

Regulation of T cell activation



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Hilary 2018

Thesis submitted for the degree of

Doctor of Philosophy in Molecular and Cellular Medicine

Acknowledgements

This four and half year PhD experience has been an 'Odyssey' and like the heroic Greek Odysseus I have managed to reach my 'Ithaca'. This would not have been possible without the help of God and many people. I am now completing 10 years outside my country and away from my family and many beloved ones.

I have been privileged to work with two great supervisors that have both been inspiring research scientists for me. I am indebted to Professor Michael Dustin for all his kindness, guidance and support from the very beginning to the end of my PhD. He has given me many and great opportunities to develop as a scientist, to work on my independence and my critical thinking. I have always admired his abilities to recall studies, his critical and forward thinking. I am also very thankful to him for allowing me to work on my research ideas and for his great support through out this PhD and for believing in me. I am also indebted to Professor Paul Bowness for his support. I have learnt a great deal from him and from his lab members especially in the field of translational medicine. I am also thankful to Dr. Simon Kollnberger for his support and collaboration at the beginning of my PhD. Dr Thibault Griseri has been an inspiring young independent scientist from whom I have also learnt a lot and I would like to thank, too.

Through out my PhD, I have met great individuals. I am indebted to Dr. David Depoil and Dr. Enas Abu Shah. They are two great scientists that have been next to me in good and bad times. I shall never forget our long times in the lab, our conversations in the lab or over food! It has been thrilling, exhausting, enjoyable, frustrating and we have always pushed forward and achieved the best of our abilities. I have learnt a lot from them and I have done my best to give back to them the best. The only way forward for community and science is through TEAM WORK and this is what David, Enas and myself practiced.

It was also an honour to have worked with all the members of the Dustin lab. Each one is a great scientist and I wish them the best. Our lab manager Elke Kurz has been there whenever I asked for help at any time. At this time, I would like to wish her good luck and quick recovery. I am grateful and honoured to have also worked closely with two great individuals Dr. Viveka Mayya and Salvatore Valvo. I have enjoyed our discussions and both of them have been very supportive at a personal and scientific level. I will never forget Salvo's Mediterranean character, which always nostalgically reminded me of home, and his ethics in helping each and everyone who sought help from him. Their support and work ethic has been outstanding. I should not forget a past member of the Dustin family; Jehan Afrose; she was the quiet essential research assistant in providing good quality reagents for our everyday bilayer experiments. It was also great working and sharing ideas with Dr Kseniya Korobchevskaya, Dr Marco Fritzsche, Liliana Barberi; especially towards the end of my PhD where time was running and key questions were yet to be addressed. Their contribution was valuable. Thank you all.

In my PhD I have met three of the kindest, most talented, brightest people I have ever met and worked with; Dr. Pradeep Kumar Sacitharan, Dr. Geoffrey Lee and Dr Anand Segar – 'the dream team', my friends. Our long hours in the lab followed by amazing discussions over food or - what was the best thing after a long frustrating day in the lab- an ice cream will always be live memories. Our conversations revealed to me the potential of human mind in thinking and dreaming! It has been inspiring! I am so lucky to have met them; they have been a treasure. Together, we created a momentum and this allowed us to push beyond our limits in the lab and in life. They were a great support to me when they were in

Oxford but they are still a great support after leaving the UK for other continents (America, Europe and Australia); 'the dream team' is always connected!

I would also like to thank my friends back home and in Oxford-London (Nectarios, Chrysostomos, Antonis, Eirini, Veronica, Dimitris, Thomas); each one helped me in different ways. They were always supportive of my work and very excited to hear about it. It was a great relief and very refreshing every time we got together back home; it was an essential break and 'pit-stop' allowing me to keep pushing forward. Thank you Dimitris and Thomas for those conversations we had over food.

Finally I would like to thank my family; my two sisters, my mother and my dad. I would have never reached and never completed this PhD without their support. I thank them for all their love, support of all types, for allowing me and supporting me in making my dreams real and for their prayers. Thank you for all your sacrifices. I have only come this far as a scientist and a human being because you have always been at my side though the years. I am also indebted to my uncle George. He was my inspiration and a person to look upon while growing from primary school to university years. He has supported me in many ways, which he might not even know. I wish I could repay them all one day! Last but not least, I cannot thank her more, is my fiancé and wife-to-be, Danae. She has seen me through the worst and best times of my PhD year. A person with unlimited patience, love and care. I am grateful to her for all her support, of many sorts, and for sticking with me thought out this period. She has sacrificed a well-balanced life back home to be next to me at all times. Despite the many difficulties and lonely times she had to go through, she has been outstanding and always at my side. I could not have achieved the best of my abilities without her support.

'The End, God is Great'

Abstract

Recognition of a cognate peptide-MHC molecule by its T cell receptor (TCR) triggers a critical cascade of protein re-organisation and clustering at the cell interface. This results in the formation of the immunological synapse (IS), concurrent with and influencing T cell activation. The glycoprotein CD2 with its ligand CD48 (mouse) or CD58 (human) has been shown to act as an important adhesion/co-stimulatory molecule influencing the T cell activation. While initial analysis of the knockout mouse revealed redundancy with LFA-1 and CD28, activity of this pathway has been correlated with enhanced pathogen clearance and autoimmunity in humans, renewing interest. Earlier analysis of CD2 dynamics in Jurkat cells revealed formation of large domains that display centralization similar to the TCR and co-stimulatory receptors like CD28. We have found that ligated CD2 displays a more complex behaviour in previously uncharacterized later stages of IS maturation in primary mouse and human CD4⁺ T cells

We observed that the CD2-CD58 complexes colocalised with TCR/pMHC microclusters during the formation of the mature IS, characterized by the central supramolecular activation cluster (cSMAC) where TCR signaling is terminated. Upon formation, the CD2-CD58-rich plaques moved outwards to form a 'corolla', a segmented ring outside the LFA-1-ICAM-1 peripheral SMAC (pSMAC). This 'corolla' is enriched in downstream active signalling molecules. We found that the CD2 'corolla' also brings with it other co-stimulatory receptors like ICOS and CD28. We suggest that CD2 rescues TCR and costimulatory signalling complexes that are destined for termination in the cSMAC to a peripheral site where signalling can be sustained, leading to enhanced signal integration. Interestingly, investigation of the classical co-inhibitory receptor PD-1, revealed that PD-1 and CD2 corolla can be segregated under specific conditions but can

also colocalise in the periphery of the IS; preliminary data suggests that this is dependent on the expression levels of PD-1. Overall, our results suggest that the expression levels of these co-stimulatory/inhibitory receptors can influence their localisation dynamics within the IS. Most importantly, CD2 expression levels are one of the key factors controlling corolla formation. Our results suggest that the expression levels of the co-stimulatory/inhibitory receptors have functional implications in the IS.

Finally, we have investigated the levels of CD2 and PD-1 in the tumour microenvironment where tumour-infiltrating lymphocytes (TILs) are failing to stop tumour growth. Our preliminary data in small cohort of eight colorectal cancer patients where surgical specimens were acquired demonstrate high levels of PD-1 and low expression of CD2 in CD8⁺ TILs compared to CD8⁺ T cells found in normal tissue adjacent to the tumour or in peripheral blood CD8⁺ T cell compartment of healthy individuals. In summary, we are proposing that CD2 acts both as a classic costimulator with strong expression the ability to enhance activation of Src family kinases and as an organiser or co-stimulatory/inhibitory signalling in the IS.

Statement of Originality

I hereby declare that this submission is my own work and to the best of my knowledge contains no materials previously published or written by another person, or which have been accepted for the award of any other degree or diploma at the University of Oxford or any other educational institution. Any contribution made to the research by others, with whom I have worked, is explicitly acknowledged in this thesis.

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Abbreviations

APC	antigen presenting cell
APL	altered peptide ligand
Ag	antigen
Akt	Protein Kinase B
BCR	B cell receptor
BSA	bovine serum albumin
C-terminal	carboxyl terminal
Ca ²⁺	calcium
CaMH	Ca ²⁺ -calmodulin-dependent kinase
CD2AP	CD2 associated protein
CD2AP-DN	CD2 associated protein-dominant negative
CD40L	CD40 ligand
CO ₂	Carbon dioxide
CRAC	Ca ²⁺ release-activated Ca ²⁺ channel
CRC	colorectal cancer
cSMAC	central supramolecular activation cluster
CTL	cytotoxic T lymphocyte
CTV	cell trace violet
CXCR5	C-X-C chemokine receptor type 5
DAG	diacylglycerol
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dSMAC	distal supramolecular activation cluster
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmid reticulum
ERK	extracellular signal-regulated kinase
Fab	Fragment antigen-binding
FBS	fetal bovine serum
FMO	fluorescence minus one
Fyn	Proto-oncogene tyrosine-protein kinase
GADS	GRB2-related adaptor downstream of Shc
GM-CSF	granulocyte-monocyte colony stimulating factor
GRB2	growth factor receptor-bound protein 2 (
GWAS	genome wide association study
hCD2FL	human CD2 full length
hCD2TM	human CD2 lacking its cytoplasmic tail
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
hr	hour/hours
ICAM-1	Intercellular Adhesion Molecule 1
ICOS	inducible costimulator

ICOS-L	inducible costimulatory ligand
IFN γ	interferon gamma
IL-1 β	interleukin-1beta
IL-2	interleukin -2
IL-4	interleukin -4
IP	immunoprecipitation
IP3	inositol 1,4,5-triphosphate
IRM	interference reflection microscopy
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
Itk	Interleukin-2-inducible T-cell kinase
kDA	kilo daltons
LAT	Linker for the activation of T cells
LDH	lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen-1
mAb	monoclonal Antibody
MAPK	mitogen-activated protein kinase
mcc	mouse cytochrome c
MFI	mean fluorescence intensity
MHC	Major Histocompatibility Complex
min	minute/minutes
ms	milliseconds
NA	normal adjacent
Nck	non-catalytic region of tyrosine kinase adaptor protein 1
NK	natural killer
OK-DMEM	Osaka Kanagawa-adjusted Dulbecco's Modified Eagle Medium
Opti-MEM	Reduced Serum Media is a modification of Eagle's Minimum Essential Media
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PD-1	programmed cell death gene-1
PD-L1	programmed cell death gene-1 ligand 1
PD-L2	programmed cell death gene-1 ligand 2
PenStrep	Penicillin-Streptomycin
PFA	paraformaldehyde
PH domain	plekstrin homology domain
PHA	phytohaemagglutinin
PHEM	PIPES, HEPES, EGTA, Magnesium sulphate
PI3K	phosphoinositide 3-kinase
PIP3	phosphoinositol 4,5-bisphosphate lipids
PKC- θ	protein kinase C-theta
pLAT	phosphorylated LAT
PLC γ 1	phospholipase- γ -1
pMHC	peptide MHC
pSMAC	peripheral supramolecular activation cluster
ROI	region of interest
RPMI	Roswell Park Memorial Institute

SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SLB	supported lipid bilayer
SLP76	Src homology 2 domain-containing leukocyte phosphoprotein of 76kDA
STORFM	Stochastic Optical Reconstruction Microscopy
TBS	Tris-buffered saline
TCR	T cell receptor
TFH	Follicular Help T cells
TIL	tumour infiltrating lymphocytes
TIRFM	total internal reflection microscopy
TNF- α	tumour necrosis factor alpha
TYR	tyrosine
V	bolts
WCL	whole cell lysate
ZAP-70	Zeta-chain-associated protein kinase of 70kDA

1 Chapter 1. Introduction

1.1 T cell activation

1.1.1 T cell receptor mediated activation of T cells.

The adaptive immune system is made up of two major lymphocyte types, T cells and B cells. Both are defined by antigen receptors that undergo recombination from gene segments to generate repertoires where each cell encodes one specificity^{1,2}. The major distinction between T cells and the B cells is that T cells recognize antigens that are expressed on the surface of other cells after a “processing” step³ whereas B cells recognize intact antigens without processing⁴. Adaptive immune responses are tightly regulated to preserve host homeostasis and protection from foreign pathogens, however, this can go wrong leading to disease. While there is a large body of knowledge regarding how T and B cells develop in our body, how they are activated to mount an immune response, and how they are stopped from attacking self antigens, there are still many gaps in knowledge. The focus of this thesis is a deeper understanding of how T cells integrate multiple signals through a structured interface referred to generically as an immunological synapse.

T cell activation requires the engagement of the TCR complex and an array of other interactions between cells that include adhesion molecules, co-stimulatory receptors and co-inhibitory receptors. There are two major types of T cells defined by use of different pairs of rearranged genes that make up the heterodimeric antigen recognition unit. The chapter will focus on T cells that express a covalent heterodimer of TCR α - and β -chains, which are each composed of 2 immunoglobulin like domains that combine to recognize a self major

histocompatibility complex proteins presenting a peptide (pMHC) from a host protein or a foreign organism⁵; the latter could be either pathogenic or non-pathogenic. There are two major types of pMHC- class I, in which the peptides are from the cytoplasm, and class II, in which the peptides are from the endocytic pathway. The other T cell type expressing a covalent heterodimer of TCR γ and δ chains, mainly recognize an array of molecular structures that are associated with sensing infection. Either of these variable TCR heterodimers lacks any signaling machinery and can only be expressed on the cell surface when combined with CD3 γ , δ , ϵ (2x), and ζ (2x) chains^{6,7}. The CD3 subunits lack enzymatic activity, but have long cytoplasmic domains with motifs containing pairs of tyrosine residues referred to as immunoreceptor tyrosine-based activation motifs (ITAMs) that are critical for TCR signaling^{6,7}. These ITAMs are phosphorylated by protein tyrosine kinases (PTK), Lck or Fyn of the Src family, upon TCR engagement by a cognate pMHC^{8,9}. These PTKs were associated either with the cytoplasmic leaflet of the membrane through lipid modifications or with the CD4 and CD8 co-receptors, which bind to MHC class II and I, respectively^{8,9}.

How TCR engagement triggers this first phosphorylation step is still a matter of debate. A number of non-exclusive mechanisms have been proposed¹⁰. These include receptor aggregation, receptor conformational change, phosphatase-exclusion/inactivation and mechanotransduction^{11–17}. A common observation is that T cell responses are significantly correlated with the kinetics of the TCR-pMHC interaction, which led to the proposal that TCR signaling could operate on a “kinetic proof-reading” principal¹⁸. Kinetic proof-reading can be combined with any of these mechanisms described above, an example being the kinetic segregation model^{14,15,19}. In brief, at steady state, kinase and phosphatases are acting on ITAMs to maintain low basal phosphorylation. For the duration of

close contacts needed for TCR-pMHC interaction, the proximity of the two membranes excludes phosphatases in a local size-dependent manner of their extracellular domains while non-receptor kinases remain at the same density in the close contact allowing a shift of equilibrium towards more phosphorylation events and initiation of triggering. If TCR-pMHC interaction sustains phosphatase exclusion for long enough to recruit down-stream kinases and allow it to act on its substrates, then signaling is triggered, whereas if TCR-pMHC interaction is of too short a duration, the phosphatases will return and reset the TCR to its pre-engagement status with no triggering. One of the challenges for the kinetic-segregation model is how the TCR-pMHC interaction is distinguished from close contact formed by some of the small adhesion molecules, which generate similar close contacts²⁰. Solution measurements of TCR-pMHC interaction kinetics provide most of our understanding, but correlations may break down in interfaces due to differential effects of force on kinetics. Efforts in this direction have begun, but there is no consensus on how the immune synapse environment impacts TCR-pMHC interaction kinetics^{21–23}.

The aforementioned phosphorylated ITAM motifs recruit the ζ -associated protein of 70kDa, ZAP-70, which in turn initiates a cascade involving its own phosphorylation^{24,25}. ZAP-70 recruits two adaptor proteins, the cytosolic adapter protein Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76kDa (SLP76) and the transmembrane adapter protein linker for the activation of T cells (LAT), both of which are key in assembling a large signaling complex required for downstream signaling leading to a cellular response^{26,27} (Figure 1.1). Phosphorylated LAT (pLAT) recruits the phospholipase- γ -1 (PLC γ 1), phosphoinositide 3-kinase (PI3K) (its p85 subunit) and the adaptor protein growth factor receptor-bound protein 2 (GRB2) and GRB2-related adaptor downstream of

Shc (GADS); reviewed in²⁸. SLP-76 also interacts with pLAT²⁹. SLP-76 itself then interacts with Vav1, non-catalytic region of tyrosine kinase adaptor protein 1, Nck, and IL-2 induced tyrosine kinase (Itk). These proteins may form distinct complexes with different sub-functions in T cell activation and this is an area of active investigation¹⁰. Importantly, the loss of LAT or SLP76 results in almost complete loss of TCR signalling and T cell development³⁰.

The non-receptor tyrosine kinase, Itk, is recruited in part through its plekstrin homology, PH, domain to sites of PIP₃ accumulation where it phosphorylates and activates PLC γ 1 which then generates inositol 1,4,5-triphosphate, IP₃, and diacylglycerol, DAG, by hydrolyzing phosphoinositol 4,5-biosphosphate lipids, PIP₃. In fact, Itk is recruited to the membrane upon TCR engagement by interacting with PIP₃, this allows Lck to phosphorylate Itk which can then bind SLP-76. DAG production induces two major signaling cascades i) the MAPK/ERK kinase cascade and ii) the NF- κ B pathway via protein kinase C- θ , PKC- θ . TCR-signalling-mediated production of IP₃ triggers an influx of Ca²⁺ ions via stimulation of Ca²⁺-permeable ion channel receptors on the endoplasmic reticulum (ER). A second wave of Ca²⁺ ions induced upon TCR signalling is via Ca²⁺ release-activated Ca²⁺(CRAC) channels in response to a depletion of ER Ca²⁺ stores³⁰. This leads to an increase in the intracellular Ca²⁺ levels and activation of downstream enzymes and signalling proteins such as calcineurin and Ca²⁺-calmodulin-dependent kinase (CaMK); these activate various transcriptional pathways downstream. For example, calcineurin activity leads to dephosphorylation of a family of transcription factors collectively known as nuclear factor of activated T cells (NFAT) and their translocation to the nucleus¹⁰.

TCR triggering can take place in the context of peripheral tolerance or mounting an immune response and thus it is insufficient on its own to determine

when an immune response should take place^{31,32}. It's now also clear that the notion that most self-reactive cells are deleted in the thymus is not correct as self-reactive T cells have been directly detected in human peripheral T cell repertoires³³. Furthermore, there are many environmental foreign antigens for which there are strongly reactive T cells in the repertoire, but against an individual must be tolerant to avoid immunopathology. The prevailing notion is that T cells must always integrate TCR signals with other information from the antigen presenting cells and tissue microenvironment in order to make an appropriate response, which may include no response or an actively tolerogenic response. This information is thought to be encoded in soluble factors including cytokines, chemokines and metabolites, and in molecules expressed on the surface of antigen presenting cells that contribute to the structure and function of the immunological synapse.

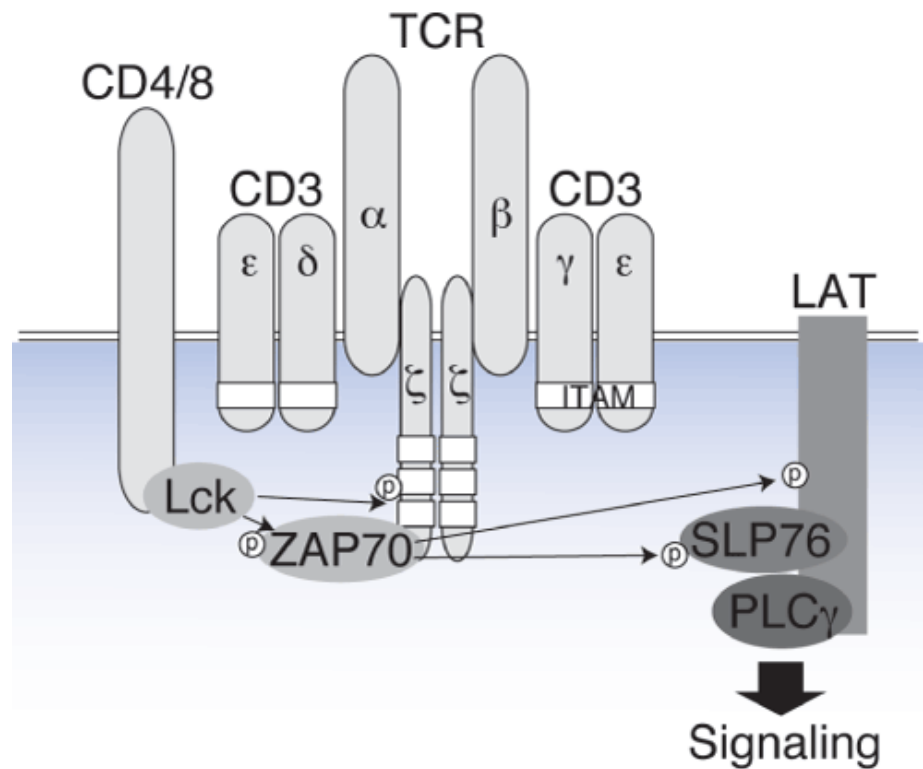


Figure 1.1 TCR/CD3 complex proximal signalling

Adapted from ³⁴ This diagram summarises some of the events taking place upon TCR/CD3 complex engagement. Lck phosphorylates TCR- ζ chains that allows ZAP-70 recruitment and its regulation by Lck and itself. These events take place also in parallel to LAT recruitment that brings in SLP76 to be activated and also PLC γ 1. These are some of the proximal events taking place upon TCR triggering.

1.1.2 The immune synapse mediated activation of T cells

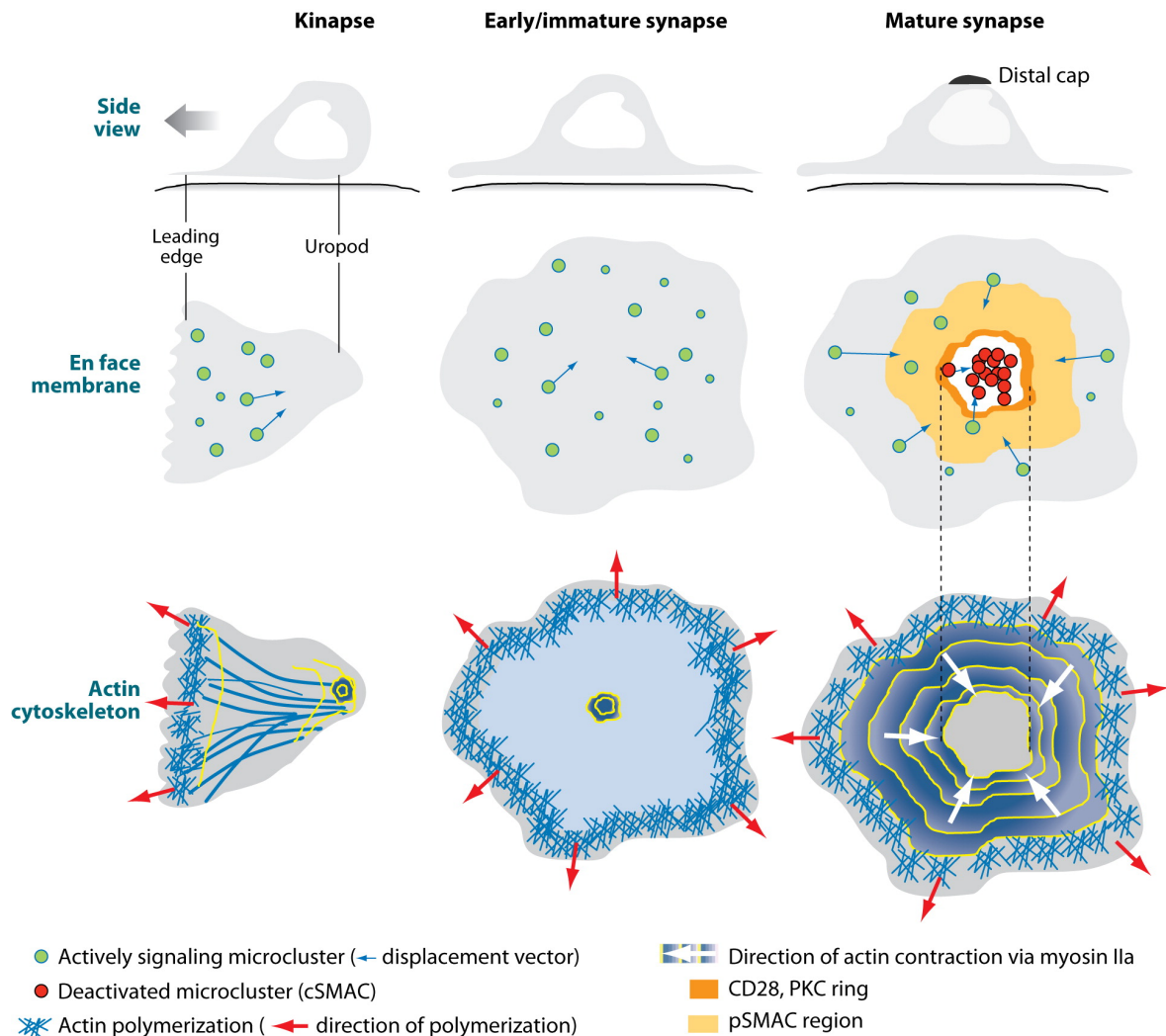
For a productive TCR engagement and TCR-signalling mediated T cell activation, a T cell needs to be efficient at recognizing the small number of activating pMHC compared to the thousands of non-activating pMHC presented by an antigen presenting cell (APC) such as a dendritic cell (DC) or a B cell. At steady state, T cells in the spleen and lymph nodes are scanning surrounding APCs and stroma cells for antigen. T cells receive various signals during that time and they have evolved to efficiently integrate them and make decisions on how to respond.

