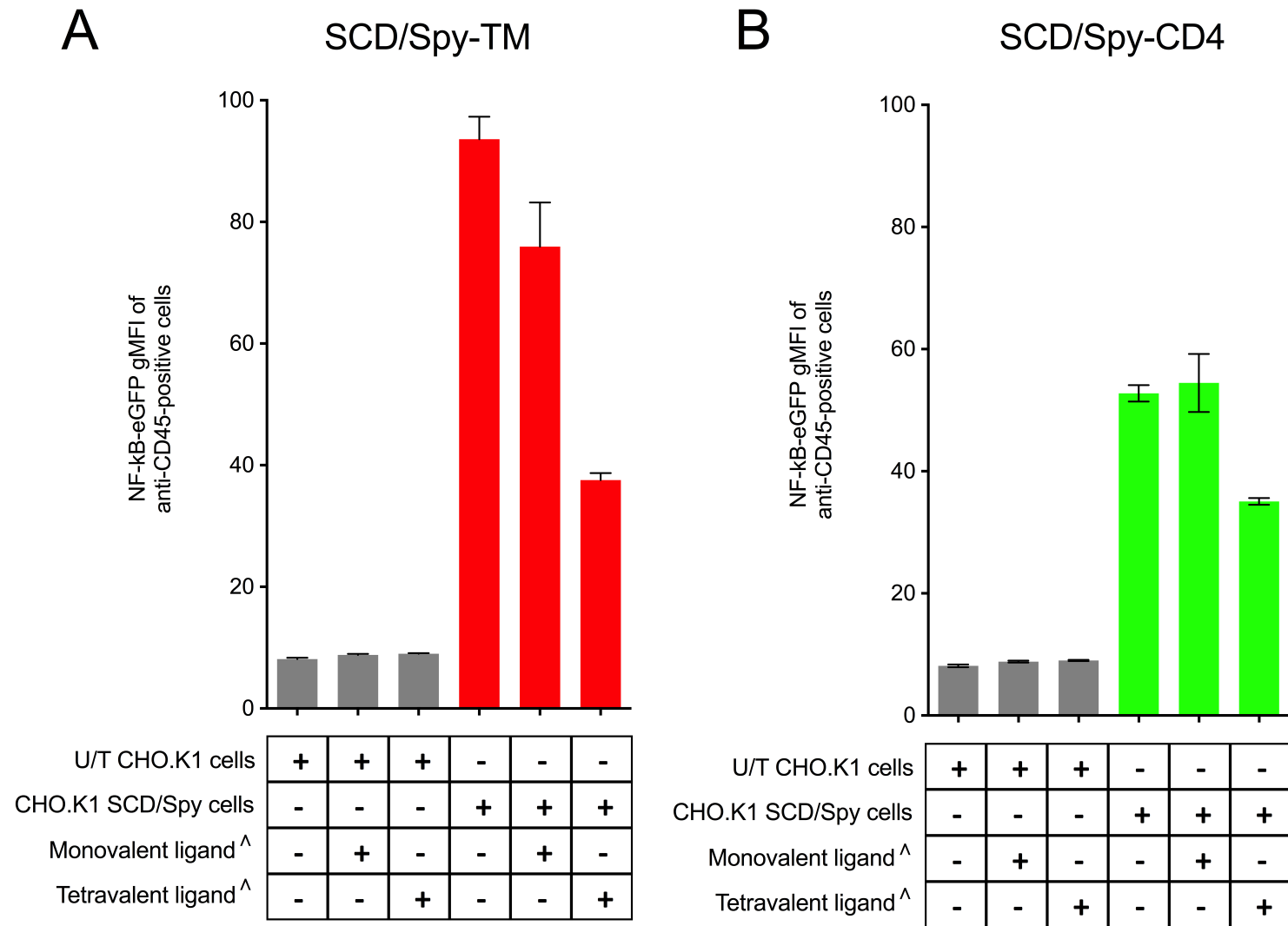


Figure B.5: Raw NF- κ B-eGFP gMFI levels from stimulation of Jurkat NF- κ B-eGFP/1G4 TCR/PD-1 Strep-tag II receptor cells with CHO.K1 SCD/Spy (A) or CHO.K1 SCD/Spy-CD4 (B) ligand cells. The Jurkat receptor cells were stimulated with untransfected (U/T) CHO.K1 or CHO.K1 SCD-based ligand cells loaded with 10 nM peptide and either uncoupled or coupled with 200 nM monovalent or tetravalent StrepTactin-SpyCatcher as indicated. NF- κ B-eGFP gMFI was measured by flow cytometry, showing mean plus range from duplicates. Results are representative of two independent experiments. [^]Ligand = StrepTactin-SpyCatcher.