Upon recognition of an activating/agonist pMHC on an APC, there is formation of the immunological synapse (IS) between the T cell and the APC interacting membranes, cell conjugates, which *in vivo* lasts ~12hrs³⁵; the advent of imaging allowed scientists to shine light on such previously impossible to address questions and provide deeper insight in the events initiating T cell activation. Imaging of fixed cell conjugates, *in vitro*, revealed the presence of a central cluster of proteins rich in TCR-pMHC, named the central supramolecular activation cluster (cSMAC), surrounded by an integrin cluster rich in LFA-1-ICAM-1, named the peripheral smac (pSMAC)³⁶. This was also observed with live imaging of T cells interacting with APC-ligand reconstituted supported lipid bilayers³⁷. A CD45-rich region was also observed surrounding the pSMAC and this became known as the distal SMAC (dSMAC)^{38,39}. Initially, the cSMAC was considered an area of enhanced TCR engagement and sustained signaling³⁷ and TCR degradation^{35,40}. It was, later, discovered that TCR signaling mainly takes place in TCR-pMHC microclusters formed in the periphery of the IS. These TCR microclusters are enriched in the signaling complexes described in the previous section, including ZAP-70 and hence they can initiate and sustain Ca²⁺ signals^{19,41,42}. These clusters then move radially towards the center of the synapse where the TCR-rich

cSMAC is formed; this is an F-actin dependent process⁴³. There is no enrichment of the above microclusters and signaling units within the cSMAC and thus it has been proposed that TCR signaling takes place in the periphery and terminates at the cSMAC¹⁹. In fact, restriction of these microclusters in the periphery allows elevated signaling^{44,45}. Replacement of membrane presented pMHC with a bivalent or monovalent anti-CD3 replicates the same type of immune synapse formation⁴⁶. More recently, Choudhuri *et al* have demonstrated that the cSMAC is a site where TCR-enriched microvesicles are formed and released, that are transferred to the APC membrane⁴⁷ and this is in line and fits with prior reports showing termination of signaling in the cSMAC via isolation of TCR microclusters into multivesicular bodies away from signaling molecules and adaptors^{19,41}; the function of these microvesicles is yet unknown as well as any other content they might carry in their cytoplasm or membrane. LFA-1-ICAM-1 interactions, leading to formation of the pSMAC, are essential for the formation of the IS and allow for ~100-fold higher sensitivity of the T cell to antigen when compared to the absence of LFA-1 ligation⁴⁸. Recently, a similar structure enriched in LFA-1 and other adhesion molecules, but of much smaller scale was described around the newly formed TCR microclusters formed at the periphery of the T cell synapse⁴⁹. The classical costimulatory CD28 also has its place in the synapse. Upon engagement of CD28 by CD80, there is formation of CD28 microclusters similarly to TCR microclusters. TCR and CD28 microclusters co-localise in the periphery of the synapse and both move to the center where TCR is sorted into the cSMAC while active CD28-PKC θ (binds the cytoplasmic domain of CD28) clusters break away and form an annular ring around the cSMAC where they are actively maintained⁵⁰. Importantly, blocking TCR clustering, with antibodies, led to disappearance of CD28-PKC θ microclusters⁵¹. The localization and signalling of other

costimulatory/inhibitory receptors is discussed in following sections. The aforementioned T cell synapse formation features are summarized in Figure 1.2

Finally, it is important to note that the final outcome of a T:APC contact depends on the productive engagement of TCR and also the signals that come from accessory molecules such as costimulatory and coinhibitory molecules and the cytokine milieu of the environment; the former factors will be discussed in the coming sections. The formation of an immune synapse is the result of a productive TCR-pMHC engagement supported by accessory molecules. However, there are also reports that suggest that an immune synapse can also form in the absence of a cognate antigen and that this could support a potential encounter with a cognate antigen⁵². A multifocal synapse, different to the bull's eye IS described above, has also been observed between T cells and dendritic cells^{51,53}. CD3, CD28 and LFA-1 accumulation in the T:DC interface was detected in patches, with CD3 sometimes in the center but mainly in the periphery while LFA-1 was also distributed in patches and not in a pSMAC-like organization^{51,53}. How the multifocal and bull's eye synapses related to each other is still an open question.



AR Fooksman DR, et al. 2010.
Annu. Rev. Immunol. 28:79–105

Figure 1.2 A Diagrammatic form of immune synapse formation

Adapted from ³⁵. It shows a diagrammatic map of synapse formation. Side (top) and en face (middle and bottom) view of the T cells when it interacts with an APC or a ligand-reconstituted SLB. The early/immature synapse consists of actively signaling microclusters found in the periphery which soon after accumulate and generate the cSMAC where signalling is terminated. Arrows show the direction of actin polymerisation. Upon antigen recognition the T cell spreads, inducing radial actin polymerisation in an outward direction. This force is counteracted by contraction of F-actin, via myosin, and centripetal movement towards the center forming the cSMAC³⁵. The localisation of CD28 and PKC-θ is depicted too as reported in⁵⁰.

1.2 Costimulation in T cells

1.2.1 CD2 in T cells

CD2 is a single type I transmembrane glycoprotein that binds CD48 in mice and CD58 in humans^{54–56}; all three proteins belong to the immunoglobulin superfamily. CD2 is expressed early in thymocyte development and is present on almost all naïve and memory mature T cells of mice, rats, humans^{56,57}. CD2 expression is higher in memory T cell subsets compared to naïve T cell⁵⁸. Loza *et al* have reported that a small population of human T cells in the periphery are CD2-/low⁵⁹. These are thought to be early thymic emigrants; they are CD3+, a mixture of double negative, single CD4/CD8+ T cell subsets, that mature in the periphery and one of their potential roles is to replenish the naïve T cell pool. The endothelium and most hematopoietic cells, in humans, express CD48 and CD58⁵⁵ and only CD48 is found in rodents. CD2 has been described as an adhesion and costimulatory molecule in both mice and humans, and to be involved in thymocyte development and peripheral function of T cells^{60–63}.

CD2 has no ITAM domains but its cytoplasmic tail has been shown to associate with the tyrosine kinases p56^{lck} and p59^{fyn}⁶⁴. Lck interacts with CD2 via its SH3 domain that binds the proline-rich motif of the CD2 cytoplasmic tail⁶⁵. CD2 has also been immunoprecipitated with the CD3-ζ chain^{66,67} and CD2 signaling is dependent on the presence of intact CD3-ζ chain⁶⁸. The importance of the proline rich motifs of the CD2 cytoplasmic tail, in the carboxyl terminal (C-terminal), in CD2 functions is underscored by mutagenesis studies of different part of these motifs^{69–72} as well as by its conservation across many species. In addition, to the ability of the cytoplasmic tail to recruit signaling molecules, it also recruits an adaptor molecule, the CD2-associated protein, CD2AP, that links CD2 to the actin cytoskeleton upon T cell activation^{73,74}. This link is required for efficient

polarization of T cell during T cell synapse formation; i.e. the redistribution of the Golgi apparatus and the microtubule-organizing center to the area of the cell:target contact⁷³. Another adaptor protein CIN85, was also described to bind the CD2 tail and link it to actin cytoskeleton; this protein binds the same motif as CD2AP⁷⁴.

A cocktail of anti-CD2 antibodies is able to induce proliferation of T cell and production of IL-2⁷⁵; at the time, this was described as an alternative pathway of T cell activation, in contrast to the classical activation through the TCR-CD3 complex⁷⁵. CD2 stimulation increases adhesion of T cells to ICAM-1 and fibronectin^{76–78}. This function was dependent on the ability of CD2 to induce activation of PI3K⁷⁹. CD2 stimulation with activating antibodies leads, within a few minutes, to an increase in intracellular calcium levels⁸⁰ and this is dependent on endoplasmic reticulum calcium stores⁸¹. In fact, CD2 mAb stimulation was linked to the production of IP₃ and DAG, that suggested a link of CD2 signalling to the activation of PLC γ 1⁸¹ that was dependent on p59^{fyn}⁸², hence this could explain the observed increase in intracellular calcium levels upon CD2 stimulation. Espagnolille *et al.* showed that upon antigen-specific stimulation of T cell with APCs, CD2-CD58 interactions are required for full activation of PLC γ 1 as opposed to TCR triggering alone⁸³ and complements aforementioned biochemical studies. Espagnolille *et al* similarly showed that CD2 and TCR signalling are also synergistic in the activation of ZAP-70 at the IS⁸³. Using imaging, it was also shown that CD2-CD58 interactions, in the IS, allow sustained increased intracellular calcium levels⁸³

In addition, to the aforementioned biochemical pathways triggered by CD2 stimulation, CD2 increases T cell sensitivity to limiting levels of antigen, by lowering T cell activation threshold, due to its adhesion properties as shown in mouse studies^{84,85}. In the context of viral infection of mice with a live virus, CD2

was found to be redundant in the generation of memory CD8⁺ T cells in mouse infection with a live virus and for clearance of the infection⁸⁶. However, more studies have investigated the role of CD2 in T cells since that study came out and have provided more insights as to the significance of CD2 in T cell functions; these are mentioned throughout this section. It has been proposed that CD2-CD58 interactions bring the T cell and APC membranes closer together allowing better pMHC recognition by the TCR, including exclusion of larger inhibitory molecules for example the phosphatase CD45⁸⁷⁻⁹⁰. In fact, there is a feedback loop between CD2 and TCR in this effort to maximize antigen recognition and signal transduction. This was shown by a study describing how TCR signalling enhances the adhesion properties of CD2 for CD58 by 3-4-fold increase in the avidity of CD2 for CD58^{91,92}; this increase in CD2 avidity is dependent on specific residues of the cytoplasmic tail of CD2, different to the motifs responsible for IL-2 production and calcium mobilisation^{77,91}. Loss of regulation of CD2 avidity also compromises the ability of CD2 to enhance T cell activation upon antigen stimulation⁹¹.

1.2.2 CD2 in T cell synapse

Fixed human cell conjugate experiments, showed the recruitment of CD2 in the T:APC interface, in the immune synapse within 5 min of interaction of a T:APC and its colocalisation with a phosphotyrosine rich central area of the IS³⁸. This study also suggested a role for CD2-CD58 interactions in an efficient exclusion of CD45 from the signalling areas of the immune synapse such as those enriched in TCR. In fact, in a similar study with human antigen specific conjugates, the colocalisation of CD2 and CD2AP was shown at the immune synapse; the recruitment of CD2AP to the synapse is CD2 engagement-dependent⁹³. Recruitment of CD2 to the synapse requires both productive TCR engagement

and the presence of its ligand such as CD58⁹³. In parallel, efficient exclusion of CD45 from the immune synapse required CD2-CD58 interactions. The same requirement was found to be needed for efficient enrichment of PKC θ in the IS⁹³. These data are different to the results obtained when T cell are incubated on ICAM-1, pMHC only reconstituted supported lipid bilayers (SLBs) where in the absence of CD2 ligation there is efficient molecular rearrangement, synapse formation and exclusion of CD45³⁷. It has been suggested that this could be explained by the nature of the different stimuli i.e. an APC as opposed to a reductionist model like a ICAM-1, pMHC reconstituted SLB.

Single molecule microscopy, revealed that in the resting Jurkat T cell line, CD2 is largely immobile and diffused in the cell membrane, with some evidence of weak clusters and that these clusters become more pronounced and discrete upon TCR triggering with anti-TCR antibodies⁹⁴. F-actin was shown to be needed for formation of these clusters but not for their maintenance. In agreement, with biochemical and immunoprecipitation assays, CD2 clusters colocalised with LAT and Lck clusters upon TCR triggering⁹⁴. A complementary study using ligand reconstituted SLBs, showed that CD2 ligation by CD58, in the absence of any TCR engagement, also results in the formation of CD2-CD58 microdomains in the periphery of T:SLB interface that move radially to form a cSMAC like cluster at the center of the interface²⁰ similar to a prior study⁷³. Formation and transport of these microdomains was an actin dependent process. Imaging, also, revealed the enrichment of Lck and Lat in these CD2 microdomains in agreement with previous studies reporting association of CD2 with Lck and Fyn in a signalling complex^{64,66,82} and an exclusion of CD45 similar to prior studies^{38,93,94} which was also obvious when the CD2 cSMAC-like structure was formed. Interestingly, the formation of CD2-CD58 microdomains was not dependent on presence of intact

LAT, in this study, while in a similar study looking at the clustering of non-ligated CD2 upon TCR-triggering was shown to be LAT-dependent⁹⁴. Even more interesting is the data showing that the CD2-CD58 microdomains are formed also in a truncated form of CD2, lacking its cytoplasmic tail but these microdomains fail to efficiently recruit activated Lck and LAT²⁰. Finally, Kaizuka *et al* showed that upon interaction of Jurkat T cells with CD58 and anti-TCR antibody only reconstituted SLBs, CD2 microdomains colocalised with TCR clusters within the first minutes of cell-SLB contact but the two signals segregated after ~5 min of cell-SLB contact into a TCR-rich cSMAC surrounded by CD2 distinct zones²⁰. These CD2 zones were also enriched in active signalling molecules such Lck and LAT; phosphorylated TCR- ζ was also enriched in these CD2 zones surrounding the TCR-enriched cSMAC²⁰.

1.2.3 CD2 in NK and B cells

CD2 is also expressed in subsets of NK cells, with CD56^{bright} NK cells having a higher expression of CD2⁹⁵. A very small subset of human mature B cells, in the thymus and periphery of healthy individuals, express CD2, too^{96,97}. A small percentage B cell malignancies have been shown to be positive for CD2 expression. In mice, CD2⁺ B cells were found not in the periphery but primarily in the bone marrow, spleen and lymph nodes at certain B cell developmental stages⁹⁸.

CD2 is enriched in NK cell synapses though much less investigated in this respect compared to T cell synapse studies on CD2^{99,100}. In NK cells, it has been suggested that CD2 signalling is dependent on the presence and antibody loading of CD16 Fc receptor¹⁰⁰. CD2 signalling synergizes with NKp46 receptor signalling, in activating resting human NK cells to produce tumour necrosis factor (TNF- α)

and interferon γ (IFN γ)¹⁰¹; CD2 triggering alone had only weak impact on intracellular calcium changes or cytokine production. IL-15 primed CD56^{bright} NK cells mount anti-tumour responses and the activation leading to this anti-tumour response was CD2-, LFA-1- and NKD2D-depenent¹⁰². Similarly, CD2 also synergizes with activating NKG2C and is crucial for antibody-triggered responses by adaptive NK cells^{103,104}.

1.2.4 CD28 in T cell synapse and activation

CD28 is a dimeric glycoprotein member of the immunoglobulin superfamily and binds CD80 and CD86 expressed on APCs. CD86 is constitutively expressed while both CD80 and CD86 are up-regulated upon activation of APCs. In humans, the majority of T cells constitutively express CD28. CD28 enhances TCR-mediated activation of T cells and specifically the production of TNF- α , IL-2, IFN- γ , granulocyte-macrophage colony-stimulating factor (GM-CSF)¹⁰⁵; this CD28-mediated enhancement requires T cell activation either through the TCR/CD3 complex or by chemical means but the actual pathways triggered are different to the TCR/CD3 induced pathways¹⁰⁵. Overall, CD28 has been described to boost T cell proliferation and cytokine production, influence T cell survival and metabolism¹⁰⁵; these are reviewed in¹⁰⁶.

Engagement of CD28 by CD80/CD86 results in tyrosine phosphorylation of its cytoplasmic tail and recruitment of p85PI3K to it followed by p110PI3K¹⁰⁷. This initiates the recruitment and activation of Akt hence the enhancement of NF κ B pathway and NFAT pathway^{106,108} Itk, which is also recruited upon TCR triggering, can also bind CD28. This interaction has been suggested to keep Itk close to Lck, that can activate Itk and thus calcium mobilization from the ER hence the observed enhanced intracellular calcium levels observed upon TCR/CD28

engagement^{10,109}. Indeed many of the pathways described are also triggered by TCR engagement thus it has been suggested that CD28 provides a quantitative change in T cell activation rather than a qualitative one¹⁰⁶; at the same time this quantitative change has been proposed to result in qualitative changes in T cell activation¹⁰.

In antigen specific T cell-APC conjugates, CD28 was detected in the interface and reported to colocalise with PKC- θ ¹¹⁰. In fact CD28 is responsible for the discrete focal pattern in the immune synapse of T cell-APC conjugates; in the absence of CD28, PKC- θ is seen diffused in the T cell-APC interface¹¹¹. Imaging T cells on APC ligand reconstituted SLB, provided further insight into the molecular dynamics of CD28 accumulation in the synapse. At the early stages of synapse formation, CD28 forms microclusters that colocalise with TCR microcluster; CD28 microcluster formation was shown to be dependent on the presence of CD28 ligand such as CD80^{50,51}. Upon TCR-rich cSMAC formation, CD28 microclusters moving to the center of the synapse form annular ring in a subregion of the cSMAC surrounded by the LFA-1-ICAM-1 pSMAC⁵⁰(also shown in Figure 1.2). The significance of this CD28 annular ring was highlighted in a study where microcontant printing of anti-CD28 antibodies in annular ring around a central anti-CD3 signal allowed enhanced IL-2 production when compared to anti-CD28 and anti-CD3 antibody central colocalisation stimulation pattern⁴⁵.

In agreement with a previous study¹¹¹, PKC- θ stable translocation to the synapse, beyond the initial few minutes of first contact, requires the interaction of CD28-CD80⁵⁰. This is also very similar to what has been reported for CD2-CD58 contribution in the organisation of PKC- θ in the IS⁹³; CD2 and CD28 signaling pathways have been compared and contrasted in^{108,112}. Using the T cell:SLB system it was shown that the stable CD28-mediated PKC- θ signal colocalises with

the CD28 annular ring⁵⁰; overall CD28 is proposed to enhance PKC- θ recruitment in microclusters and at the later stages of SMAC formation. In fact, the PKC- θ annular ring was shown to be CD28-dependent and finally PKC- θ was co-immunoprecipitate with CD28 from T cell lysates of previously chemically activated T cells; there is yet no evidence of direct interaction of PKC- θ with CD28⁵⁰. A negative regulator of CD28 is CTLA-4 and its mechanism relies on competing with CD28 for the same ligands¹¹³. A second negative regulator of CD28 is PD-1 which is discussed in the following sections.

1.2.5 Inducible costimulator (ICOS) in T cell activation and synapse

ICOS belongs to the family of CD28 members and the immunoglobulin superfamily. It is a homodimer protein and in contrast to CD28, it is inducibly expressed upon T cell activation and it is of similar potency and function to CD28¹¹⁴. ICOS was found to bind B7-H2 also known as ICOS-ligand (ICOS-L)^{115,116}. It was later found, that ICOS-L also binds CD28¹¹⁷.

ICOS cytoplasmic tail also recruits p85 hence it can activate PI3K/Akt pathway similarly to CD28; however minor differences in CD28 and ICOS cytoplasmic tails are suggested to explain some of their functional differences¹¹⁸. Key differences of ICOS compared to CD28, are its inability to enhance IL-2 production but ICOS instead induces production of high levels of IL-10, IL-4 and IL-5 that are only weakly induced by CD28 signalling¹¹⁴. It has been shown that a subset of T cells found in tonsils, also known as T follicular helper cells, T_{FH}, closely associated with B cells in the light zone of germinal centers, express high levels of ICOS¹¹⁴; it was also found in a small percentage of T cells from the spleen and thymus. T_{FH} are key regulators of germinal center formation and maturation; many of their functions are reviewed in¹¹⁹. ICOS is a major contributing

receptor in the generation of humoral responses especially in the absence of CD28¹²⁰. ICOS costimulation induces IL-21 production and up-regulates CD40-ligand (CD40L) on T_{FH} that is an essential molecule providing T cell help to B cells^{114,121}; ICOS-induced IL-21 is a key contributor in T_{FH} expansion¹²¹. T_{FH} express C-X-C chemokine receptor type 5 (CXCR5) a chemokine receptor that contributes to the positioning of T_{FH} within the germinal centers and express also high levels of programmed cell death gene-1, PD-1^{114,122}(discussed in the next section).

ICOS accumulates in the synapse of T:APC conjugates¹²³. The dynamics of ICOS accumulation in the synapse have been poorly investigated compared to CD28. ICOS costimulation results in increased accumulation of CD40L in the T_{FH} IS formed in contact with ligand reconstituted SLB and increased LFA-1-ICAM-1-area i.e. an LFA-1-ICAM-1 enriched pSMAC covering a larger area of the synapse¹²⁴. This ICOS-induced effect has been suggested to likely play a role in T:B cell entanglement phenomenon previously reported *in vivo*, in mice¹²⁵. This entanglement phenomenon was shown to be important for efficient surface engagement between T_{FH} and B cells that promotes differentiation of germinal center B cells to bone marrow plasma cells¹²⁵. In summary, we can see that ICOS is involved in inducing downstream signalling and transcriptional changes in T cells that also influence the T cell synapse structure and function.

1.3 Co-inhibitory receptors in T cell activation and synapse

1.3.1 CD45

A major regulator receptor in T cell activation that has both activatory and inhibitory mechanisms is the phosphatase CD45; reviewed in^{69,126}. For example,

CD45 can desphosphorylate the inhibitory domain of Lck allowing subsequent autophosphorylation of Lck that results in its activation and ability to phosphorylate adaptors and receptors downstream of TCR engagement^{69,127,128}. At the same time, CD45 can dephosphorylate the active domain of Lck and reduce its activity^{128,129}. TCR/CD3 and CD2 microclusters have both been reported to exclude CD45 due to its long extracellular domain compared to the shorted TCR and CD2 extracellular domains^{15,20,94}; an event described in the kinetic segregation model of TCR triggering. CD45 exclusion is suggested to allow enrichment of kinases in TCR and CD2 microclusters hence favouring initiation of downstream signaling cascades. At later stages of IS formation when the pSMAC and cSMAC are formed, CD45 rich areas have been detected in the outer periphery of IS in the dSMAC^{38,39,93}. CD45-mediated regulation of T cell activation and other immune cells has been and remains a field of great interest and uncovered mysteries.

1.3.2 PD-1 in T cell activation and T cell synapse

Programmed cell death gene-1, PD-1, was discovered as a gene up-regulated upon programmed cell death, apoptosis, in a T cell hybridoma¹³⁰. PD-1 is another member of the CD28 family along with ICOS. The negative regulatory function of PD-1 on immune responses and subsequently in T cells was first described in PD-1 knock out mice that developed lupus-like autoimmune diseases¹³¹. PD-1^{-/-} T cells showed significantly enhanced proliferation in response to antigen stimulation. PD-1 binds PD-L1¹³² and PD-L2¹³³; PD-L1 also binds CD80 expressed on activated T cells to transduce negative signals for T cell activation¹³⁴.

PD-1, PD-L1/2 can be found in various cell subsets of the immune system and in non immune cells; expression profiles are summarised in ¹³⁵. Naïve T cells

do not express PD-1 as PD-1 is expressed upon T cell activation¹³⁶. PD-L1/2 expression can be variable between immune cells and the same for nonhematopoietic cells, it varies between humans and mice and it can be regulated by cell activation and cytokines¹³⁵. The expression of these ligands has attracted a lot of interest especially in the field of cancer immunotherapy due to the increased expression of these in the tumour microenvironment.

PD-1 signalling can reduce TNF- α , IFN- γ and IL-2 production upon T cell activation, T cell proliferation and metabolism, in both mice and humans^{137–140}. It is suggested that PD-1 compromises T cell expansion because of its effect on IL-2 production as expanding T cells remain responsive to exogenous IL-2¹³⁹. A study in human T cells, showed that the aforementioned effects were dependent on PD-1 expression levels. For example low PD-1 levels were needed to inhibit the TCR-pMHC-mediated expansion as well production of IL-2, TNF- α but higher levels were needed to suppress IFN- γ and cytotoxicity; PD-1 could always suppress TCR-induced intracellular Ca²⁺ calcium increases in a titratable manner ¹⁴⁰. Interestingly, high levels of PD-1 have been reported in “exhausted” T cells found in chronic viral infections but also in the tumour microenvironment¹⁴¹; we discuss this further in the next section. How the titration levels used in the above study relate to the levels seen in exhausted T cells was not addressed.

PD-1 is uniformly distributed on the T cell membrane and has been shown to accumulate in IS of T:DC conjugates in a ligand dependent manner^{142,143}. PD-1 engagement recruits primarily the phosphatase, SHP-2, and to a weaker extent SHP-1, at its cytoplasmic tail which immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosin-based switch motifs (ITSM) and these are phosphorylated on the tyrosine residues upon PD-1-PD-L1/2 interaction^{143–145}. PD-1-SHP2 was shown to directly suppress TCR signaling by desphosphorylating

both the TCR- ζ chain and ZAP-70^{143,145}; in effect PD-1-SHP-2 reduced ZAP-70 recruitment to the TCR- ζ chain¹⁴⁵. High resolution imaging of mouse T cells on ICAM-1, pMHC, PD-L1 reconstituted SLBs, revealed the close localization of ligated TCR and ligated PD-1 in peripheral microclusters at the early stages of IS formation¹⁴³. Upon TCR-rich cSMAC formation, PD-1-PDL-1 clusters were organized primarily around the cSMAC and more weakly within the cSMAC, similar to a CD28 annular ring¹⁴³. It was shown *in vivo* that PD-1-PD-L1 interactions counteract the TCR-induced stop signal that accompanies IS formation¹⁴⁶. This was reproduced in a T:SLB setting by Yokosuka *et al*, in the aforementioned study¹⁴³. A stop signal, *in vitro*, translates to a stable IS with a cSMAC in the center of the T:SLB interface in a symmetrical manner that breaks and changes to a crawling T cell as the time passes. Signals interfering with this TCR-induced stop signal result in broken cSMAC symmetry and a migrating T cell at earlier time points. PD-1-PDL1 interaction is one such signal^{143,146}. PD-1-SHP-2 also targets the cytoplasmic tail of CD28¹⁴⁷ and attenuates recruitment of PKC- θ ¹⁴³ and PI3K¹⁴⁷ to the cytoplasmic tail of CD28. Importantly, both ligated PD-1 and CD28 microclusters were recruited to TCR microclusters at the early stages of IS and upon cSMAC formation were mainly in an annular ring arrangement with weak accumulation in the center of the cSMAC. Finally, Hui *et al* have suggested that PD-1 primarily targets CD28 and to a lesser extent TCR^{147,143,147}. A suggested explanation for the final stronger effect of PD-1 on CD28 rather than TCR is that, an *in vitro* reconstitution system, Lck, that phosphorylates both CD28 and TCR- ζ , has a six-fold higher catalytic rate for TCR- ζ compared to CD28 for phosphorylation, hence, making CD28 more sensitive to PD-1-SHP1 mediated inhibition¹⁴⁷.

1.4 T cell Costimulation in Disease

The immune system has evolved to maintain host homeostasis by eliminating infectious pathogens, cancerous cells and clearing cell debris upon cell death. When it fails to do so, either directly or indirectly, diseases such as chronic viral and parasitic infections arise that could lead to host death. Cancer is another disease often strongly associated with a failure of the immune system recognize and efficiently clear cancer. On the other side of the coin, the immune system can go on overdrive and mount an exacerbated response that might lead to development of allergies or autoimmune diseases such rheumatoid arthritis, Crohn's disease and multiple sclerosis. T cells and T cell costimulation have been implicated in both cancer and autoimmune disease. In the following sections, we will, briefly, discuss the costimulatory receptor phenotype of T cells in disease, with a focus in cancer, and how this is or might be implicated in disease pathogenesis.

1.4.1 CD28 and PD-1 in Cancer

Costimulatory receptors such as CD2, CD28 and ICOS in cancer, especially in the tumour microenvironment, have been overlooked as the field has primarily focussed in the expression and blockade of coinhibitory T cell receptors like PD-1 and CTLA-4. Two studies have reported an increased percentage of CD28-CD4+ alone or both CD28-CD4+ and CD28-CD8+ T cells in peripheral blood of cervical cancer patients compared to healthy controls^{148,149}. A study in B cell leukaemia and a study in primary breast cancer patients have reported an increased percentage of CD28-CD4+ and CD28-CD8+ T cell in the periphery^{150,151}. None of the above studies compared the levels of CD28 expression within the CD28+ T

cell compartment. CD28⁻ T cells have been generally considered as antigen experienced T cells and this subset increases with age especially within the CD8⁺ T cell compartment. CD28⁻ T cells have reduced proliferation capacity upon antigen stimulation but they show enhanced cytotoxicity and regulatory functions¹⁵². Leitner *et al* have reported that in CD28-CD8⁺ T cells have high levels of CD2 and that CD2 costimulation is their main costimulatory pathway in boosting cytokine production, proliferation and effector functions when compared to other costimulatory receptors like 4-1BB, ICOS, CD40L¹⁵³. CD28-CD8⁺ T cells belong to the memory T cell population. It is thought that CD28 expression in CD8⁺ T cells is compromised over time due to repetitive or chronic viral infections. Loss of CD28 and CD27 are phenotypic features of T cell senescence, too^{154,155}. It has been suggested that T cell senescence has a potential role in cancer development but how exactly the link is made has been poorly explained mainly because it is based on a few assumptions and theoretical associations and the lack of studies in addressing this exact link¹⁵⁶.

CD28 expression has attracted even more interest these days after the recent study, by Kamphorst *et al*. This study reported the need for CD28 stimulation in “exhausted” T cells to exit their exhaustion phenotype after PD-1/PD-L1 checkpoint blockade and clear a chronic viral infection or cancer¹⁵⁷. ‘Exhausted’ T cells are described phenotypically as CD127^{LOW}PD-1^{HI} T cells, have sustained expression of other coinhibitory molecules like CTLA-4 and TIM-3, compromised TNF- α , IL-2 and IFN- γ production as well as reduced cytotoxicity. ‘Exhausted’ T cells can also show loss of CD28, in humans, similar to senescent T cells¹⁵⁸. Senescent T cells and exhausted T cell have phenotypic similarities but also differences including the mechanisms by which they are generated; often the terms are used interchangeable but the extend of similarity and differences is not

clear¹⁵⁸. A potential main difference is that senescent T cells have reduced intrinsic proliferative capacity while 'exhausted' T cells can proliferate normally provided they don't receive coinhibitory signalling via the array of coinhibitory receptors they express upon TCR/costimulatory-mediated activation. PD-1 and PD-L1 blockade interventions and their clinical outcome in cancer immunotherapy underscore the role and significance of PD-1-PD-L1 in inhibiting T cell anti-tumour responses^{159–161}. PD-L1 expression has been described on several types of tumours^{162,163}; PD-L1/2 can be expressed by both tumour cells and immune populations found in the tumour microenvironment such as APCs and T cells themselves^{135,162}.

In summary, dysregulated CD28 and/or PD-1 costimulation renders T cell inefficient in tumour clearance. Both receptors have been looked into the context of immunotherapy with PD-1/PD-L1 blockade being nowadays used extensively in the clinic¹⁶⁴ while CD28 agonists are on halt due to the adverse effects of the first human clinical trial¹⁶⁵. Only a percentage of cancer patients respond to PD-1/PD-L1 blockade and some responders relapse showing a non-durable response to this checkpoint blockade. For these reasons, combination therapies are trialled where more than one inhibitory pathway is blocked. There is yet significant room for improvement in the current immunotherapy treatments. A deeper understanding of the tumour microenvironment investigated by multiple studies these days will probably allow the design of more efficacious treatments.

1.4.2 CD2 in Cancer

The significant contribution of CD2-CD58/CD48 interactions in cytotoxic T lymphocyte (CTL) cytotoxicity against cancer cell lines such as melanoma, was revealed in the 90s^{60,166}, and also recently highlighted by a study that utilised the

CRISPR-CAS9 system to screen for the essential genes for CTL cytotoxicity against tumours; CD58, the ligand of CD2 was amongst the top ten essential genes¹⁶⁷. Studies looking at the expression of CD58 by tumour cells have revealed the recently recognized extreme heterogeneity that can be exhibited by tumours. Melanoma cells lines and a renal cell carcinoma cell line, generated *in vitro*, from patients, expressed CD58 while a study on adenocarcinoma revealed that 5% of the tumours lack CD58¹⁶⁸. A limitation of the, *in vitro*, generated cell lines is that the presence/absence of a surface molecule as well as the levels of expression might be regulated by the *in vitro* culture system hence not represent the tumour microenvironment state of the tumour cells. A study reported CD58 mutations in Hodgkin lymphoma cell lines and in two of the three lines tested had low to almost null CD58 expression¹⁶⁹. In primary lymphoma cells, loss of CD58 was only seen in a small fraction, 11%, of patients whose disease relapsed; tumour cells from primary tissue of non-relapse patients were positive for CD58 expression. Similarly, Challa-Malladi *et al*, reported mutations in the CD58 gene of diffuse large B-cell lymphoma cells resulting in loss of CD58 expression and immune evasion¹⁷⁰. Interestingly, this study also reported similar mutations in the β 2-microglobulin and hence loss of human leukocyte antigen (HLA) -1, expression in these patients. Their results suggested a co-selection of CD58 and β 2-microglobulin mutations as a way of escape of cancer cells from immunosurveillance¹⁷⁰. In conclusion, loss of CD58 expression has been reported mainly in blood malignancies.

The presence and expression levels of CD2 in the tumour microenvironment have been poorly investigated. *In vitro* cultures of TILs from seven melanoma patients, showed that TILs expressed CD2, including CD3, CD58 and LFA-1¹⁶⁶. To our knowledge, decreased expression of CD2 has only

been reported in three case reports of patients with CD2 immunodeficiency^{171–173}. One patient presented with Epstein-Barr virus-associated lymphoproliferative disorder¹⁷¹, the other with a systemic *Rhodococcus* infection due to severe lymphopenia and similarly the third patient with disseminated *Mycobacteria avium* infection along with the presence of anti-DNA and anti-cardiolipin antibodies associated with the patient's venous thrombosis recurrent events¹⁷³. All three case reports suggested that the results provide an *in vivo* evidence of the role of CD2 in normal T cell function. A complication of those reports is that the T cells of those patients also lacked either the TCR α/β complex¹⁷² or CD4/CD8 coreceptors^{171,173}, too.

Finally, a renewed interest has emerged in the role of CD2 in T cells in disease after the study by McKinney *et al* proposing that CD2 costimulation contributes to the prevention of an 'exhaustion' phenotype in CD8+ T cells¹⁷⁴. Chronic, *in vitro*, stimulation of T cells with anti-CD3/CD28 antibody activating beads resulted in 'exhaustion' of T cells, with high levels of PD-1 and low levels of CD127. Addition of CD2 costimulation to the bead activation cocktail prevented development of an 'exhausted' T cell phenotype, though, PD-1 signaling counteracted this CD2-induced effect. In addition, they reported an association of the CD2-costimulation gene signature in CD4+ T cells with autoimmunity and a better clearance of pathogens due to reduced CD8 T cell exhaustion gene signature. These findings suggested that both the CD2 and PD-1 pathways could be harnessed in a disease setting to enhance or dampen T cell function, accordingly.

1.5 Total Internal Reflection Microscopy (TIRFM) and confocal airyscan microscopy

TIRFM¹⁷⁵ allows imaging at increased axial resolution, ~300nm, and also enables restriction of the illuminated area in the z-axis that allows exclusion of background signals and thus increased contrast. Both live and fixed single molecule TIRFM can be performed. A schematic of our main tool used in our study i.e. the use of supported lipid bilayers (SLBs) on a coverslip, followed by incubation of T cells on these and imaging of the interface between the T cell and the lipid bilayer where receptor-ligand interactions take place is shown below.

Airyscan microscopy is a method develop recently that allows increased spatial resolution and signal-to-noise ratio compared to conventional confocal microscopy without the need for increased laser power¹⁷⁶; it involves the use of multiple detectors to detect light coming from the object from different angles, integration of them followed by post-acquisition processing.

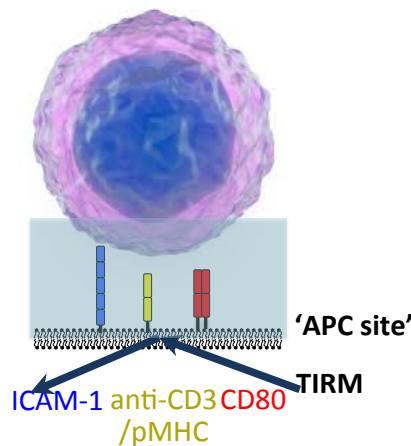


Figure 1.3 TIRFM and SLB setup

A diagram of the set up of our main tool used in this study. TIRFM allows increased axial resolution (~300nm) and the evanescent wave created (light blue and arrows and shade) allows imaging in a very short z-plane (~100nm). T cells are incubated on supported lipid bilayers presenting ligands normally presented by antigen presenting cells (APC) such as ICAM-1, CD80 and peptide MHC (pMHC) or anti-CD3 Fab as a polyclonal stimulus. Receptors on T cells are engaged by these ligands, followed by protein re-organization in clusters in the T:SLB interface resulting in the formation of the IS

2 Chapter 2. Materials and Methods

2.1 Preparation of supported lipid bilayers

2.1.1 Preparation of supported lipid bilayers (SLBs) in 6 well IBIDI chambers.

Nexterion coverslip glass D (Schott Jena, 1098576), were incubated for 20 min covered in Piranha solution prepared by mixing in hydrogen peroxide and sulphuric acid at a final 30% and 70% final concentration, respectively. Coverslips were washed in plenty of running milliQ water. Coverslips were dried using a airpump and cleaned further with 5 min incubation in a plasma cleaner. Synthetic lipids previously prepared by our research assistant were used to prepare the mixing required for the experiment. We are using three type of lipids: 18:1 DGS-NTA(Ni), NTA-lipids, (Stratech, Avanti Polar Lipids, 790404C-AVL) that bind HIS-tag proteins, CapBio 18:1 Biotinyl Cap PE (Stratech, Avanti Polar Lipids, 870282C-AVL), CapBio-lipids, and 18:1 (Δ^9 -Cis) PC, DOPC-lipids, (Straterch, Avanti Polar Lipids, 850375C-AVL). Lipids were prepared in stock concentrations of 15% (4mM) NTA-lipids, 4mM CapBio-lipids and 4mM DOPC-lipids. For experiments NTA-lipids were used a final concentration of 7.5% and CapBio-lipids were used at a concentration previously determined to give 30 molecules/ μm^2 of monobiotinylated anti-CD3 Fab or monobiotinylated peptide MHC.

After plasma cleaning the coveslips, they were firmly attached on 6 well, sticky-slides VI 0.4 (IBIDI, 80608) and made sure all air is removed from interface. 30 μl of lipid mixture was added to each well and incubated for 20 min. This was followed by a wash step with flow cell buffer, 1 ml per well. The flow cell buffer was previously prepared as follows HEPES-buffered saline containing human serum albumin, HSA, 50ml of 10x HBS [200mM HEPES, pH 7.2, 1.37 M NaCl, 50 mM KCl, 7mM Na₂HPO₄, 60mM D-glucose], 20 ml of 25x HSA, 500 μl 1M CaCl₂, 1ml

MgCl₂ and distilled H₂O to bring a total volume to 500ml and then filtered with 0.22µm filter also allowed to remove any air bubbles. From this point onwards, lipids on this coverslip chambers will be referred to as supported planar lipid bilayers (SLBs).

SLBs were blocked by using approximately 120µl of 5% casein (previously prepared by our research assistant), supplemented with NiSO₄ at a final concentration of 100µM for each well. After this 20 min incubation step, each well was washed as described above. SLBs were then incubated with 150µl/well of 20µg/ml unlabeled or labeled streptavidin (depending on the experiment) when biotinylated ligands were to be used; in the case of no such ligands we went straight to the next step. Excess streptavidin was washed as initial washing step. HIS-tag and biotinylated ligands (see table of reagents) were mixed and prepared in flow cell buffer at concentrations required to give the final density of ligands required (previously determined in titration experiments with lipid bilayers on beads described in next section). 100µl of protein mixture was added per well and incubated for 20 min. Finally SLBs were washed and incubated for at least 15 min at 37°C. All incubation experiments were performed at room temperature.

In the case of live imaging experiments, SLBs were transferred to a pre-heated incubator on top of the TIRF microscope and cells were added in the well at a density of 0.5x10⁶/150µl per well. In the case of fixed experiments, SLBs were transferred on a 37°C heating block and cells were added at 0.5x10⁶/150µl per well, incubated for a specific time depending on the experiment and then fixed with pre-warmed at 37°C, 2% paraformaldehyde prepared from 4% % paraformaldehyde and 2x PHEM buffer [18.14g PIPES, 6.5g HEPES, 3.8g EGTA, 0.99g MgSO₄, pH 7.0]. Fixation was performed while the SLB-IBIDI chamber was on the 37°C heating block. Fixation agent was washed with flow cell buffer. In

some experiments, intracellular staining for PKC- θ , or phosphor proteins such as pPLCy1 were performed as follows; 150 μ l of 0.1% TRITON-X100 were prepared in flow cell buffer were added per well for 2 min to permeabilise the cells. This was followed by a washing step with flow cell buffer. 150 μ l of 5% casein (supplemented with serum from the host in which the secondary antibody was raised) was added per well and incubated at room temperature for 1hr. This was washed and 150 μ l of primary antibody prepared in same blocking buffer as above were added per well and incubated at 4°C overnight. This was washed with flow cell buffer the next day and 150 μ l of secondary antibody were added and incubated for 45-60 min before washed off with flow cell buffer. Cells on SLB were either immediately imaged with TIRF or fixed with same fixation agent as above for 2-4 min at room temperature before washing and storing at 4°C. The concentration and details of primary and secondary antibodies can be found in Table of Reagents.

2.1.2 Determination of ligand densities on supported lipid bilayers

Silica microspheres (SS05003/SS05N) of size (4.63 or 5.04 μ m diameter size; year batch based), (Bangs Laboratories) were used for SLB preparation. In order to simulate the surface area used in our SLBs prepared in 6-well chamber IBIDI slides, we used the number of beads that gives us the same surface area as the surface area covered by the SLBs prepared as described in 1.1.1. (well surface area is approximately 68.4x10⁶ μ m²/well. So for one IBIDI well and subsequent protein reconstitution, 1 or 0.9 μ l of silica beads was needed; a number of proteins and/or titrations experiments were performed so beads were batch processed for most steps until the point of ligand-reconstitution. Silica bead vial was vortexed briefly to uniformly resuspend silica beads. The final calculated volume of beads

was transferred to a clean 1.5ml Eppendorf tube and 1ml of PBS was added to wash the beads by spinning beads down at 1500rpm for 2 min. This was repeated twice. In the meantime the required mixture of lipids (DOPC+/- NTA and/or Cap-bio) was used as described in 1.1.1; 3 μ l of lipid mixture was per one bead conditions for example 30 μ l of lipids for 10 μ l of initial volume silica beads. Silica beads were incubated with lipids at room temperature for 20 min; they were resuspended every 5 min as silica bead settle at the bottom. Silica beads were washed, twice, with addition of 1ml of flow cell buffer (see 1.1.1) and spinning at 1500 rpm for 2 min to remove supernatant. Silica beads were blocked for 20 min, at room temperature with casein/10 μ M NiSO₄ (50 μ l/bead condition); beads were transferred to a 15ml Falcon tube depending on volume requirements and resuspend as described above to prevent settling of beads to bottom. Silica beads were spun down and washed, twice, by addition of flow cell buffer of equal volume to the final volume of casein/10 μ M NiSO₄ used in previous step. If titration of biotinylated proteins was used, cap-bio lipids were titrated instead of the protein itself, hence silica bead with different lipid mixtures were prepared and processed separately. If biotinylated proteins were titrated, streptavidin, 50 μ l/bead condition (20 μ g/ml, saturating concentrations for our SLB system) was added to the silica beads for 10 min at room temperature. Silica beads were washed as previous washing step.

Silica beads were resuspended in flow cell buffer, 200 μ l/bead condition, and 200 μ l of bead suspension were added per well of a 96-well V-bottom plate. Plate was spun at 1500rpm for 2 min, room temperature and supernatant removed by flicking the plate. If HIS-tag proteins were titrated, 100 μ l of a protein (serial dilution protein concentrations were used with 5 or 10 μ g/ml protein used as a starting point). If biotinylated proteins were tested, for example, biotinylated anti-

CD3 Fab, then 5µg/ml were used. Proteins were incubated with silica beads for 20 min at room temperature on a plate shaker (300-500 rpm) to avoid beads from settling to bottom of well.. Silica beads were washed, twice, with addition of 200µl of flow cell buffer per well. If proteins were already fluorescently-tagged with a known dye-protein ratio then the SLB-covered beads were resuspended in 200µl of flow cell buffer and run on a flow cytometry machine (LSII or FORTRESSA) along with standard beads, Quantum Alexa Fluor 488 MESF or Quantum Alexa Fluor 647 MESF (Bangs Laboratories, 488, 647). If unlabelled proteins were tested, silica beads were stained with 5µg/ml antibodies (100µl/bead condition i.e. 100µl/well) against the ligand reconstituted on the SLB-coated beads. Silica beads with no protein or DOPC-covered silica beads were prepared as controls, too. Bead standards were used to translate the mean fluorescence intensity of protein-coated SLB-silica beads to number of dye per bead. Then using the dye-protein ratio of the ligand tested, we calculated the mean number of ligands per beads. The surface area of a bead was known hence the density of ligands was calculated by dividing the mean number of ligands per bead by the surface area of the bead. If ligands were detected with anti-ligand mAb, then, we used either the dye-protein ratio of the mAb and assumed a 1:2 ratio for mAb:ligand to determine the mean number of ligand per bead. If the dye-protein ratio of antibody was not known, the Quantum Simply Cellular anti-mouse IgG kit was used to make a calibration standard curve (Bangs Laboratories, 815). Please see also section 2.2.1 with regards to the use of this kit.

2.1.3 Preparation of supported lipid bilayers (SLBs) in 96 well plate.

A Brooks 96-well flat bottom plate (Brooks, MGB096-1-2-LG-L) was used and 100µl of 1% Hellmanex III, 50% Isopropanol were added per well (carried by our

research assistant) and incubated overnight. Wells were then rinsed with 5ml of water using an automatic plate washer and plates dried by centrifugation at 4000rpm for 1 min. 100µl of 3M sodium hydroxide were added per well for 1hr at room temperature. This was washed as per above washing step and wells dried as above. Then 50µl of lipid mixture was prepared as in section and then all the steps that followed as the same as in section 2.1.1. The only difference is that all reagents from this point onwards were prepared at 2-fold higher concentrations as in this plate format a significant 100µl residual volume is left in the well to avoid SLB oxidation.

2.2 Quantification of proteins using antibody capture beads

(Bangs)

Quantum Simply Cellular anti-Mouse IgG was used to determine the level of specific proteins such as CD2, CD28, PD-1 etc on cells or supported lipid bilayers (Bangs, 815). The protocol provided by the company was followed. In brief, A drop of beads or 10µl of bead suspension was added in a 1.5ml eppendorf tube followed by addition of 50µl of flow cytometry staining buffer (PBS/5% bovine serum albumin, BSA). The saturating concentration of antibodies was first determined by titration of antibodies. One batch (Lot Number 13039), of this kit was used for the entire of this study. The saturating concentration for this LOT was a final concentration of monoclonal antibody (mAb) at 4µg/ml. The mAb was added, the tube was briefly mixed by tapping and then incubated for 30 min as per protocol. Beads were finally washed three times by addition of 1ml of PBS/5%BSA. Beads were finally resuspended in 200µl of PBS/5%BSA ready to be run on a flow cytometry machine. The calculation were performed as per company's instructions.

2.2.1 Re-calibration of Quantum Simply Cellular anti-Mouse IgG

Some of our data suggested that the aforementioned calibration beads might be overestimating the antibody binding capacity of the beads and hence this resulted in overestimation of the proteins in T cells tested. After communicating with Bangs company, we were informed that these calibration beads were calibrated using polyclonal mouse IgG. They assumed that monoclonal antibodies of any IgG isotype would be with equal capacity; this was not confirmed experimentally. We had the opposite hypothesis so we tested this. We had commercially available Alexa Fluor 647 conjugated mAbs of different isotypes with know dye-protein ratio. We used this along with AF647 MESF beads (Bangs), also used in 2.1.2. The MESF beads were used to generate a standard curve for number of dye molecules and the corresponding mean fluorescent intensity they produce on a flow cytometry machine. This allowed us to determine the mean number of dye molecules on each bead population of the Quantum Simply Cellular anti-Mouse IgG kit. Knowing the mean number of dye molecules on each bead population and the dye to protein ratio of each mAb allowed us to calculate the number of antibodies captures by each bead population hence determine the antibody binding capacity of each bead population for the different IgG isotypes tested.

The calculations are shown below in Figure 2.1. We have determined the average correction factor by taking an average of all the Expected MFI:Detected MFI and used this to re calibrate the ABC values of each bead population provided.

A

Antibodies	Clone	Isotype	Cat No.	F/P
anti-human 4-1BB	4B4-1	Mouse IgG1, κ	B226372	5.2
anti-human OX40	Ber-ACT35 (ACT35)	Mouse IgG1, κ	B214966	5
anti-human CD107a (LAMP-1)	H4A3	Mouse IgG1, κ	B220036	4
anti-human HLA-A/B/C	w6/32	Mouse IgG2a, κ	B214282	4.5
anti-human CD32	FUN-2	Mouse IgG2b, κ	B194956	4.9

B

mAb	bead population	Expected No of dye molecules based on given ABC values	Expected MFI	Detected MFI	Expected MFI/ Detected MFI
anti-41BB	1	63736.4	3322.546004	527	6.30464
	2	378274	19719.23057	3514	5.61162
	3	1473472	76811.34338	12966	5.92406
	4	4609368.4	240284.0223	37855	6.34748
anti-OX40	1	61285	3194.755773	574.8	5.55803
	2	363725	18960.79862	3568.8	5.31293
	3	1416800	73857.06094	12894.8	5.72766
	4	4432085	231042.3291	37518.8	6.15804
anti-CD107a	1	49028	2555.804619	553	4.62171
	2	290980	15168.6389	3122	4.85863
	3	1133440	59085.64875	11207	5.27221
	4	3545668	184833.8633	31913	5.7918
anti-HLA-A/B/C	1	55156.5	2875.280196	475	6.05322
	2	327352.5	17064.71876	2662	6.41049
	3	1275120	66471.35485	9802	6.78141
	4	3988876.5	207938.0962	28402	7.32125
anti-CD32	1	60059.3	3130.860658	608	5.14944
	2	356450.5	18581.58265	3440	5.40162
	3	1388464	72379.91972	12579	5.75403

Figure 2.1 Re calibration of the ABC of antibody capture beads

A) The table shows the information on the antibodies tested on the antibody capture beads. All most all of the antibodies used in this study where of IgG1 isotype. B) The table shows the expected values for the number of dye molecules based on given the antibody binding capacities (ABC) from the company. This was calculated using a calibration curve of AF647 MESF standard beads from Bangs.

2.3 Isolation and culture of human mononuclear cells

2.3.1 Isolation of total T cell and T cell subpopulations

Human peripheral blood leukocyte cones were obtained from the John Radcliffe Hospital Blood and Transplant Department. RosetteSep™ Human CD4+ T Cell Enrichment Cocktail (Stem Cell, 15062) or RosetteSep™ Human CD8+ T Cell Enrichment Cocktail (Stem Cell, 15063) were used as per company's protocol for isolating the human total CD4+ or CD8+ T cells from leukocyte cones; the only deviation was the use of 1.6ml of kit/ leukocyte cone was used). For downstream isolation of naïve or memory T cell populations the following kits were used and the company's protocol was used. The kits used are: EasySep™ Human Naïve CD4+ T Cell Isolation Kit (Stem Cell 19555), EasySep Human naïve CD8 T cell enrichment kit (Stem Cell, 19158), EasySep Human naïve CD8 T cell isolation kit (Stem Cell, 19258), EasySep™ Human Memory CD4+ T Cell Enrichment Kit (Stem Cell, 19157), EasySep Human Memory CD8+ T cell enrichment kit (Stem Cell, 19159). Examples of the purities achieved are shown below in Figure 2.2. Only isolated T cells with >92% purity for naïve or memory T cells were used in downstream experiments.

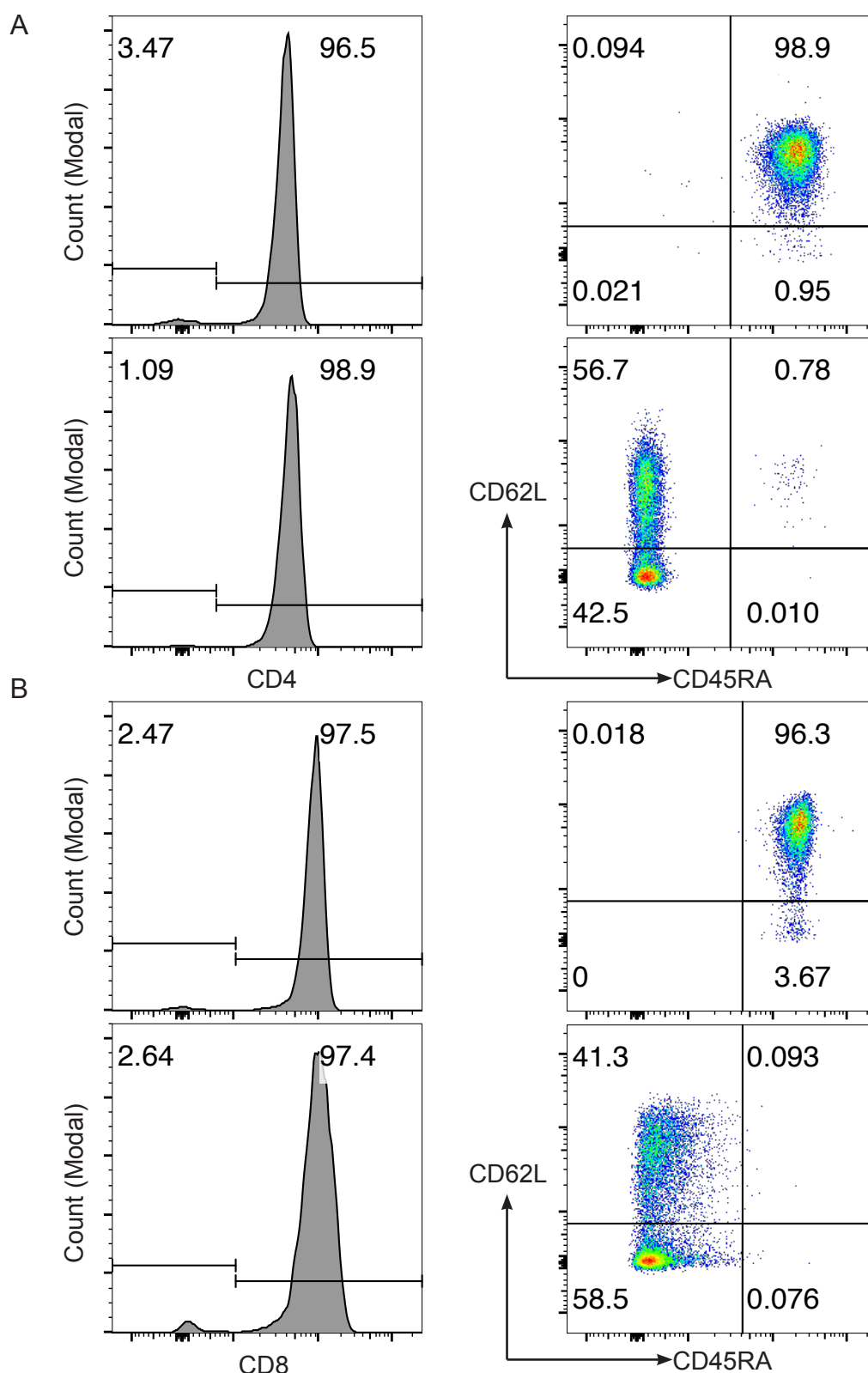


Figure 2.2 Isolation of human naïve and memory CD4+/CD8+ T cells from leukocyte cones.

A) Representative plots of purity checks for naïve (top) or memory (bottom) CD4+ T cells isolated from human peripheral blood leukocytes cones. Cells were first gated on live FSC-SSC, single cells and then CD4 positive, followed by CD62L and CD45RA.

B) Representative plots of purity checks for naïve (top) or memory (bottom) CD8+ T cells isolated from human peripheral blood leukocytes cones. Cells were first gated on live FSC-SSC, single cells and then CD4 positive, followed by CD62L and CD45RA.

2.3.2 Isolation of CXCR5^{HI} and CXCR5⁻ T cells from tonsils

Human Tonsils were cut with 2-4mm small pieces with scalper while being soaked in a petri dish with media (Hanks' Balanced Salt solution with phenol red, supplemented with 2% fetal bovine serum, FBS, 5% Penicillin-Streptomycin, Penstrep (Gibco, 15140122), 5µg/ml kanamycin, 1x antibiotic-antimycotic (Gibco, 15240062). Before cutting in small pieces, tonsils were washed in above media to remove any blood remains from the surgery. Small pieces of tonsil tissue were smashed over a 70µm cell strainer on top of a 50ml Falcon tube and washed twice with media to release as many mononuclear cells as possible. When processing two human tonsils, the isolated cells from previous steps were diluted in 200-300 ml of media and layer over Ficoll (VWR international, 17-1440-03); 15ml of Ficoll and 30ml of cell suspension. The cells were centrifuged at 1200g for 20 min, with no acceleration and no brake. The mononuclear cell layer was recovered with a Pasteur pipette and washed twice with media. The recovered cells were enriched for CD4⁺ T cells using the Easysep Human CD4⁺ T cell Enrichment Kit (Stem Cell, 19052) as per company's protocol. Following this enrichment, cells were stained for CXCR5 and because this was a collaborative process, cells were also stained for CD126 and CD25 for sorting Tregs for the purpose of a colleague's project. For this study CXCR5^{HI} and CXCR5⁻ cells were sorted. An example of our sorting gating is shown below in Figure 2.3.

A

BD FACSDiva 8.0.1

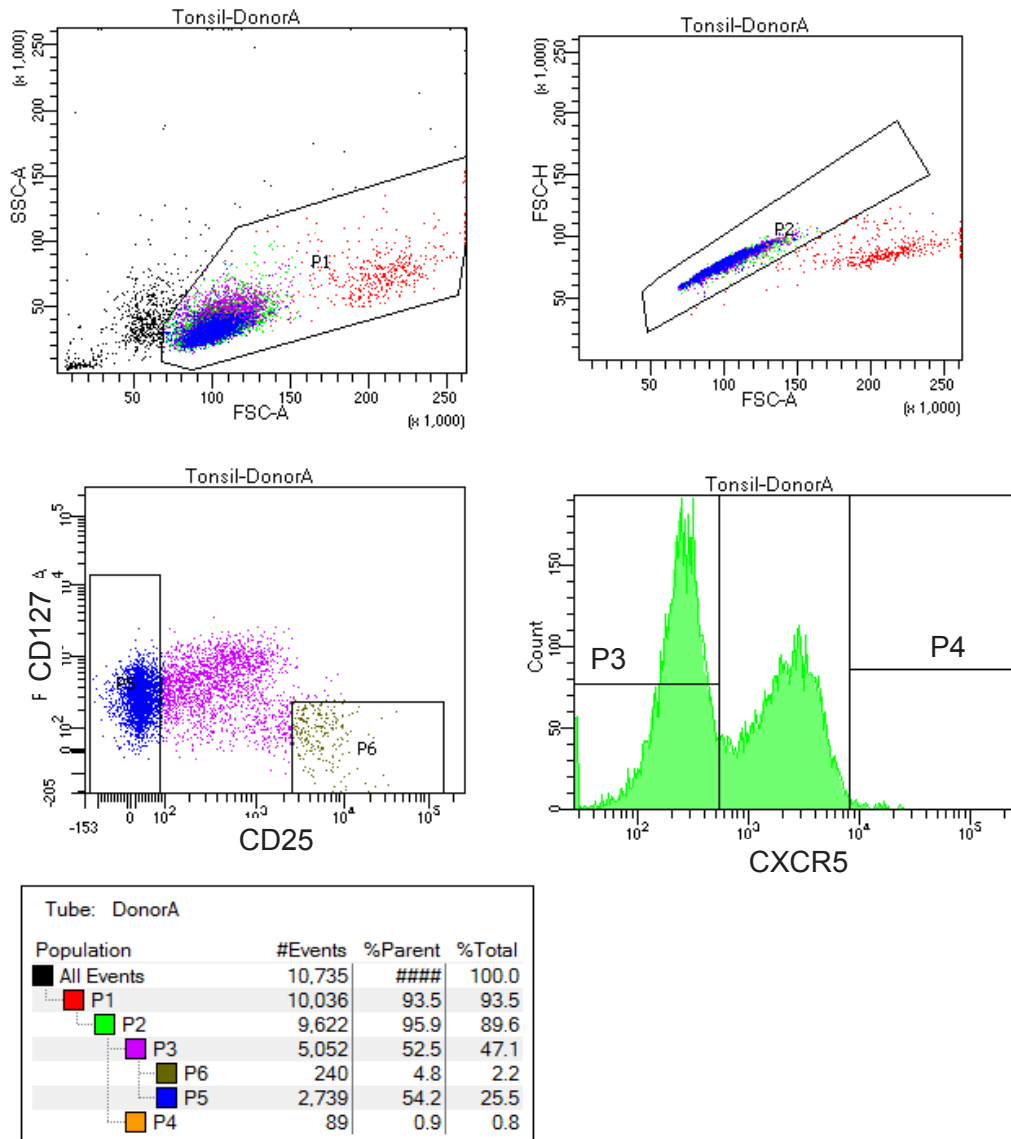


Figure 2.3 Sorting human T cell populations from lymphoid organs such as tonsils.
 A) Shown here is an example of the sorting strategy used to isolated CXCR5^{hi} (P4 gate) and CXCR5 negative CD4⁺ T cells (P3 gate) from previously enriched with magnetic sorting for CD4⁺ T cells. The magnetic sorting purities were >98% CD4⁺ T cells (not shown here).

2.3.3 Culturing isolated human T cells in resting conditions.

Isolated human T cells were cultured in resting conditions, with no IL-2, at 37°C, 5% CO₂, in RPMI 1640 (Roswell Park Memorial Institute) medium (Life Technologies UK, 31870074) supplemented with 10% FBS, 5% Penicillin-Streptomycin, Penstrep (Gibco, 15140122), 1x MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific, 11140035), 10mM HEPES (Life Technologies, UK, 15630056), 1mM Sodium Pyruvate (Thermo Fisher Scientific, 11360070). T cells were cultured at 2x10⁶ cells/ml. Human T cells isolated from tonsils were used within a maximum of 24 hr post isolation and human T cells isolated from leukocytes cones were kept in culture resting conditions up to 3 days. No T cells, in resting conditions, were used in experiments beyond day 3 of culture.

2.3.4 Activation of human T cells with activation beads to generate human T cell blasts.

Human T cells isolated as described above were used to generate T cells blasts using the Dynabeads Human T-Activator CD3/CD28 for T cell expansion and Activation kit (Life Technologies, 1132D) as per company's protocol. In brief, T cells were prepared at 1x10⁶/ml in the media described in 2.3.3, supplemented with recombinant human interleukin-2, IL-2, at 30U/ml (Peprotech, 200-02) and cultured in 24-well plates, at 1-2ml/well, for two days at 37°C, 5% CO₂ incubator. On Day 3, magnetic activation beads were removed with a magnet, as per company's protocol, T cells were counted and resuspended at 1x10⁶/ml, supplemented with new IL-2, at 30U/ml. On day 4 and/or day 5 cells were counted and when cell densities were beyond 2x10⁶/ml, cells were resuspended again at 1x10⁶/ml, supplemented with new IL-2, at 30U/ml. T cells blasts were used in experiments on Days 6-7. No IL-2 was supplemented in media the day before an experiment.

2.3.5 Generation of *in vitro* exhausted T cells

Total or Naïve CD4⁺ or CD8⁺ T cells were prepared at 1x10⁶/ml in the media described in 2.3.3, supplemented with recombinant human interleukin-2, IL-2, at 30U/ml (Peprotech, 200-02) and cultured in 24-well plates, at 1-2ml/well, for two days at 37°C, 5% CO₂ incubator. Cell numbers were monitored and so were media colour changes by resuspending cells on Day 2/3. Dilute cells again down to 1x10⁶/ml and supplemented with IL-2, 30U/ml and new activation beads (Life Technologies, 1132D) by calculating how many more beads to add to keep a ratio of beads to cell, 1:1, plus 50% more beads, to account for any beads 'inactivated' as they could be probably covered by cell membrane debris, microvesicles and exosomes generated by dividing T cells. This step was repeated on Day 4 cells were split on Day 2 or on Day 5 if cells were split on Day 3. On the day of the SLB experiment, activation beads (magnetic) were removed in the morning to allow some rest from activation stimuli and used on SLBs in the afternoon. Cells were used in SLB experiments on Day 5 or Day 6.

2.3.6 Activation of human T cells with APC

1G4 TCR transfected T cells (see 2.4.1) were cultured with peptide or no peptide loaded APC, CF996 B cell line, at 1:1 ratio, 30 000 T cells and 30 000 APCs, in media described in 2.3.3; no IL-2 was supplemented in media. Cells were co-cultured in a final volume of 200µl of media in 96-well U-bottom plate in a 37°C, 5% CO₂ incubator for 24 hr before staining for cell death and surface markers. In the case of CD58 blocking experiments, APCs were incubated for 20 min, at room temperature with either 20µg/ml anti-CD58 (TS2/9 clone) or isotype control (monoclonal mouse IgG1). Then, T cells were added and plate was transferred to the culture incubator; it must be noted that addition of T cells results in dilution of

added antibodies to 10µg/ml. For surface staining of activation markers etc see section 2.3.10.

2.3.7 Labelling and loading of APCs with peptides

APCs in resting media (see 2.3.3) were pelleted and resuspended at 2×10^6 /ml of PBS (Thermofisher, 10010023/0.5µM cell trace violet (CTV, C34571) and incubated at 37°C, 5% CO₂ for 10-15 min. 10% FBS was added to quench dye and cells washed twice in above media. APCs were resuspended in media at final cell density of 2×10^6 /ml, supplemented with the final concentration of peptide. Peptide was prepared in 2x concentration and so where the APCs and then mixed together to give the final concentration of peptide and cell density.

2.3.8 Cell conjugate experiments and staining for CD2 and LFA-1.

APCs were prepared as described in section 2.3.7. For cell conjugate experiments used to visualise the T cell synapse, APCs were loaded with 1µM NY-ESO-9V peptide. APCs were washed once and resuspended at $1-2 \times 10^6$ cells/ml. 1G4 TCR transfected T cells at the same cell density (see section 2.4.1) were added the APCs; 100-200µl of each cell type was used and transferred to a sterile 5ml round bottom tube. Cells were briefly mixed and pelleted to the bottom of the tube at 1000 rpm for 1 min, at room temperature. All above steps were prepared at room temperature but in previously pre-warmed, at 37°C, culture media (see section 2.3.3. Tubes were immediately transferred to a 37°C water bath for a 20-30 min incubation.

Cell pellets were then smoothly dislodged with a Pasteur pipette and transferred to a poly-L-lysine coated µ-slide 8 well glass bottom chambers.

Chambers were briefly spun at 800 rpm for 1 min at room temperature, transferred to a 37°C incubator for 2 min to allow cells to adhere to the bottom of the wells. An equal volume, to the cell volume added per well, of pre-warmed, at 37°C, 4% paraformaldehyde was added to the cells and incubated at 37°C for 10 min. Cells were washed with PBS/0.5%BSA. Cells were permeabilised for 4-5min at room temperature with 0.1% Triton-X100 prepared in PBS/0.5%BSA. Cells were washed with PBS/0.5%BSA and blocked with PBS/5%BSA/5% donkey serum for 1hr at room temperature. Cells were washed with PBS/0.5%BSA and incubated with unlabeled, non-blocking anti-CD2 (CD2.1 clone)^{177,178} prepared at 10µg/ml in PBS/0.5%BSA to be used at a final concentration of 10µg/ml. There is always some residual buffer left in the well hence reagents are prepared at 2x to account for the final dilution in the well. This anti-CD2 primary antibody was incubated with cells overnight at 4°C. Cells were washed and incubated in secondary antibody, AF647 conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories, 715-586-150) for 45 min at room temperature. Cells were washed and fixed for 5 min with a final concentration of 2% PFA. Cells were washed and incubated for 1hr, at room temperature, with FITC-conjugated anti-CD11a (TS2/4) at a final concentration of 5µg/ml prepared in PBS/0.5%BSA. Cells were washed and fixed for 5 min with a final concentration of 2% PFA. Finally, cells were loaded with Vectashield antifade mounting medium (H-1000) to minimize the movement of cell conjugates during imaging.

2.3.9 Isolation of monocytes and generation of monocyte-derived DCs

Monocytes were isolated from peripheral blood leukocyte cones using the Rosette Human Monocyte Enrichment Cocktail kit (Stem Cell, 15608). In brief, 400µl of the kit were added to 0.5-1ml of leukocyte cone blood, mixed and incubated for 20min at room temperature followed by addition 700µl of PBS/2%FBS to dilute the blood. Blood was layered on a 3ml Ficoll layer and centrifuged at room temperature, 1200g for 20 min with no acceleration and no brake. The cell layer between ficoll and media was recovered and washed twice before resuspending in culture media (see section 2.3.3) and culturing in 2ml /well in 12 well plates for at least 4hr at 37°C to allow for adhesion of monocytes. Culture supernatant was resuspended and removed. Cells were washed twice with fresh media before adding culture media supplemented with 0.1µg/ml , granulocyte-monocyte colony stimulating factor, GM-CSF (Immunotools, 11343125), and 0.05µg/ml IL-4 (Peprotech, 200-04). Cells were incubated for 24hr in the above conditions at 37°C and 5% incubator. Culture supernatant was supplemented the day after with a final concentration of 0.1µg/ml prostaglandin E, 0.04µg/ml TNF- α , 0.02µg/ml IL-1 β and 0.02µg/ml IFN γ . The cells were activated and ready for use after 24hr of incubation with above cocktail. An example of the activation of DCs is shown in Figure 2.4

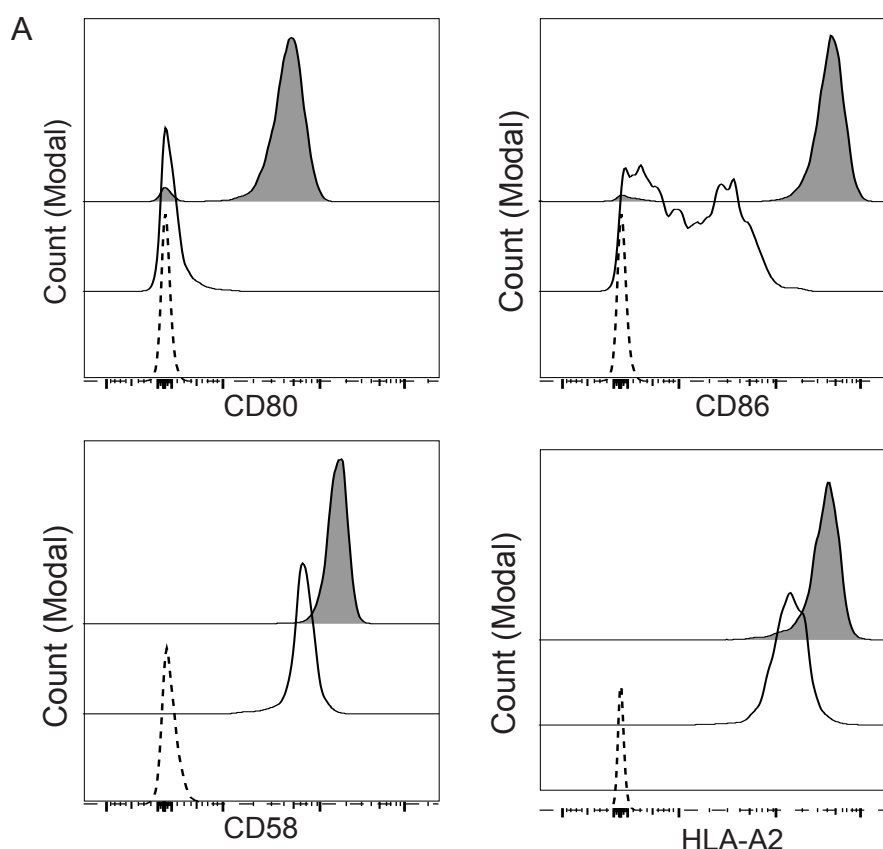


Figure 2.4 Cytokine activation of monocyte-derived Dendritic Cells (DCs)

A) (top) The expression levels of CD80 and CD86 are shown for monocytes (empty solid histograms) and cytokine differentiated and activated DCs (grey solid line histograms); empty dashed line histograms represent isotype control stained cells. (Bottom) The expression levels of CD58 and HLA-A2 are shown for monocytes (empty solid histograms) and cytokine differentiated and activated DCs (grey solid line histograms); empty dashed line histograms represent isotype control stained cells.

2.3.10 Flow cytometry staining for surface markers

1×10^6 peripheral blood mononuclear cells, PBMCs, or 100 000 cells (T cells, B cell line, monocyte or DCs) were stained in 96-well U-bottom plates. Cells were stained in 50 μ l of fixable viability dye efluor 780 (eBioscience, 65-0865-18) prepared in ice cold PBS/0.5%BSA, incubated for 10 min on ice or 4°C fridge. Cell were washed by addition of 200 μ l of PBS/0.5%BSA and centrifugation at 1300rpm, 5 min. For staining PBMCs, monocytes, DC and B cells, an Fc block step was performed by resuspending cells in 50 μ l of Fc block prepared in PBS/5%BSA (room temperature) using 5 μ l of Human TruStain FcX (Biolegend, 422302) per sample i.e. 5 μ l/50 μ l of PBS/0.5%BSA. Cell were incubated at room

temperature for 10 min. Cells were pelleted at 1300 rpm for 5 min and then resuspended in 50µl of antibody mix prepared in ice cold PBS/0.5%BSA (and Fc block in the case of PBMCs, monocytes, DC and B cells). The antibodies used in flow cytometry staining and their details can be found below in 2.15. Cells were analysed on an LSRII or Fortessa machine. Analysis of acquired data was performed on FlowJo 10.4.2 and GraphPad Prism 7.

2.4 RNA transfection of T cells with fluorescently coupled receptor/adaptor RNA constructs

Freshly isolated T cells (human or mouse) or T cells in culture (human or mouse) were harvested and washed three times in Opti-MEM media (Reduced Serum Media is a modification of Eagle's Minimum Essential Media, LifeTechnologies, 31985070) which time pelleting the cells at 300g for 10 min. Depending on the expression levels to be achieved and the number of available cells, T cells were resuspended between 1×10^6 - 2.5×10^6 per 100 μ l. Each 100 μ l cell suspension was mixed with the RNA preparation and transferred to a Gene Pulser/Micropulser Electroporation Cuvette, 0.2 cm gap (BIO-RAD, 1652086). Cells were electroporated at 300V, 2ms (a previously determined optimized setting) in an ECM 830 Square Wave Electroporation System (BTX, 45-002). 0.5ml of pre-warmed culture media at 37°C, supplemented RPMI for human T cells as described in section 2.3.3. or OK-DMEM, with supplemented IL-2, 50U/ml (see section 2.5) for mouse AND T cells, was added in the cuvette to recover the cells in two steps of 250 μ l media each. Depending on the cell number the transfected cells were transferred to a well containing enough media to achieve a cell density between 1 - 2×10^6 cells/ml. Cells were incubated for at least 12hr in 37°C, 5% CO₂ incubator before testing expression levels and transfection efficiency. An example of RNA transfection of human CD2-Ruby in AND T cells and an example of PD-1 transfection in human T cells are shown in Figure 2.5. For overexpression experiments up 20-40 μ g RNA were used.

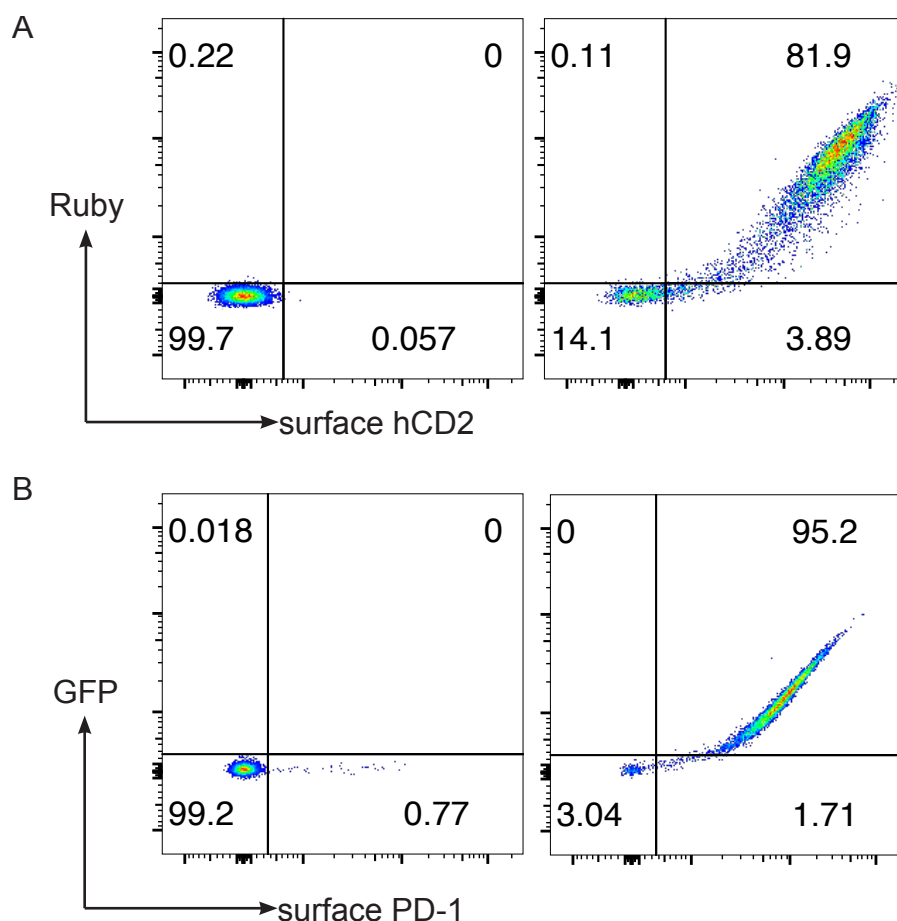


Figure 2.5 RNA transfection in mouse and human T cells

A) The plots show an RNA transfection example of human CD2-Ruby construct in mouse AND T cells; stained untransfected cells, left and stained transfected cells, right.

B) The plots show an RNA transfection example of PD-GFP construct in human naïve CD8⁺ T cells; stained untransfected cells, left and stained transfected cells, right.

2.4.1 TCR RNA transfection of T cells

TCR RNA transfection was performed as described in 2.4 above. We used 5 μ g of each TCR α - β chain and 5 μ g of CD3 construct in 100 μ l of cell suspensions, 25 $\times$ 10⁶ cells/ml (an example is shown in Figure 2.6). BTX cuvettes, 2mm gap (VWR, 732) were used here instead of the Bio-Rad cuvettes.

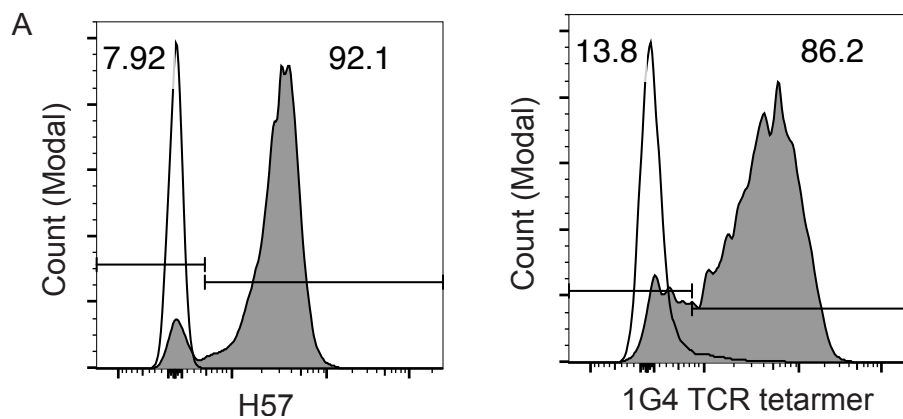


Figure 2.6 TCR RNA transfection in human T cells

A) (Left) An example of 6F9 TCR (human-mouse hybrid TCR) RNA transfection in human CD4⁺ T cells, detected with anti-mouse TCR β staining (H57 clone); Empty histogram represents stained untransfected T cells. (Right) An example of 1G4 TCR transfected CD8⁺ T cells stained with 9V-loaded HLA-A2 tetramers; Empty histogram represents stained untransfected T cells.

2.5 In vitro preparation on AND T cell blasts

Two spleens and lymph nodes, (mesenteric, inguinal, axillary and branchial) were harvested from sacrificed AND TCR transgenic mice that have CD4⁺ T cells recognizing the moth cytochrome c, MCC, 880103 peptide presented by I-E^k 179. APLs have been generated for this TCR, too. Harvested material was transferred into ice cold media (see below). Harvested spleen and lymph nodes were crashed together over a 70 μ m cell strainer to make single cell suspensions. Cells were pelleted and resuspended in 3-5 ml of ACK lysing buffer (LifeTechnologies, A1049201) and incubated at room temperature for 3 min. Reaction was stopped by topping up to 30 ml with media. Cells were washed twice in media to remove dead cells and ACK lysing buffer. Cells were finally resuspended at 10x10⁶/ml in Osame Kanagawa-adjusted Dulbecco's Modified Eagle Medium, OK-DMEM media [for 2L preparation in house, 26.74g DMEM powder was dissolved in milliQ water, supplemented with 0.55mM L-arginine hydrochloride, 0.24mM L-Asparagine, 24mM sodium bicarbonate, 10mM HEPES, 1mM Sodium Pyruvate,

1.5mM L-Glutamine, 5% PenStrep, 50 μ M 2-mercaptoethanol, filtered sterile with a 0.22 μ m filter in sterile bottles followed by addition of heat inactivated FBS] supplemented with 50U/ml human IL-2 and 1 μ M T102S peptide, an altered peptide ligand derived from moth cytochrome c peptide for which AND are reactive too¹⁷⁹. Cells were counted on day 2 and if above 2x10⁶/ml they were diluted with fresh media, supplemented with 50U/ml IL-2, to 1-1.5x10⁶/ml. This was repeated on Day 4 and AND T cell blasts were used on Day 5-7 for experiments. An example of AND T cell blasts generated on Day 6 is shown in Figure 2.7

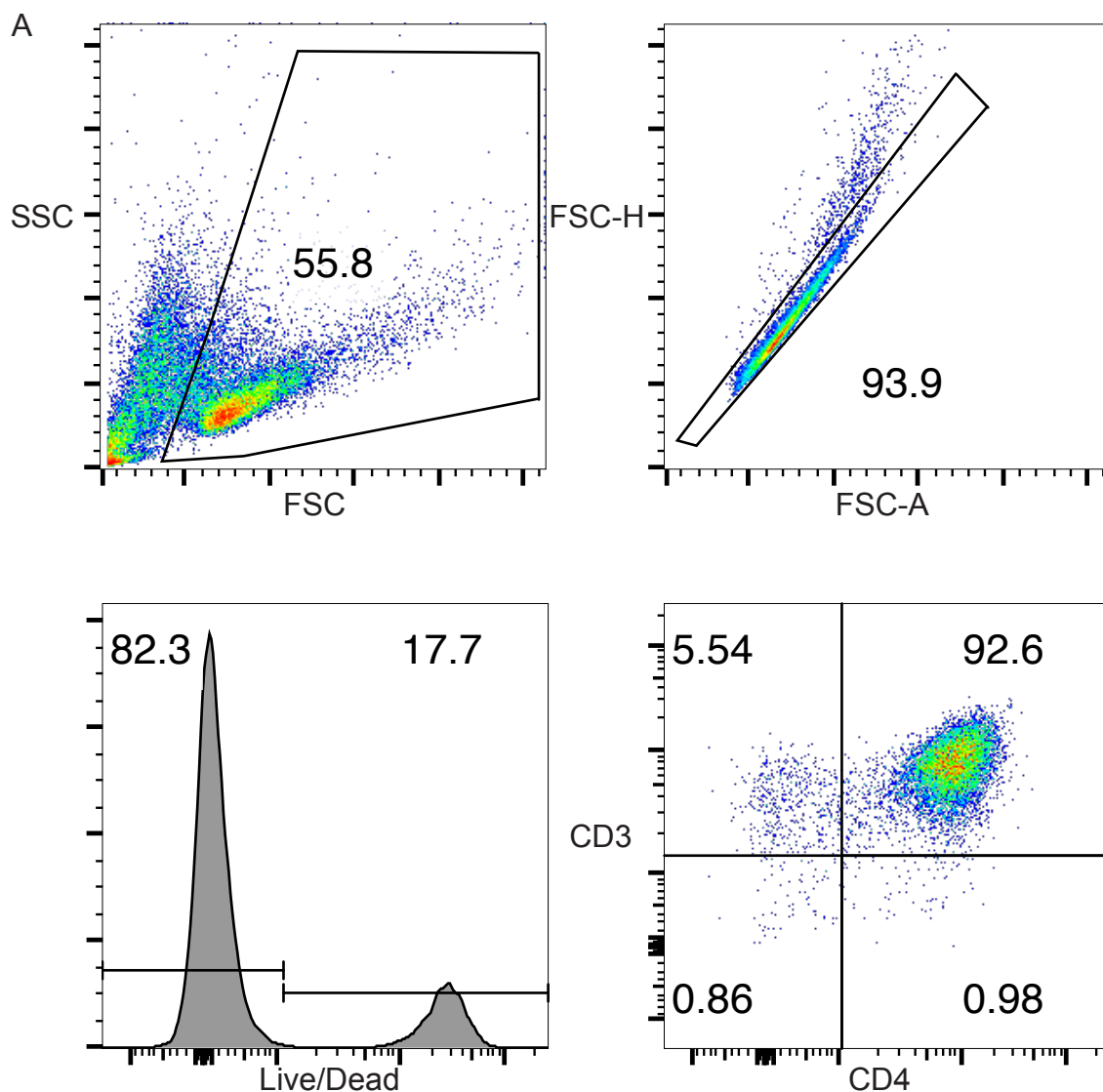


Figure 2.7 *In vitro* activation of AND T cells

A) AND T cell blasts were generated by *in vitro* co culture with APCs from lymph nodes and spleen. Shown here is the purity of AND T cells on Day 6 of expansion procedure.

2.6 1G4 CTL clone *in vitro* propagation.

1G4 CTL clone was kindly provided by Dr Ji-Li Chen (Cerundolo Lab, WIMM, University of Oxford). The clone was propagated *in vitro* with the help of Elena Brenna (McMichael Lab, NDM, University of Oxford). Allogeneic feeder cells were generated by irradiation PBMCs from donors and then mixing them together. T cells were seeded at $1-2 \times 10^6$ cells/well in a 24-well plate along with 0.5×10^6 feeder cells/ml in culture media (see section 2.3.3) with human serum (Life Science Group, S-101B-FR) instead of FBS, additional 1% beta-mercaptoethanol and finally the media was supplemented with 500U/ml IL-2 and $1 \mu\text{g/ml}$ of phytohaemagglutinin (PHA) and incubated in a 37°C , 5% CO_2 incubator. Cell clustering was monitored and cells were split at 1:2 whenever we observed significant clustering and proliferation under the microscope. Cells were also counted and diluted to 1.5×10^6 cells after the first two initial culture splits.

2.7 Processing of colorectal cancer patient tissue samples

Tumour and matched normal adjacent tissue collected from colorectal cancer patients that underwent colectomy at the Churchill Hospital, Oxford. The samples were collected under the Ethics 16/YH/0247 as a part of a collaboration with Fiona Powrie lab members, the medical staff of BRC Oxford GI Biobank and BRC Oxford IBD Cohort, supported by NIHR Biomedical Research Center, Oxford. The views expressed are those of the author/s and not necessarily those of the NHS, the NIHR or the Department of Health. Powrie lab members kindly processed the samples and single cell suspensions were provided for downstream flow cytometry staining as described in section 2.3.10. Briefly, tumour tissue and an approximately equal size piece of matched normal adjacent tissue to the tumour

were cut into small pieces and digested with Collagenase A, 1.5mg/ml, (Sigma Aldrich) prepared in RPMI supplemented with 1% PenStrep on a magnetic stirrer at 37°C, shaking for 1hr. Half way through the incubation, the tissue was homogenized by passing it through a 18G needle. At the end of 1hr incubation, the digested tissue was passed again through a 18G needle, a 21G needle and a 70µm mesh filter sequentially to generate a single cell suspension. Cells were washed in 1XHBSS/1%PenStrep (500g, 8 min). Resuspending the cell pellet with ACK lysing buffer was used to lyse red blood cells. Cells were washed and resuspended in RPMI/10%FBS/1%Penstrep. At this point, the cells were counted and used for surface staining and flow cytometric analysis.

2.8 DNA constructs generated

Human CD2 full length and human CD2 lacking its cytoplasmic tail were kindly provided by Prof. Simon Davis (WIMM, University of Oxford). These were transferred with addition of restriction sites to pGEM-64A-Ruby backbone DNA plasmid. Below we provide the sequence of the inserts:

Insert: Human CD2 full length sequence coupled to RUBY fluorescent protein:

```
taatacgactcactatagggagacagcttacgcgtgccaccGCGTGCCACCATGAGCTTTCATGTAAA
TTTGTAGCCAGCTTCCTTCTGATTTTCAATGTTTCTTCCAAAGGTGCAGTCTCCAAAG
AGATTACGAATGCCTTGGAAACCTGGGGTGCCTTGGGTCAGGACATCAACTTGGACA
TTCCTAGTTTTCAAATGAGTGATGATATTGACGATATAAAATGGGAAAAAACTTCAGA
CAAGAAAAAGATTGCACAATTCAGAAAAAGAGAAAGAGACTTTCAGGAAAAAGATACA
TATAAGCTATTTAAAAATGGAACCTCTGAAAATTAAGCATCTGAAGACCGATGATCAGG
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GATTTGAAGATTCAAGAGAGGGTCTCAAAACCAAAGATCTCCTGGACTTGTATCAACA
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AGCCTGTCAGCTGTCCAGAGAAAGGTCTGGACATCTATCTCATCATTGGCATATGTG
GAGGAGGCAGCCTCTTGATGGTCTTTGTGGCACTGCTCGTTTTCTATATCACCAAAA
GGAAAAAACAGAGGAGTCGGAGAAATGATGAGGAGCTGGAGACAAGAGCCCACAGA
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CTCCTGCTCCGTCCGGGCACACAAGTTCACCAGCAGAAAGGCCCGCCCTCCCCAGA
CCTCGAGTTCAGCCAAAACCTCCCCATGGGGCAGCAGAAAACTCATTGTCCCCTTCC
TCTAATTAtctagaggatccaccgggtcgccaccatggtgtctaaggggcgaagagctgatcaaggaaaatgcgtatg
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gacaatgaaatgtcgtagtacaacgcgaacacgcagttgccaagttcgccgggcttggtggtgggatggacgagctgtac
aagtaagcggccgcggatccccgggtaccgagctcgaattcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaactagtggcgcc
```

Features :

T7 promoter sequence

HindIII restriction site

MluI restriction site

Kozak sequence

CD2 full length sequence

XbaI restriction site

64A polyA sequence

RUBY fluorescent protein sequence

Insert: Human CD2 sequence lacking its cytoplasmic tail coupled to RUBY fluorescent protein:

taatacgactcactatagggagacagccttaccggtgccacatgagccttccatgtaaattgtagccagcttctctgatttc
aatgtttcttccaaagggtgcagtcctcctcctggaacctgggtgcttgggtcaggacatcaactg
gacattcctagtttcaaatgagtgatgatattgacgatataaaatgggaaaaaactcagacaagaaaaagattgcacaatt
cagaaaagagaaagagacttcaaggaaaaagatacatataagctatttaaaatggaactctgaaaattaagcatctgaa
gaccgatgatcaggatatctacaaggatcaatatatgatacaaaaggaaaaaatgtgttgaaaaaatattgattgaaga
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caagtaagcggccgcgatccccgggtaccgagctcgaattcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaactagtggcgcc

Features :

T7 promoter sequence

HindIII restriction site

MluI restriction site

Kozak sequence

CD2 sequence lacking the cytoplasmic tail

XbaI restriction site

64A polyA sequence

RUBY fluorescent protein sequence

Insert: Human PD-1 full length sequence coupled to GFP fluorescent protein:

```
taatacgactcactatagggagacaggttgcaccATGCAGATCCCACAGGCGCCCTGG
CCAGTCGTCTGGGCGGTGCTACAACTGGGCTGGCGGCCAGGATGGTTCTTAGACTCCCCA
GACAGGCCCTGGAACCCCCCACCTTCTCCCCAGCCCTGCTCGTGGTGACCGAAGGGGAC
AACGCCACCTTCACCTGCAGCTTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTAC
CGCATGAGCCCCAGCAACCAGACGGACAAGCTGGCCGCTTTCCCCGAGGACCGCAGCCAG
CCCGGCCAGGACTGCCGCTTCCGTGTACACAACTGCCCAACGGGCGTGACTTCCACATG
AGCGTGGTCAGGGCCCGGCGCAATGACAGCGGCACCTACCTCTGTGGGGCCATCTCCCTG
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GCCGTCTCTGTCTCCCGGGCCGCACGAGGGACAATAGGAGCCAGGCGCACCGGCCAGCCC
CTGAAGGAGGACCCCTCAGCCGTGCCTGTGTTCTCTGTGGACTATGGGGAGCTGGATTTC
CAGTGGCGAGAGAAGACCCCGAGCCCCCGTGCCTGTGTCCCTGAGCAGACGGAGTAT
GCCACCATTTGTCTTTCCTAGCGGAATGGGCACCTCATCCCCGCCCGCaGGGgCTCAGCC
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ttcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaaaactagtggcgcc
```

Features :

T7 promoter sequence

HindIII restriction site

Kozak sequence

PD-1 full length sequence

XbaI restriction site

EGFP sequence

PD-1 full length sequence : this part was determined to the intracellular domain using the TMHMM Server v. 2.0 online tool

Insert: Human PD-1 sequence lacking its cytoplasmic tail coupled to GFP fluorescent protein:

```
taatacgactcactatagggagacaaagttgccaccATGCAGATCCCACAGGCGCCCTGG
CCAGTCGTCTGGGCGGTGCTACAACTGGGCTGGCGGCCAGGATGGTTCTTAGACTCCCCA
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CGCATGAGCCCCAGCAACCAGACGGACAAGCTGGCCGCTTTCCCCGAGGACCGCAGCCAG
CCCGGCCAGGACTGCCGCTTCCGTGTACACAACCTGCCCAACGGGCGTGACTTCCACATG
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ctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccacc
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ttcaagtccgccatgcccggaaggctacgtccaggagcgcaccatcttcttcaaggacgac
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aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaactagtggcgcc
```

Features :

T7 promoter sequence

HindIII restriction site

Kozak sequence

PD-1 sequence lacking its cytoplasmic tail.

XbaI restriction site

EGFP sequence

2.9 Preparation of RNA

Constructs in pGEM plasmids were linearised with *SpeI* restriction enzyme at 37°C for 1-2 hrs. DNA precipitation and *in vitro* RNA transcription and *in vitro* polyadenylation were performed exactly as described in the protocol of the mMESSAGE mMACHINE T7 ULTRA Transcription kit (Thermofisher Scientific, AM1345). RNA was purified as per instructions of MEGAclear Transcription Clean-up kit (Thermofisher Scientific, AM1908). RNA was finally aliquoted at 5µg per PCR vial and stored in -80°C freezer. RNA was only thawed once and used immediately.

2.10 Culture of cell lines

The CF996 EBV-transformed B cell line (Sigma Aldrich, 9094), was maintained in supplemented RPMI (as described in section 2.3.3) at cell densities of $0.75-1 \times 10^6$ cells/ml in 6- or 12-well plates in a 37°C, 5% CO₂ incubator. Trombelli melanoma cell line was maintained DMEM media (commercially available), supplemented with 10% FBS/1% Penstrep in flask and allowed to grow to 80-90% confluence before splitting. Cells were maintained in a 37°C, 5% CO₂ incubator. Melanoma cell line was used in experiments between 3-7 passages.

2.11 TIRF and confocal microscopy and image analysis.

An Olympus IX83 inverted TIRF microscope was used to acquire most of the experiments performed with T cell on ligand reconstituted SLB. An Olympus UAPON 150x NA 145Oil TIRF microscope objective was used. The instrument was also fitted with 405 nm, 488 nm, 561 nm, 633 nm laser lines, an MT20 equipped with a 150W xenon-mercury arc burner used to create internal reflection

microscopy patterns during adhesion of T cells on SLBs. A Photometrics Evolve 512 Delta EMCCD camera was used for image acquisition. Analysis was performed in Fiji (ImageJ, version 2.0.0) and GraphPad Prism 7. T cells on SLBs were also sometimes imaged on a GE InCell 6000 with a 40x NA 0.75 objective (GE Healthcare).

A Zeiss inverted LSM 880 AiryScan confocal microscope was used for imaging in xyz fixed T:APC cell conjugates in the airyscan mode. Airyscan analysis was performed by the ZEISS ZEN software used to operate the microscope and the airyscan settings were used in the automated mode. This was chosen as the airyscan mode provides a 2-fold increase in z-resolution compared to conventional confocal microscopy. Due to emission filter restrictions we would image in 405, 488 and 647 channels or 405, 488 and 568 channels but not 568 and 647 channels. The instrument was equipped with a Plan-Apochromat, 63x, NA. 1.40 Oil objective. Analysis and 3-D rendering was performed in Imaris software followed by cropping of images for presentation purposes in Fiji.

2.12 Live widefield microscope of Ca²⁺ signalling in T cells on ligand reconstituted SLBs

0.5-1x10⁶ T cells in culture were pelleted at 1300rpm for 5 min and resuspended in 250-500µl of 5µg/ml Fura-2 dye (LifeTechnologies, F1221) prepared in RPMI/2%FBS. Cells were incubated at room temperature on a rotator for 20 min. Cells were washed twice by addition of 1ml of RPMI/2%FBS each time and centrifugation at 1300 rpm for 5 min. Cells were finally resuspended in 120-240µl of prewarmed flow cell buffer, incubated in a 37°C water bath for 10 min and then

cells were ready to be incubated on SLBs for monitoring their intracellular calcium level changes.

Imaging was performed on the Olympus TIRF microscope in the widefield mode at 37°C, we recorded the emission spectra of Fura-2 dye when excited at 340 and 380 nm at 5s intervals for 20 min on a Hamamatsu ORCA flash 2.8 version C11440-10C. The MT20 xenon-mercury arc burner on the Olympus TIRF microscope was used as the excitation source for Fura-2.

Analysis of experiment was performed as follows: The raw image data were exported as 340/380 ratio. Cells were tracked using the 'TrackMate' plugin in Fiji. A collaboration with the Fritzsche Lab (Kennedy Institute, University of Oxford) was set up to analyse our data using the CalQuo software¹⁸⁰. In brief, the format of data exported from Fiji, was adjusted so that the CalQuo software can process it. This was done using a Matlab code written by our collaborators. The Calquo software was then used to assign calcium profile responses as single peak and oscillating peaks and also differentiate between profiles that described a T cell that was triggered/activated and non-triggered T cells. In addition, we selected an extra criterion that only the calcium profiles of tracked cells with more than 100 frames will be used in the analysis i.e. we would process data from T cells that were tracked based on their ratio value for more than 8 min and regardless of the calcium levels profile. Calcium profiles were, then, aligned on the maximum peak found within the first frames of a calcium change response. Data was exported from CalQuo software. Excel and Originlab software were used to normalize all calcium profiles by the maximum ratio value found when comparing all experimental condition of one donor and generate average ratio profiles for each condition along with error bars representing standard error of mean (SEM).

2.13 Western blot analysis and co-immunoprecipitation

T cells were incubated on ligand reconstituted SLB prepared in 96-well flat bottom plates as described in section 2.1.3 or co-cultured with APCs as described in section 2.3.8 but instead of dislodging the cell pellet, we removed the supernatant and washed cells twice in ice cold PBS (1300 rpm, 5 min, 4°C) before cell lysis. In the SLB experiment, the plate was transferred on an ice cold metal block and cells were lifted off SLB by addition of ice cold PBS/2%FBS/2mM EDTA and incubated at 4°C for 15 mins; at 7-8 min the buffer of each well was pipetted up and down to help detach cells off the SLBs. Cells were harvested and washed in ice cold PBS twice (1300 rpm, 5 min, 4°C). Cells were lysed in 1X cell lysis buffer (Cell Signalling, 9803) supplemented with 1x Protease/Phosphatase Inhibitor Cocktail (Cell signalling, 5872) and 1mM Sodium Orthovanadate (NEB, P0758) for 15-20 min on ice. Cell debris was pelleted at maximum rpm for 15 min and cell free whole cell lysate (WCL) recovered.

In the meantime, protein G sepharose (VWR, 17-0618-01) was washed in PBS twice (3000 rpm, 5 min). WCL was incubated with protein G sepharose and mouse IgG isotype for 1-2 hr on a rotator at 4°C to remove any unspecific binding by the sepharose and antibody. Sepharose pelleted and WCL was recovered. WCL was incubated with mouse anti-human CD28 mAb (9.3 clone) or mouse IgG control and incubated overnight on a rotator at 4°C. The next day, freshly washed sepharose was added to the WCL-Ab mix and rotated at 4°C for 1-2hr. Sepharose was pelleted and culture supernatant harvested to be use for detection of pAKT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in WCL. Sepharose was washed twice in 1X cell lysis buffer, resuspended in PBS supplemented with 1x protein orange loading buffer (Li-Cor, 928-40004) and beta-mercaptoethanol and heat denatured for 10 min at 95°C. Sepharose was pelleted and the

supernatant was recovered and loaded on a 4-15% Mini-PROTEAN TGX-gel (Bio-Rad, 456-1084), at 90V for 1-1.5 min using 1X Tris/Glycine/SDS buffer prepared in from 10X Tris/Glycine/SDS (sodium dodecyl sulphate) (Bio-Rad- 161-0732) buffer in distilled water. Protein bands were wet transferred onto a nitrocellulose membrane (Bio-Rad, 162-0115). Nitrocellulose membrane was blocked for 1 hr at room temperature with Li-Cor Odyssey blocking buffer (TBS), (Li-Cor, 927-50000) supplemented with 5% donkey serum. The membrane was washed three times for 5 min in TBS/2%Tween-20 at room temperature and rinse three times in PBS before addition of primary rabbit anti-human antibodies (for example anti-CD28 or anti-PKC- θ , see table at the end for antibody details) for an overnight incubation at 4°C. Membranes were washed three times as described above. Membranes were finally blotted with IRDye 800 CW donkey anti-rabbit secondary antibody (Li-Cor, 926-32213) and incubated at room temperature for 45 min. Membranes were washed three times for 5 min in TBS/2%Tween-20 at room temperature and rinse three time in PBS before imaging on a Li-Cor machine.

WCL generated as described above or directly from lysed cells (with no preceding immunoprecipitation) were loaded on a protein gel and blotted for pAkt, GAPDH, total Akt similarly to above steps.

For a second round of staining with primary antibodies, membranes were incubated for 20 min at room temperature in NewBlot IR stripping buffer and washed as described above before repeating blocking and staining with primary and secondary antibodies.

2.14 Lactate dehydrogenase (LDH) assay for determining CTL cytotoxicity.

30 000 Trombelli melanoma cells, target cells, were seeded in a 96-well flat-bottom plate and allowed to adhere overnight. 1G4 CTL clone cells, effector cells were added at 0.5:1, 1:1 and 3:1 ratio of effector:target cell. In an initial time course experiment, culture supernatants were harvested at 4hr and 24 hr post co-culture of effector and target cells. The LDH Cytotoxicity Detection kit (Takara Bio, MK401) was used to determine the percentage of cytotoxicity by quantifying the levels of LDH released by dying target cells. We performed quantification of released LDH as instructed by the kit manual including control wells with media alone, target cells alone and effector cells alone. In brief, the percentage cytotoxicity was determined by calibrating cell death with two reference points the maximum cell death achieved by incubation of target cells with a final concentration of 0.1 or 1% Triton-X100 and untreated cells representing the background cell death of target cells.

2.15 Antibody Reagents

Flow cytometry

anti-human Ab

Surface Marker	Fluorophore	*FC (µg/ml)/Dilution	Company	Clone	Cat No
CD3	BV650	1	Biolegend	UCHT-1	300468
CD4	AF700	1	Biolegend	A161A1	357418
CD8	PB	1	Biolegend	HIT8a	300928
CD62L	AF647	4	Biolegend	DREG-56	304818
CD45RA	FITC	1 in 50	BD Bioscience	HI100	555488
CD2	PE	5	Biolegend	RPA2.10	300208
CD28	PE	5	Biolegend	37.51	102105
PD-1	PE	5	Biolegend	29F.1A12	135205
TCRα/β	PE	5	Biolegend	IP26	306708
CD3	PE	5	Biolegend	UCHT-1	300456
CD11a	PE	5	Biolegend	TS2/4	350605
CD18	PE	5	Biolegend	TS1/18	302107
CD56	BV711	1	Biolegend	HCD56	318335
CD56	BV785	1	Biolegend	5.1H11	362549
isotype control	PE	5	Biolegend	MOPC-21	400112
CD2	PE-Cy7	5	Biolegend	RPA2.10	300222
CD2	APC	5	Biolegend	RM2-6	100112

Cell Conjugates

CD2	unlabelled	5	in-house	CD2.1	N/A
CD11a	FITC	5	Biolegend	TS2/4	350604

Blocking experiments

CD58	unlabelled	10 to 20	Biolegend	TS2/9	330912
isotype control	unlabelled	10 to 20	Biolegend	MG1-45	401404

Patient samples

CD45	BV510	1	Biolegend	HI30	304035
CD45	BV785	1	Biolegend	HI30	304048
CD3	BV650	1	Biolegend	UCHT-1	300468
CD4	AF700	1	Biolegend	A161A1	357418
CD8	FITC	1	eBioscience	HIT8a	11-0089-42
CD127	PE/Dazzle594	5	Biolegend	A019D5	351336
CD127	PE-TexasRed	5	BD Biosciences	HIL-7R-M21	562397
PD-1	PE	5	Biolegend	29F.1A12	135205
CD28	eFluor750	3	eBioscience	CD28.2	48-0289-42
CD28	BV421	3	Biolegend	CD28.2	302929
CD2	PE-Cy7	5	Biolegend	RPA2.10	300222
isotype control	PE	5	Biolegend	MOPC-21	400112
isotype control	PE-Cy7	5	Biolegend	MOPC-21	400126

Monocytes/DCs

CD80	AF647	5	Biolegend	2D10	305216
CD86	AF647	5	Biolegend	IT2.2	305416
HLA-A2	PE	5	Biolegend	BB7.2	343306
CD58	AF647	5	BD Biosciences	1C3	563567

IP/WB/IHC

CD28 (IP)	unlabelled	3-5µg in 100-250µl of WCL	BioExcel	9.3	N/A
CD28	unlabelled	as per datasheet	Cell Signalling	D2Z4E	38774S
p85	unlabelled	as per Cell Signalling datasheet	Cell Signalling	N/A	4292S
PKC-θ	unlabelled	as per Cell Signalling datasheet	Cell Signalling	E1I7Y	13643
GAPDH	unlabelled	as per Cell Signalling datasheet	Cell Signalling	D16H11	5174S
pAkt(Ser473)	unlabelled	as per Cell Signalling datasheet	Cell Signalling	D9E	4060S
Akt (pan)	unlabelled	as per Cell Signalling datasheet	Cell Signalling	C67E7	4691S
pPLCγ1 (Tyr783)	unlabelled	as per Cell Signalling datasheet	Cell Signalling	N/A	2821S
pLAT (Tyr171)	unlabelled	as per Cell Signalling datasheet	Cell Signalling	N/A	3581S
pSrc(Tyr416)	unlabelled	as per Cell Signalling datasheet	Cell Signalling	D49G4	6943S
mouse IgG	unlabelled	3-5µg in 100-250µl of WCL	EMD Millipore		PP54
PKC-θ	unlabelled	1 in 50	Santa Cruz	C-19	sc-1875

Flow cytometry

anti-mouse mAb

Surface Marker	Fluorophore	*FC (µg/ml)/Dilution	Company	Clone	Cat No
CD4	AF700	1 in 50	Biolegend	RM4-5	100536
CD3	BV421	1 in 50	Biolegend	17A2	100237
TCRβ	APC	5	Biolegend	H57-597	109218
TCRβ-Fab	AF647 (labelled in house)	1 in 200	BioExcel	H57-597	N/A
TCRβ-Fab	AF647 (labelled in house)	1 in 200	Biolegend	H57-597	N/A

*Final concentration

3 Chapter 3. CD2, LFA-1 and TCR in the T cell synapse

3.1 Introduction

A number of studies have looked into the biochemical properties of CD2 engagement in T cells. Our lab measured the two-dimensional dissociation constant for interaction of CD2 and laterally mobile ligands CD58 (human) and CD48 (rodent) in supported lipid bilayers^{89,181–185}. The CD2-CD58 interactions in this setting were highly dynamic, consistent with the fast off-rate measured in solution by Davis and van der Merwe^{70,186}. Engaging CD2 with a multivalent form of CD58 failed to induce T cell signaling, unless combined with a non-function blocking anti-CD2 monoclonal antibody, which induced TCR dependent activation^{178,187}. Grakoui *et al* showed in supplemental data that in mature immunological synapses (~ 30 minutes), CD2-CD48 and TCR-pMHC interactions segregated³⁷. Douglass and Vale found that CD2 marked domains in the T cell plasma membrane that transiently interact with Lck during T cell activation by surface immobilized anti-CD3, further suggesting a dynamic connection between CD2 and TCR⁹⁴. Kaizuka *et al* CD2-CD58 microdomains excluded CD45 and triggered weak pMHC independent TCR signalling²⁰. Furthermore, they demonstrated that CD2 and TCR initially (~ 2min) colocalise when co-engaged by laterally mobile CD58 and anti-CD3 on SLBs, but then segregated at later time points (>5 min), consistent with the supplemental data from Grakoui *et al*. This finding suggested two phases of CD2 actin in immune synapse formation, but didn't incorporate ICAM-1 to fully put the result in context. Despite this intensive study over 30 years, there remain many gaps in understanding of CD2 function

and recent work implicating CD2 and CD58 in human autoimmune diseases lends greater urgency to closing these gaps.

We, therefore, sought to investigate the localisation dynamics of ligated CD2 during the formation of a classical 'bull's eye' T cell immunological synapse (IS) on SLBs reconstituted with CD58, ICAM-1 and anti-CD3 or pMHC. Preliminary work from our lab on the rodent model confirmed a unique distribution pattern for ligated CD2 in the mature immunological synapse. This study, thus, looks further into it in with the human system and investigates what is driving this unique CD2 pattern and potential significance for autoimmunity and tumour immunology.

3.2 Hypothesis

Ligation of CD2 results in a unique distribution of CD2-CD58 clusters upon T cell synapse formation. This unique distribution is a novel way by which CD2 enhances T cell activation.

3.3 Results

3.3.1 Localisation dynamics of CD2 during T cell synapse formation

We investigated the localisation dynamics of ligated CD2 receptors during the formation of the T cell synapse on SLBs. Human T cells, isolated from PBMCs, were incubated on SLBs reconstituted with fluorescently labelled ICAM-1, anti-CD3 Fab' and CD58 to track the engaged LFA-1, TCR/CD3 and CD2, respectively. Therefore, when we refer to LFA-1, TCR or CD2 microclusters this is based on tracking the accumulated ligands. Live time-lapse movies were recorded for up to 20-30 min. At the early stages of synapse formation (1-2min), TCR&CD2 combined microclusters were formed upon spreading of T cells on the SLB (Figure 3.1A), which appeared co-localised at ~300 nm resolution (Figure 3.1B). The TCR&CD2 combined microclusters segregated from LFA-1-ICAM-1 accumulations in the interface, and excluded free ICAM-1, as indicated by dark areas in the ICAM-1 channel. TCR and CD2 combined microclusters moved towards the center of the cell-SLB interface, and by 2 min the co-localization of TCR and CD2 decreases with some anti-correlated domains appearing. At 3-5 min of synapse formation, CD2-CD58 microclusters re-emerge in the LFA-1-ICAM-1-enriched pSMAC and outside of the pSMAC i.e. in the dSMAC (Figure 3.1A). It must be noted, that even when CD2 clusters were observed within the pSMAC, there was again exclusion of ICAM-1 by those clusters (Figure 3.1B).

From 10 min onwards, the majority of the CD2-CD58 signal had segregated from TCR-enriched cSMAC. At these later time-points of synapse formation, CD2-CD58 was arranged in clusters around the pSMAC, forming a peripheral ring-like structure around the classical LFA-1-ICAM-1 enriched pSMAC i.e. in the dSMAC (Figure 3.1A,B). Often, this pattern was also seen as a ring within the outer border

of the pSMAC; ICAM-1 was always excluded in those regions. We have termed this ring, the CD2 corolla, resembling a crown or the petals of a flower.

CD2 corolla formation was observed not only with the anti-CD3 driven polyclonal activation system (Figure 3.1A) but also in antigen-specific systems based on more physiological pMHC (Figure 3.1C). CD4⁺ and CD8⁺ T cell, isolated from PBMCs, were transfected with 6F9 (MHC-II restricted TCR) and 1G4 (MHC-I restricted TCR), respectively. 6F9 TCR recognizes an HLA-DPB1*04:01-restricted MAGE-A3 melanoma antigen¹⁸⁸. To facilitate expression of the 6F9 TCR the constant Ig domains were replaced with the corresponding mouse constant Ig domains. The 1G4 TCR recognizes an HLA-A*0201(HLA-A2)-restricted NY-ESO-1 cancer-testis antigen¹⁸⁹. Each transfected T cell subset was incubated on ICAM-1, CD58 and corresponding pMHC reconstituted SLBs. Control peptide loaded SLB experiments were also performed to prove the antigen-specific of the system (see 3.3.3). Once the CD2 corolla was fully formed around 5-10 min, i.e. the majority of the CD2-CD58 signal is in the dSMAC. The CD2 corolla structure is dynamic as shown by the 10s interval snapshots from a live time lapse TIRFM movie (5s/frame) in Figure 3.1.D

The aforementioned experiments were performed using human PBMC-isolated CD4⁺ and CD8⁺ T cells. We sought to investigate also if T cells, found in human lymphoid organs, like the tonsils, also have this ability to arrange ligated CD2 in a corolla structure. CXCR5^{HI} and CXCR5 negative (CXCR5⁻) CD4⁺ T cells were isolated and sorted from human tonsils. CXCR5^{HI} and CXCR5⁻ CD4⁺ T cells were incubated on ICAM-1, anti-CD3 Fab and CD58 reconstituted SLBs, for 15 min, before fixation and imaging with TIRFM. Both T cell populations organised their CD2-CD58 clusters, in the peripheral ring-like structure that we have termed the CD2 corolla (Figure 3.1E).

Finally, we sought to investigate whether the CD2 corolla is also a structure present in T:B cell conjugates. Human CD4⁺ and CD8⁺ T cells isolated from PBMCs, were transfected with 6F9 or 1G4 TCR, respectively. MAGE-A3 or NY-ESO-9V peptide loaded CF996 EBV-transformed B cells were conjugated to TCR transfected CD4⁺ and CD8⁺ T cells, respectively, for 25-30 minutes. CD2 and CD11a, the α -subunit of LFA-1, staining revealed the presence of a CD2 signal outside an LFA-1 enriched pSMAC or within the pSMAC with observable signs of exclusion of LFA-1 by CD2 (Figure 3.1.F). We sought to detect the CD2 localisation relative to the TCR-enriched cSMAC. Attempts to label the TCR and/or CD3 did not result in any staining for the former and no staining in the interface of the conjugates for the latter. This may result from failure of TCR/CD3⁺ extracellular vesicles that make up the cSMAC remaining in the synapse center for 30 minutes⁴⁷. Nonetheless, these images demonstrate that CD2 corolla formation is observed in T cell:antigen presenting cell conjugates formed by both CD4⁺ or CD8⁺ T cells.

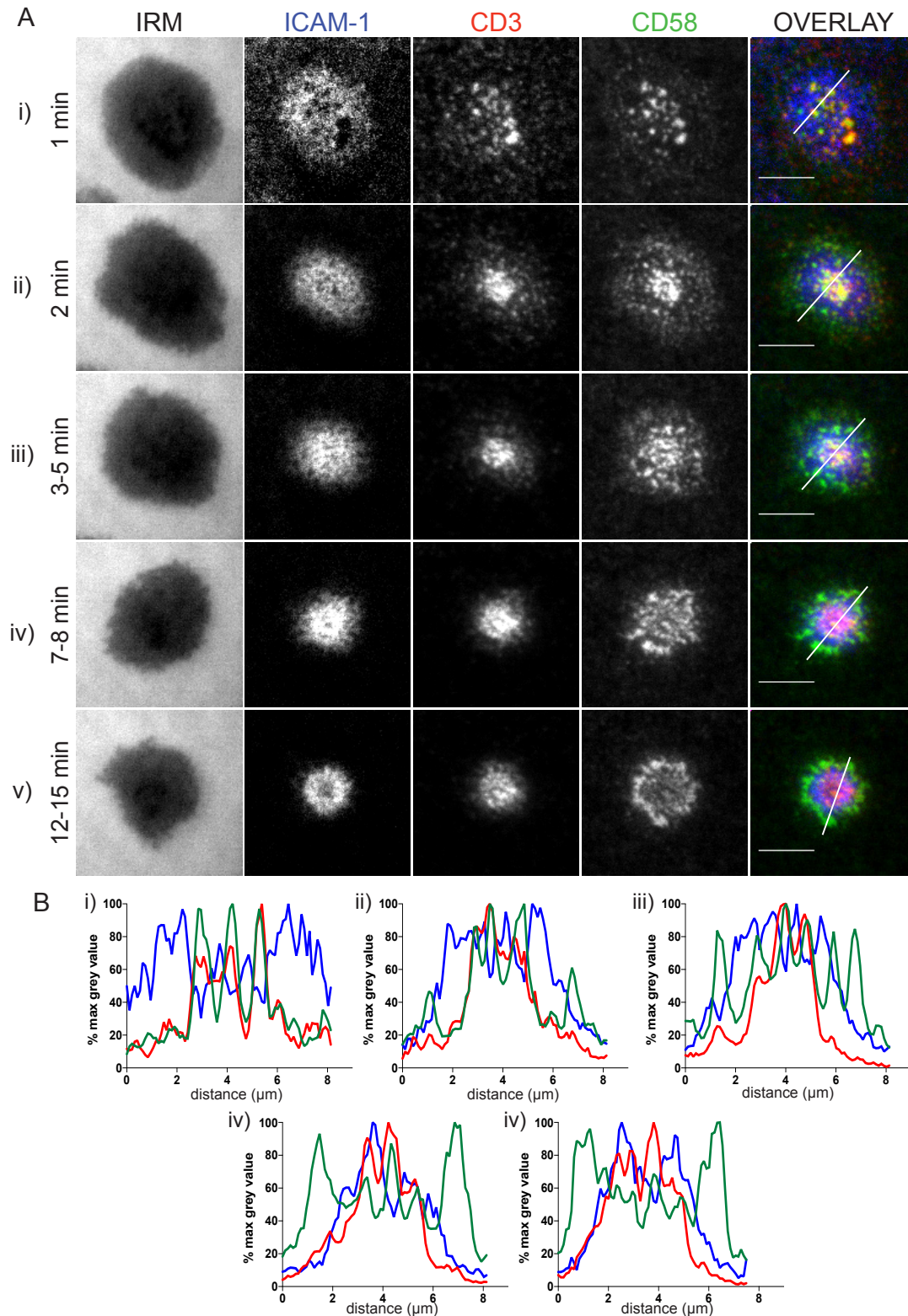
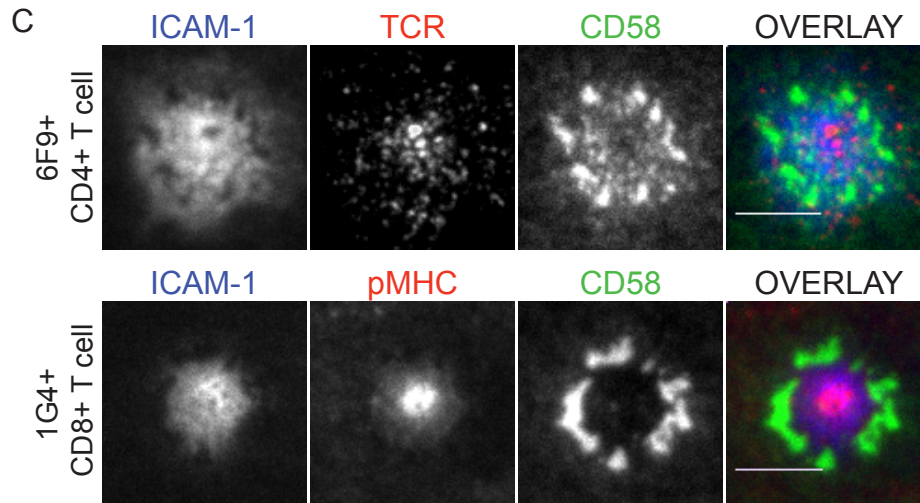
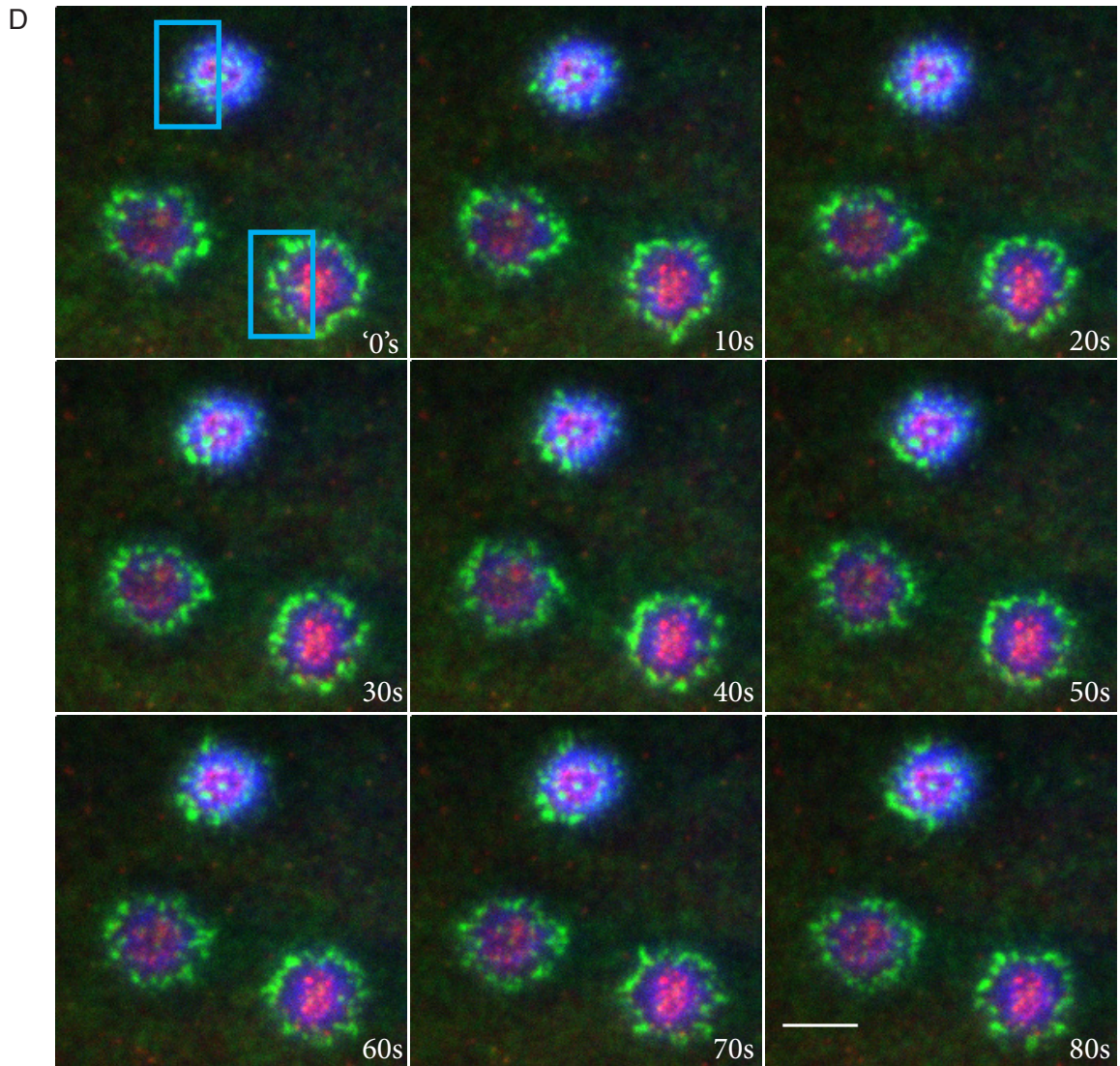


Figure 3.1 Unique CD2-CD58 supramolecular activation cluster, ‘CD2 corolla’ in the T cell synapse.

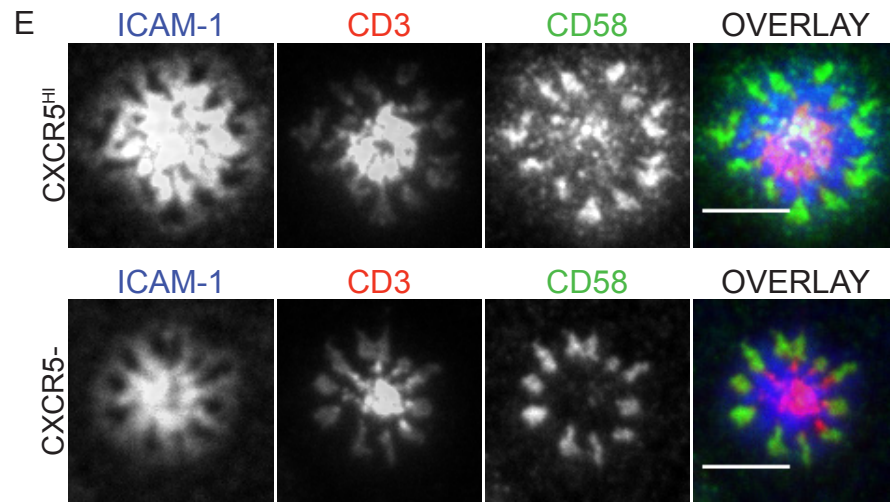
A) Resting human CD4⁺ T cells, isolated from PBMCs, were plated on ICAM-1, blue, ($200/\mu\text{m}^2$), anti-CD3 Fab, red, ($30/\mu\text{m}^2$), CD58, green, ($200/\mu\text{m}^2$) containing SLB. Interference reflection microscopy, IRM, signal shows the spreading phase of the cell. Cells were imaged at 4s intervals with TIRFM. Images from 1 to 12-15min after initial contact are shown. Scale bar, 5 μm . Histograms in (B,i)-iv) depict the intensity profiles on the diagonal white lines in overlay images shown in (A,i)-iv), respectively. y-axis; raw pixel intensity signal normalized to maximum intensity pixel of each channel. A representative experiment of at least three independent experiments with either CD4⁺ or CD8⁺ T cells.



C) Resting 6F9 TCR transfected CD4+ T cells, 6F9+, (top panel) and 1G4 TCR transfected CD8+ T cells, 1G4+, (bottom panel), previously isolated from PBMCs were incubated on ICAM-1 ($200/\mu\text{m}^2$), MAGE-A3 peptide loaded HLA-DP4, ($30/\mu\text{m}^2$) and NY-ESO-9V peptide loaded HLA-A2 ($30/\mu\text{m}^2$), and CD58 ($200/\mu\text{m}^2$) containing SLBs for 15 min before fixation and imaged with TIRFM. 6F9+ TCR CD4+ T cells were also stained with a fluorescently labeled anti-mouse TCR β Fab (H57 clone) prior to SLB incubation. Fluorescently labelled streptavidin was used to track the biotinylated NY-ESO-9V peptide loaded MHC. Data are from one representative experiment out of three. Scale bar, 5 μm .

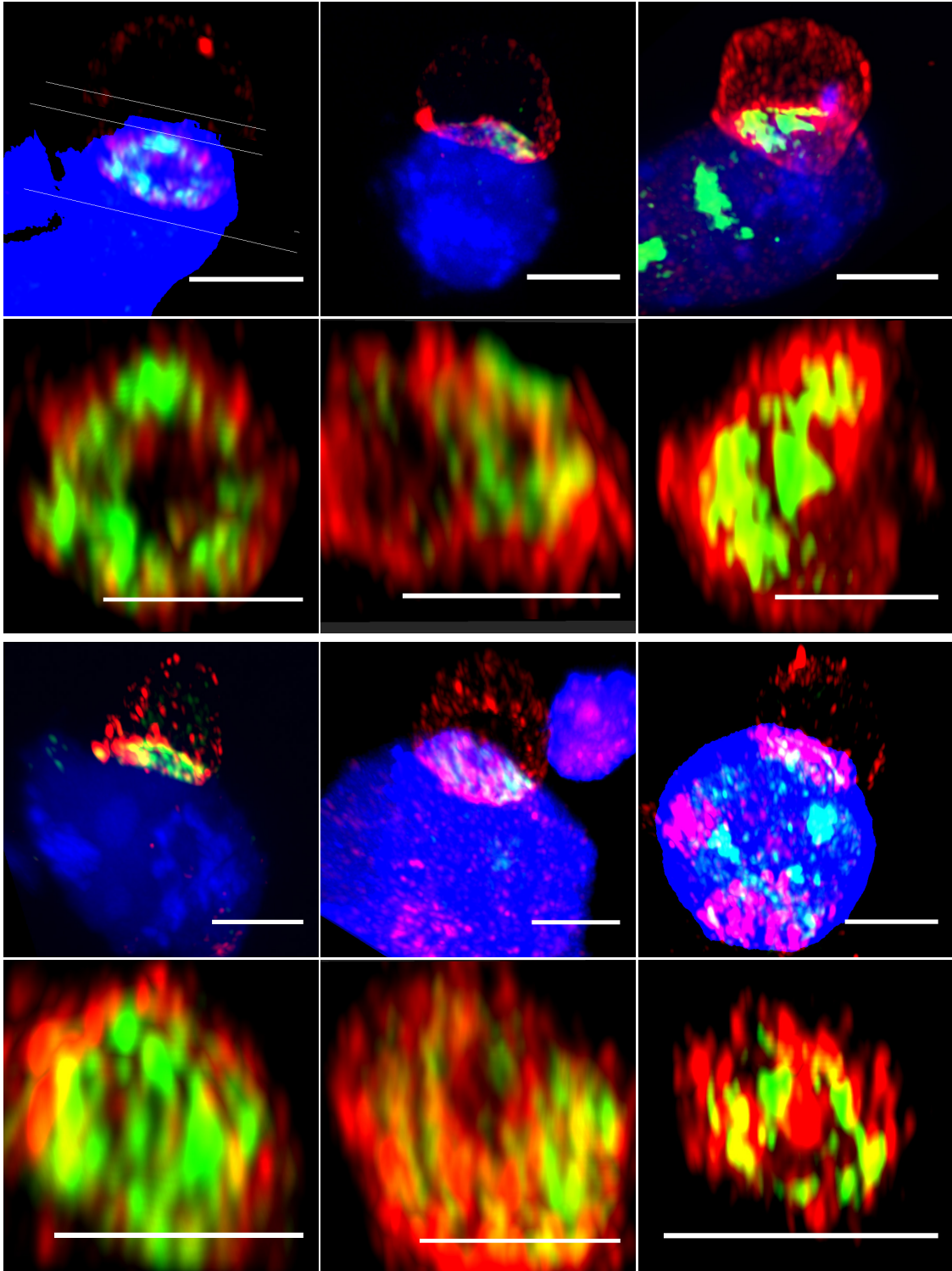


D) The dynamic changes of a fully formed CD2 corolla. Resting 1G4+ CD8+ T cells, previously isolated from PBMCs were incubated on ICAM-1 ($200/\mu\text{m}^2$), NY-ESO-9V peptide loaded HLA-A2 ($30/\mu\text{m}^2$), and CD58 ($200/\mu\text{m}^2$) containing SLBs. Fluorescently labelled streptavidin was used to track the biotinylated NY-ESO-9V peptide loaded MHC. Cells were imaged at 5s intervals with TIRFM. The interval between images is 10s. Data are from one representative experiment out of two. Scale bar, 5 μm .



E) CD2 corolla formation during T cell synapse is also observed in T cells isolated from lymphoid tissue such as human tonsils. Human CD4⁺ T cells isolated from tonsils and FACSsorted in CXCR5^{HI} (top panel) and CXCR5 negative (CXCR5⁻) (bottom panel) T cell populations were incubated on ICAM-1 (200/ μm^2), anti-CD3 Fab (30/ μm^2), CD58 (200/ μm^2) containing SLBs for 15 min, before fixation and imaged with TIRFM. Shown here, are representative images of both T cell subsets, at least 50 cells for each subset were captured, from one representative experiment out of three. Scale bar, 5 μm .

F



F) The CD2 corolla in human T:APC conjugates. Resting 6F9+ CD4+ T cells, first two images, and 1G4+ CD8+ T cells, previously isolated from PBMCs were conjugated for 25-30 min at 37°C with a EBV-transformed B cells (blue-volumetric dye) pulsed with 1μM MAGE-3A or 1μM-NY-ESO-9V peptide, respectively. Cells were fixed, permeabilized and stained with anti-CD2 mAb, CD2.1 clone, (red) and anti-CD11a mAb, TS2/4 clone, (green). Shown, here, in 3-D rendering (IMARIS software), are T:B cell conjugates (top panels) and 1μm thick slice of the T:B cell interface shown in the bottom panels. Images were captured on an Airy-Scan Confocal Microscope (ZEISS). Representative images are shown from two independent experiments. Scale bar, 5 μm.

3.3.2 CD2 corolla is enriched in activated signalling molecules

It was reported, that CD2 microclusters generated in a SLB system are enriched in signalling molecules²⁰. We, thus, investigated whether the CD2 corolla is enriched in such signalling molecules, too. Staining for the active forms of Src kinases, Lck and Fyn, (pSrc), for phosphorylated PLC γ 1 (pPLC- γ) and phosphorylated LAT (pLAT), at 15 min of synapse formation, showed an enrichment of these signalling molecules in the CD2 corolla. In fact, these signalling molecules are detected in clusters colocalised with CD2-CD58 clusters around the pSMAC or at the outer border of the pSMAC (Figure 3.2 A-C). The localization of pSRC kinases, pPLC γ 1 and pLAT were also determined in the absence of CD2 ligation, at 15 min of synapse formation. In this case, the pSrc, pPLC γ 1 and pLAT signal was primarily observed in microclusters, found within and around the pSMAC, with little to no signal in the cSMAC. The levels of pSrc, pPLC γ 1 and pLAT, at 15 min of synapse formation, were quantified in the presence and absence of CD2 corolla. In the presence of the CD2 corolla, there was a statistically significant increase in pSrc, pPLC γ 1 and pLAT levels by approximately 2-fold when compared to no CD2 ligation (Figure 3.2 D).

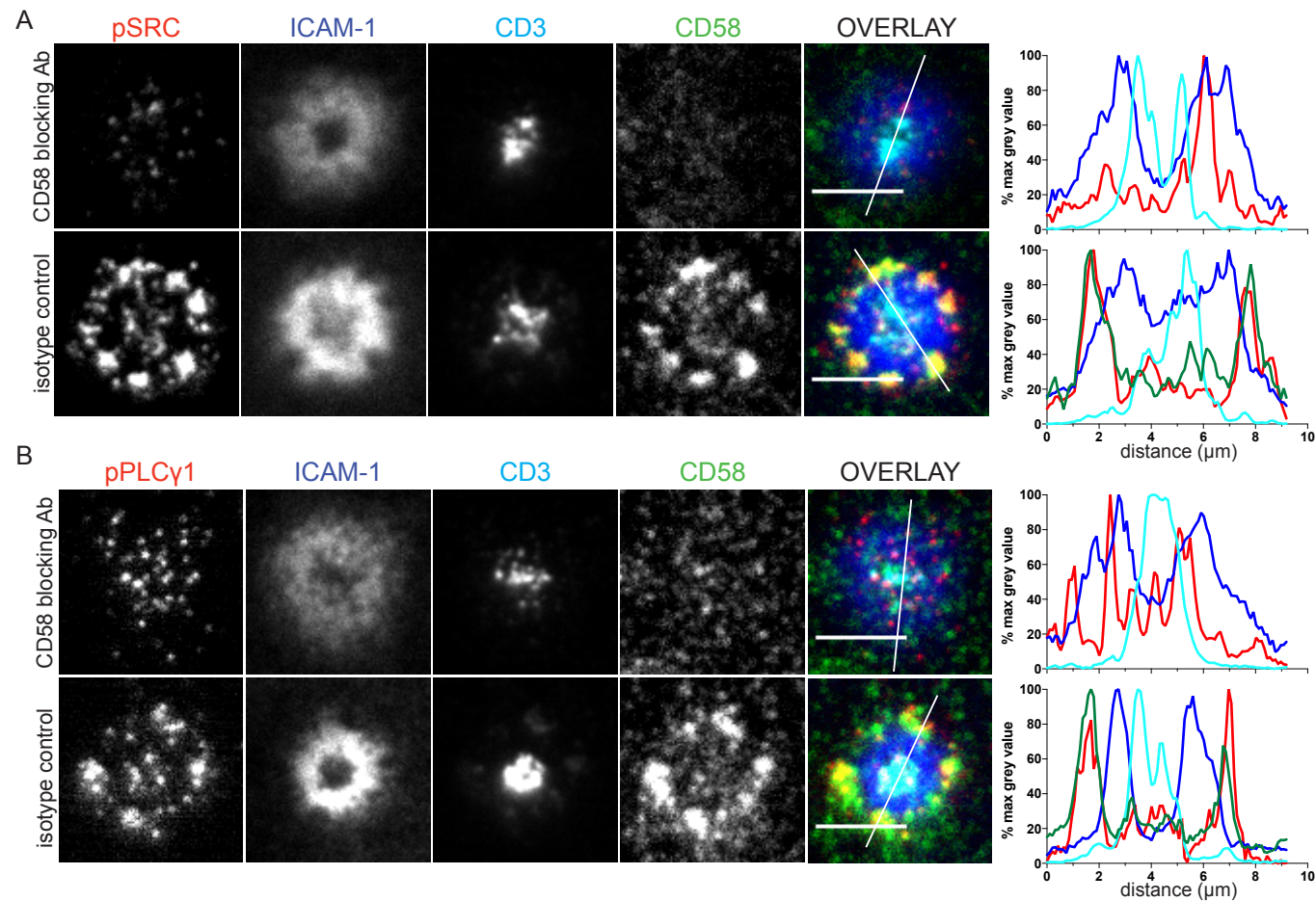
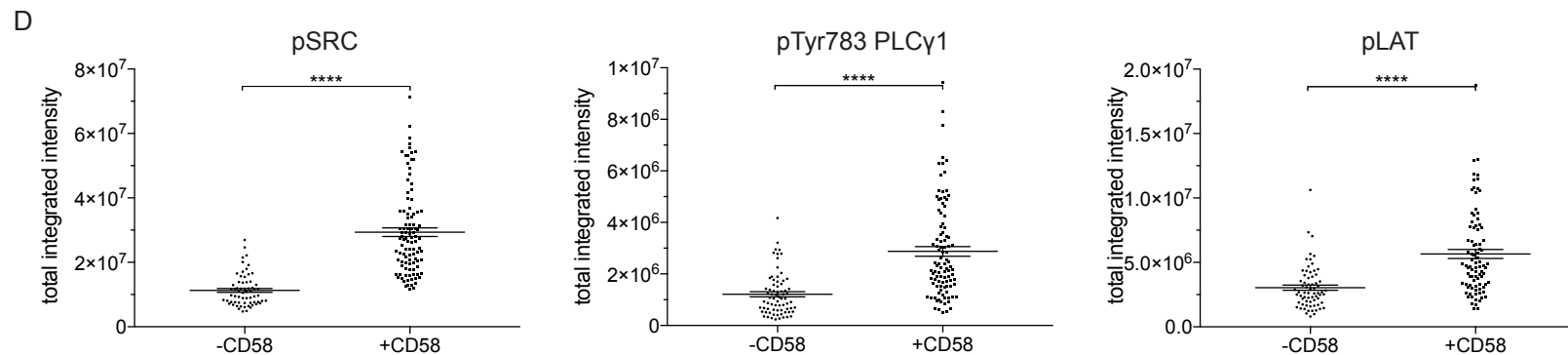
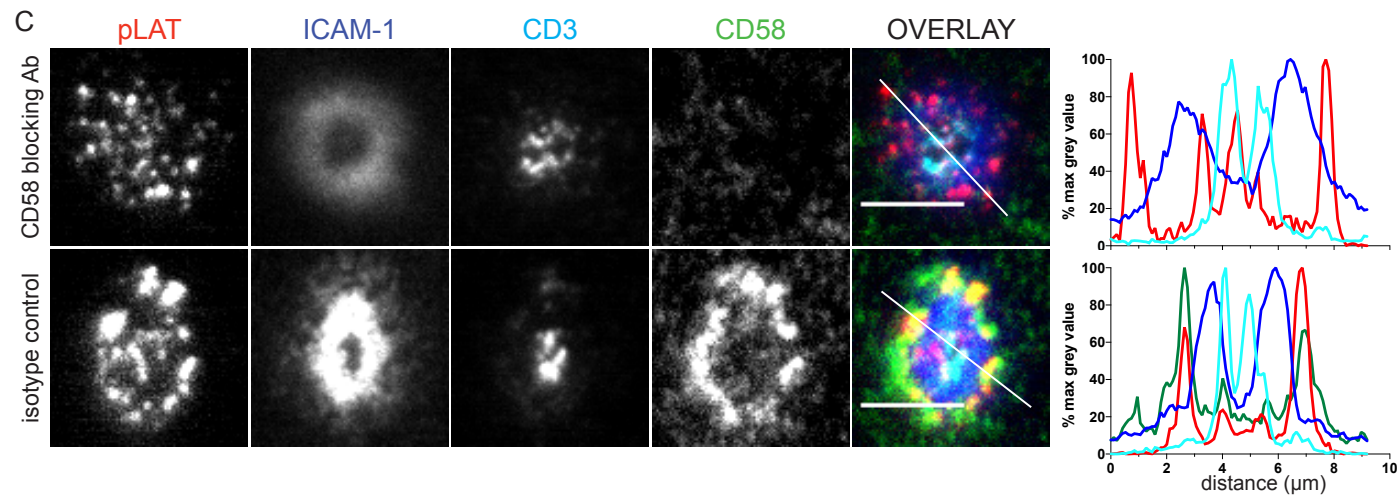


Figure 3.2 CD2 corolla enhances enrichment of classical TCR signalling-linked molecules in the T cell synapse.

Resting human CD8⁺ T cells, isolated from PBMCs, were plated on ICAM-1, blue, ($200/\mu\text{m}^2$), anti-CD3 Fab, cyan, ($30/\mu\text{m}^2$) and CD58, green, ($200/\mu\text{m}^2$) containing SLBs for 15 min before fixation, followed by permeabilisation, blocking and intracellular staining for phosphorylated Src kinases, pTYR416Src, pSrc, red,

(Lck and Fyn in T cells) in (A), pTYR783PLCγ1, pPLCγ1, in (B) or pTYR171LAT, pLAT in (C). SLBs were previously incubated with blocking anti-CD58 mAb (TS2/9 clone) or an isotype control. Same experiment was repeated by omitting CD58 on SLB in the condition where no CD2 engagement is



required, rather than using a blocking agent to prevent CD2-CD58 interactions; the same CD2-induced effects were obtained. Cells were imaged with TIRFM. Scale bar, 5 μ m. Histograms depict the intensity profiles on the diagonal white lines in overlay images. y-axis; raw pixel intensity signal normalized to maximum intensity pixel of each channel. The CD58 signal was omitted in CD58 blocking conditions for the ease of reading the plots clearly. D) The graphs show the levels of pSrc, pPLC γ 1 and pLAT for 60-100 cell/condition shown in A-C without and with addition of CD58 on the SLB. ****, p value < 0.0001 with non-parametric Mann Whitney *U*-test. A representative of three experiments is shown. Error bars represent standard error of mean, SEM.

3.3.3 TCR engagement is necessary for CD2 corolla formation

We sought to investigate what are the essential requirements for formation of the CD2 corolla. 1G4 TCR transfected (1G4+) CD8+ T cells were incubated on ICAM-1, CD58 reconstituted SLBs with either one of NY-ESO-9V loaded HLA-A2 and anti-CD3 Fab or GAG-SL9 loaded HLA-A2 (as a control peptide for 1G4 TCR). At 15 min of synapse formation, as expected, there was formation of the CD2 corolla upon recognition of the NY-ESO-9V cognate antigen or upon CD3 crosslinking with anti-CD3 Fab (Figure 3.3A-B). In the presence of a non-cognate (null) pMHC, there was no CD2 corolla formed. Rather, CD2-CD58 interactions tended to accumulate in a single, trailing macrocluster in cells that appeared to be migrating on ICAM-1 by 15 min (Figure 3.3C). This suggested that productive TCR engagement is needed for the formation of CD2 corolla. Control experiments with ICAM-1, CD58 only reconstituted SLB were also performed with same results as described for null pMHC. Further work in our lab, using an Lck inhibitor, confirmed that TCR signalling, and not just TCR engagement, is also necessary for driving CD2 corolla formation (data not shown).

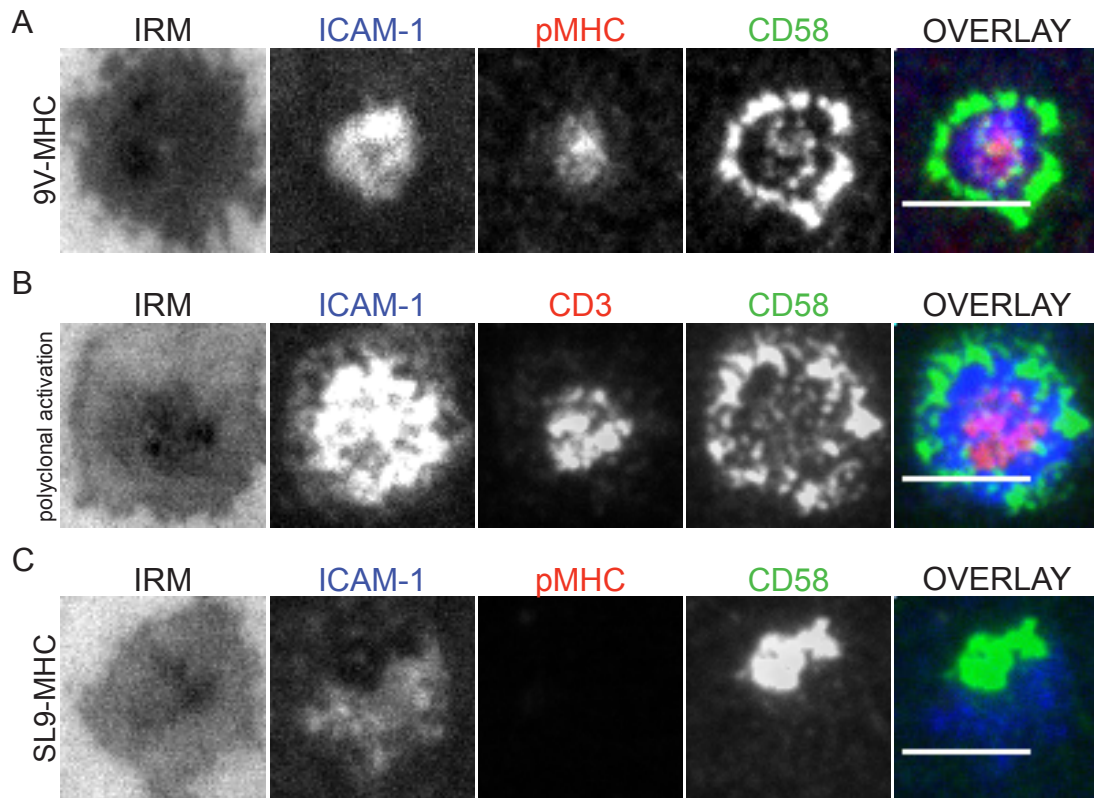


Figure 3.3 TCR signalling is necessary for CD2 corolla formation.

Resting 1G4+ CD8+ T cells, previously isolated from PBMCs were incubated on ICAM-1 ($200/\mu\text{m}^2$), CD58 ($200/\mu\text{m}^2$) containing SLBs in addition to one of A) NY-ESO-9V peptide loaded HLA-A2 ($30/\mu\text{m}^2$) and B) anti-CD3 Fab ($30/\mu\text{m}^2$) or C) GAG-SL9-peptide loaded HLA-A2. Fluorescently labelled streptavidin was used to track the biotinylated NY-ESO-9V peptide-loaded MHC. Cells were imaged with TIRFM. Scale bar, 5 μm . A representative experiment of three is shown.

3.3.4 The role of the actin cytoskeleton in the formation of CD2 corolla

We investigate, further, whether the segregation of ligated CD2 and its localisation in the pSMAC and dSMAC in a corolla structure is an active process, driven by the actin cytoskeleton, acting directly on CD2. One connection between the CD2 cytoplasmic tail and the actin cytoskeleton is the adaptor protein, CD2AP, which interacts with Cortactin/HS-1 and CAPZ to regulate branched actin dynamics, the type of F-actin structure abundant in the dSMAC. Expression of the first N-terminal SH3 domain of human CD2AP (known as CMS, and it has the same protein sequence as the corresponding domain of mouse CD2AP) attached to the coiled coiled region of mouse CD2AP (CD2AP-DN), (kindly provided by Dr Marion Brown, University of Oxford), using RNA transfection of T cells, was used as a way to outcompete the endogenous WT CD2AP and hence uncouple CD2 from its link to the F-actin regulation; we coupled CD2AP-DN to the fluorescent protein, Clover, to allow its tracking during imaging. Overexpression of the soluble form of Clover alone was used as a transfection control (Figure 3.4C and Figure 3.4G). This was performed in, both, human and mouse CD4⁺ T cells; mouse T cells also form a CD2 corolla upon CD2 interaction with CD48. Overexpression of the CD2AP-DN, in either mouse CD4⁺ T cells (Ag-specific SLB), (Figure 3.4A-B), or human CD4⁺ T cells (polyclonal activation SLB), (Figure 3.4E-F) did not impact CD2 corolla formation and resulted in the same percentage of cells forming a CD2 corolla (% corolla+) when compared to the control (Figure 3.4D and Figure 3.4H).

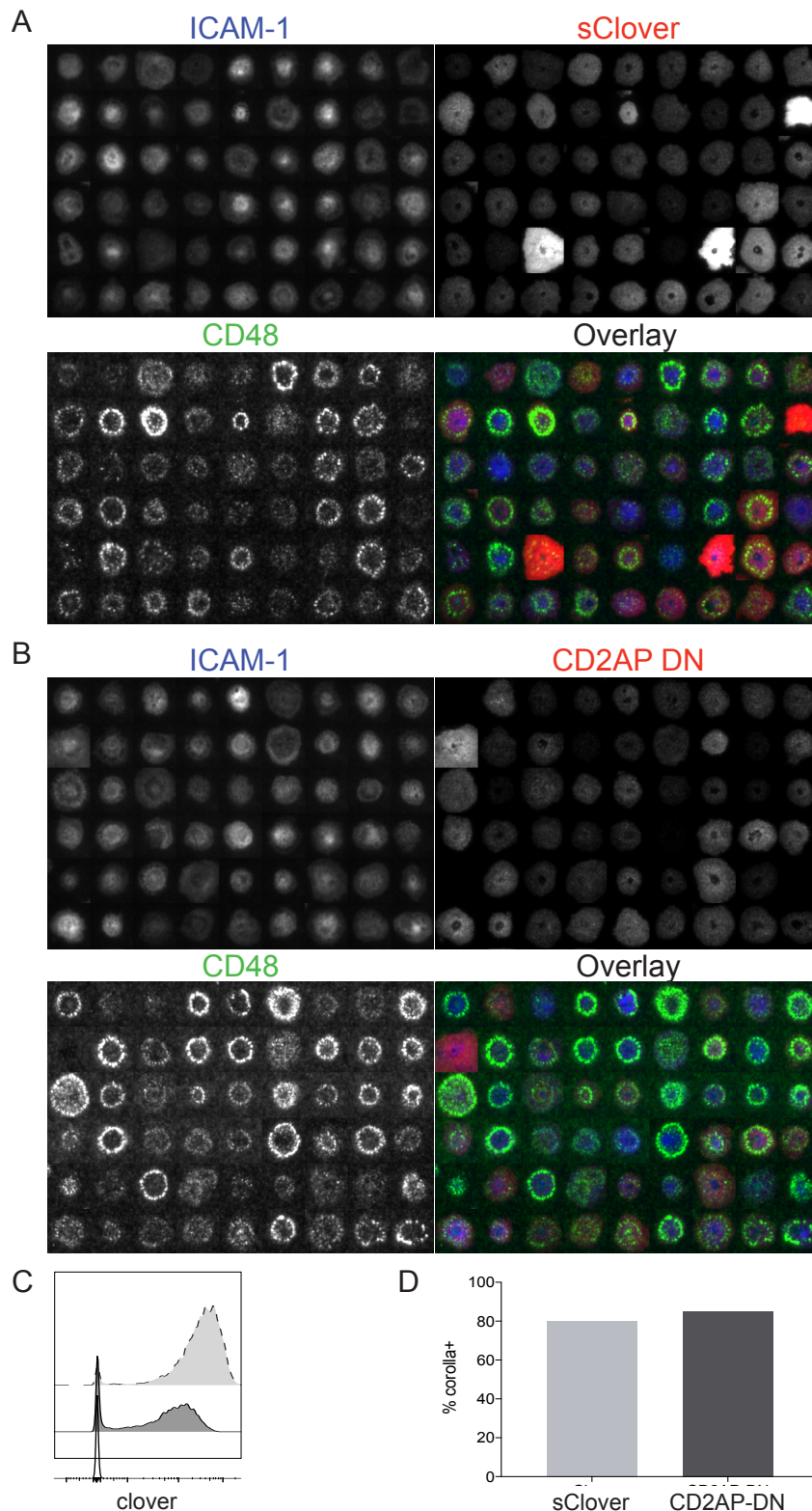
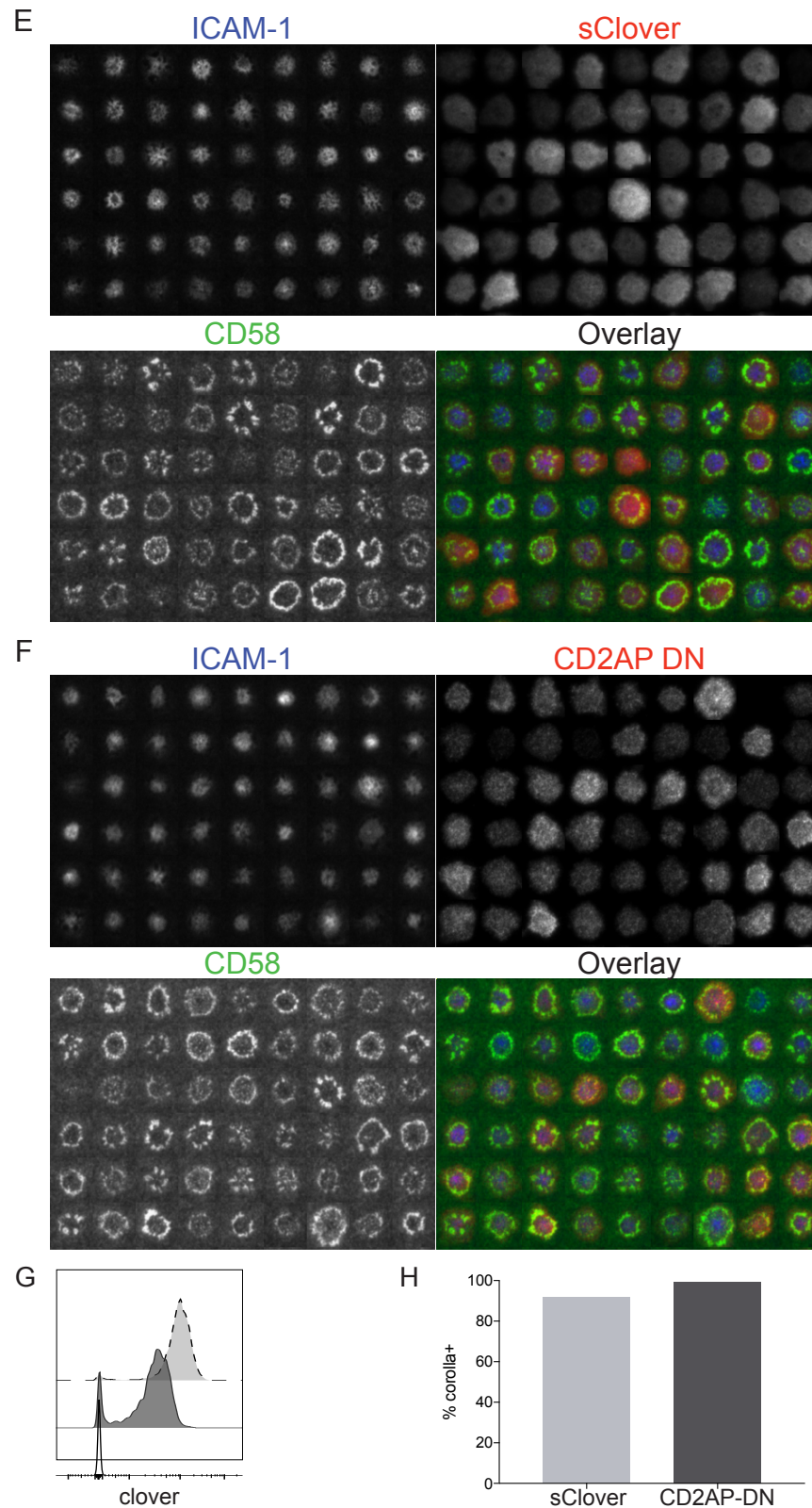


Figure 3.4 CD2 coupling to actin cytoskeleton is not essential for CD2 corolla formation.

AND T cells transfected with either soluble Clover (sClover) (A) or CD2AP-dominant negative-Clover (CD2AP-DN) (B) were incubated on SLB containing ICAM-1 ($200/\mu\text{m}^2$), MCC-I-E^k ($30/\mu\text{m}^2$) and CD58 ($200/\mu\text{m}^2$) for 15 mins followed by fixation. Cells were imaged with TIRFM. The ICAM-1, CD58 and clover signals in the IS of a random selection of AND T cells are shown for each condition. C) The plot shows the transfection levels of sClover (light grey) and CD2AP-DN (dark grey). D) The graph shows the percentage of AND T cells that formed a CD2 corolla amongst clover positive cells, in each condition (% corolla+); 90-100 cells were imaged per condition.



Resting human memory CD4⁺ T cells transfected with either soluble Clover (sClover), (E), or CD2AP-dominant negative-Clover (CD2AP-DN) (F) were incubated on SLB containing ICAM-1 (200/ μm^2), anti-CD3 Fab (30/ μm^2) and CD58 (200/ μm^2) for 15 mins followed by fixation. Cells were imaged with TIRFM. The ICAM-1, CD58 and clover signals in the IS of a random selection of T cells of each transfected condition are shown. G) The plots show the transfection levels of sClover (light grey) and CD2AP-DN (dark grey). H) The graph shows the percentage of T cells that formed a CD2 corolla amongst clover positive cells, for each condition (% corolla+); 90-100 cells were imaged per condition.

3.3.5 The effect of CD2 signalling on the formation of CD2 corolla

The use of RNA overexpression of CD2AP-DN might not have been sufficient to outcompete the endogenous WT CD2AP to uncouple CD2 from the actin cytoskeleton. We, thus, made use of a CD2 construct lacking its cytoplasmic tail effectively removing any direct link of CD2 to the actin cytoskeleton. We coupled the human CD2 construct missing its CD2 cytoplasmic tail (hCD2TM) and the full-length hCD2 (hCD2FL) to a fluorescent protein to allow its tracking with imaging (both parent constructs were kindly provided by Simon Davis, University of Oxford). Mouse AND+ T cells were used as an empty T cell host for expressing the different forms of human CD2 to investigate the role of the CD2 cytoplasmic tail in CD2 corolla formation; i.e whether CD2 signalling and/or coupling to actin cytoskeleton are required for CD2 corolla formation. Both constructs were expressed to levels similar to endogenous CD2 in human T cells (Figure 3.5C). hCD2FL+ or hCD2TM+ AND T cells were incubated on ICAM-1, pMHC, CD58 reconstituted SLBs. Both types of hCD2 resulted in CD2 cluster formation and organisation of these in a corolla structure at 15 min of synapse formation (Figure 3.5A).

We determined the efficiency of the transfected AND T cells in forming a CD2 corolla by quantification of the number of cells that formed a corolla amongst the cells that formed a synapse and were also positive for hCD2 (% corolla+ cells). In two of four experiments, hCD2TM was as almost as efficient as hCD2FL (1st experiment, 98%-100%, 81%-61% respectively). However, in the other two experiments, hCD2TM was 3-fold less efficient when compared to hCD2FL, although still able to form corollas. It must be noted, that in all cases of hCD2+ AND T cells, there was accumulation of CD2-CD58 clusters in the synapse, which were either arranged in a corolla formation or between the cSMAC and pSMAC.

There was a systematic difference between the two sets of experiments. The first two experiments were performed using previously frozen activated AND T cells and the next two experiments were performed with freshly made activated AND T cells. For unknown reasons, the fresh AND T cells were less efficient at corolla formation and revealed a quantitative role of the TM domain in efficiency of corolla formation. Overall, we could conclude from the four experiments that the CD2 cytoplasmic domain is not required for corolla formation, but some activity of the tail contributes to the efficiency of the process. The cytoplasmic tail enables CD2 signalling and coupling to the actin cytoskeleton so both of these factors could be involved in parallel.

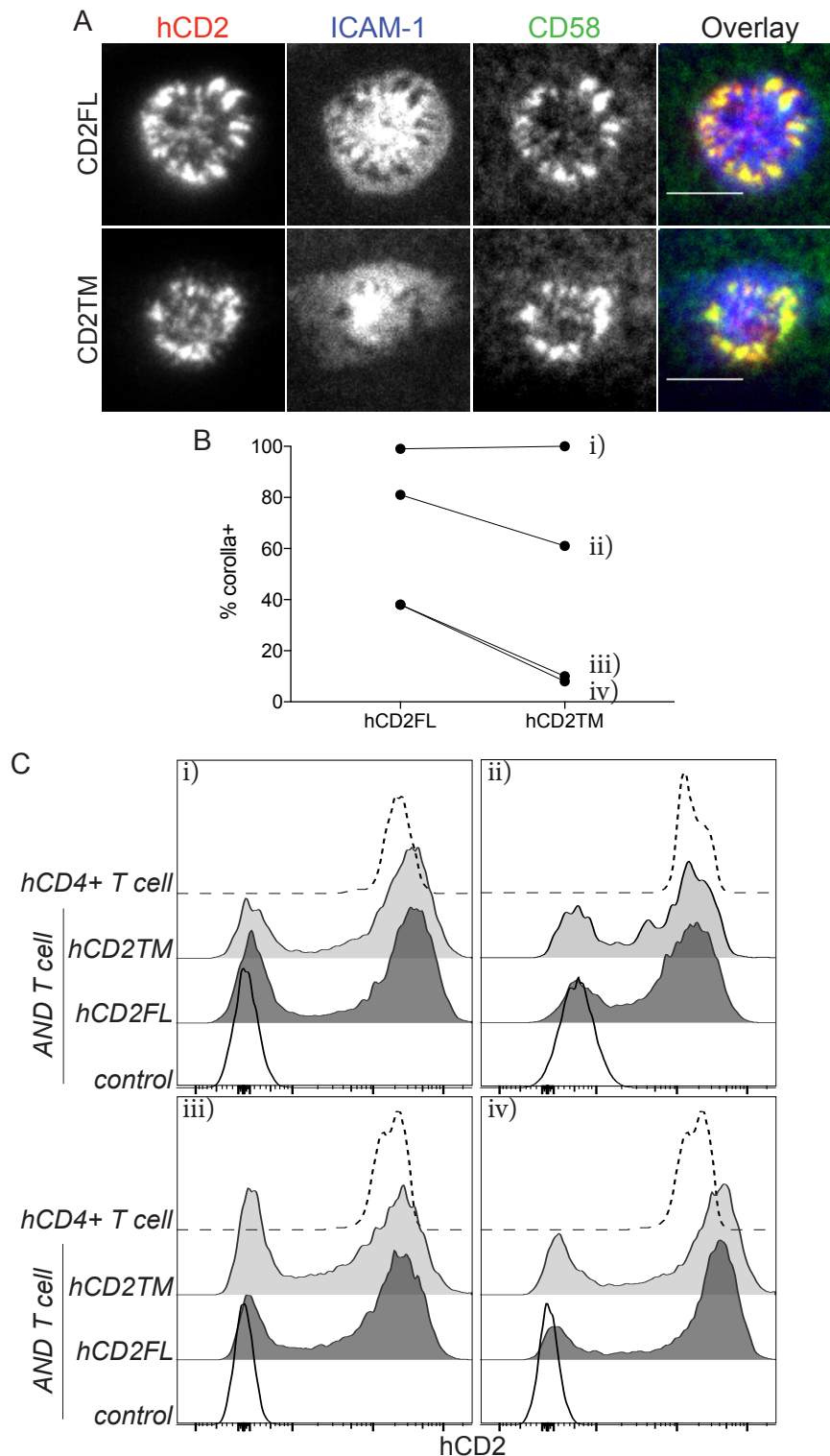


Figure 3.5 The role CD2 cytoplasmic tail in CD2 corolla formation

AND T cells transfected with either human CD2 full length (hCD2FL) or hCD2 lacking the cytoplasmic tail (hCD2TM) at levels comparable to CD2 levels found in human peripheral blood T cells were incubated on ICAM-1 ($200/\mu\text{m}^2$), MCC-I-E^k ($30/\mu\text{m}^2$) and CD58 ($200/\mu\text{m}^2$) for 15 mins followed by fixation and imaged with TIRFM. A) Representative images of hCD2FL transfected T cells (top) and hCD2TM transfected T cells (bottom). One of four representative experiments is shown. B) The graph shows the percentage of transfected T cells that formed a CD2 corolla (% corolla+) in these four experiments; hCD2FL:hCD2TM i) 99%v:100%, ii)81%:61%, iii) 38%:10% iv)38%:8%.60-100 cells were collected per condition D) The CD2 levels of hCD2FL and hCD2TM in transfected AND in the above four experiments; control are stained untransfected AND T cells.

3.3.6 The levels of CD2 in human T cells and their role in CD2 corolla formation

It has been reported previously that naïve and memory T cells subsets express different levels of CD2⁵⁸. This was reproduced, in our hands, and we also performed an absolute quantification to determine the mean number of CD2 molecules expressed in each naïve and memory CD4⁺ and CD8⁺ T cells subset (Figure 3.6A-B). CD2 levels ranged from 2.8×10^4 to 7.6×10^4 molecules per cell, in the different T cell subsets (Figure 3.6B). This probed us to ask the question whether naïve and memory T cells varied in their response in forming a CD2 corolla. When comparing naïve (CD62L⁺CD45RA⁺) and memory T cells (CD62L⁺/-CD45RA⁻) from the same donor, memory T cells accumulated large clusters in the periphery in a corolla arrangement, often found in a complete ring around the pSMAC, resulting in a more intense and thicker CD2 corolla (Figure 3.6C-D). Naïve CD4⁺ T cells had the least intense CD2 corolla with smaller clusters and often forming an interrupted or spotted ring. In fact, in naïve CD4 T cell subset, some of cells did not arrange the CD2-CD58 clusters in a corolla arrangement (Figure 3.6C, top panel). We, thus, determined, the percentage of cells that form a corolla in each T cell subset. All subsets, with the exception of naïve CD4⁺ T cells, had a mean of 85-95% of corolla⁺ T cells. Naïve CD4⁺ T cells had an efficiency of around 60% (Figure 3.6E). Naïve CD4⁺ T cells express the lowest levels of CD2 molecules, 2.9×10^4 molecules/cell, amongst the T cell subsets investigated above, while memory T cells express on average 1.5-2.6-fold higher levels of CD2 molecules. This let us to investigate whether the levels of CD2 on human T cells is a factor controlling the formation of CD2 corolla (see section 3.3.7).

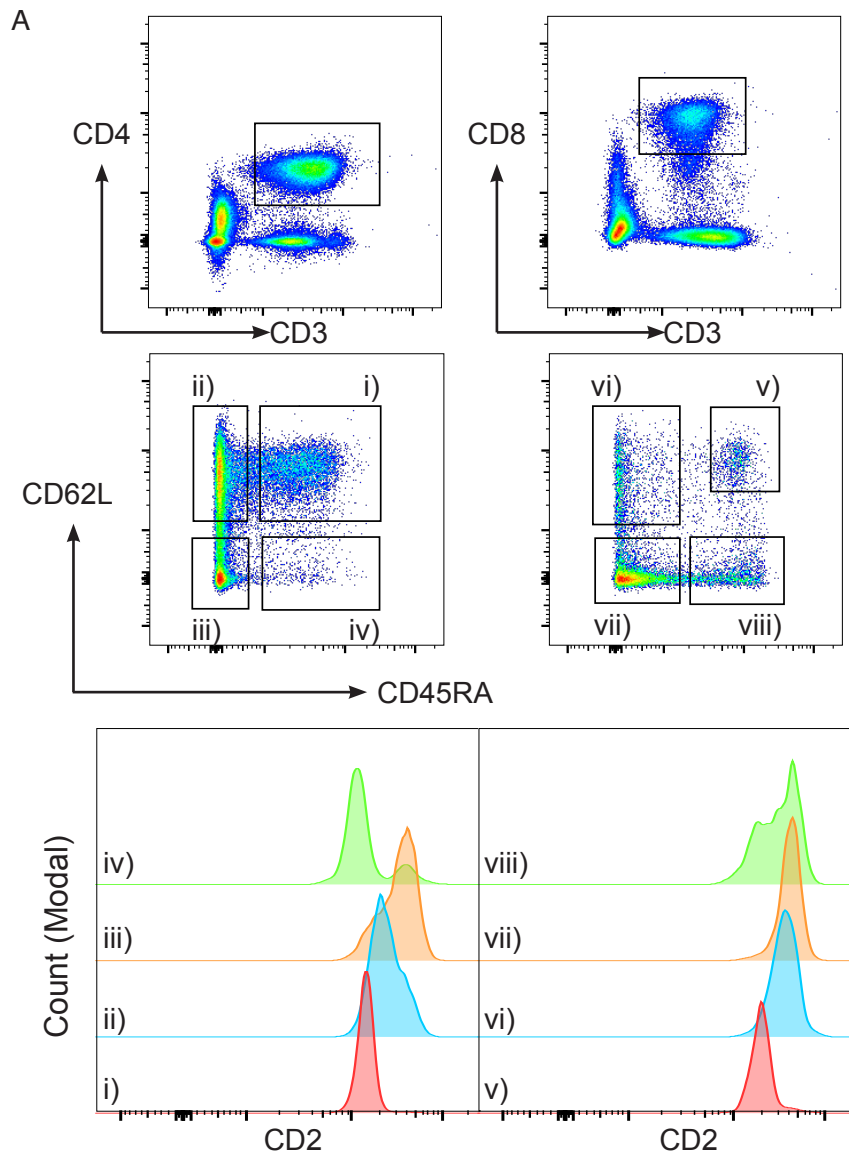
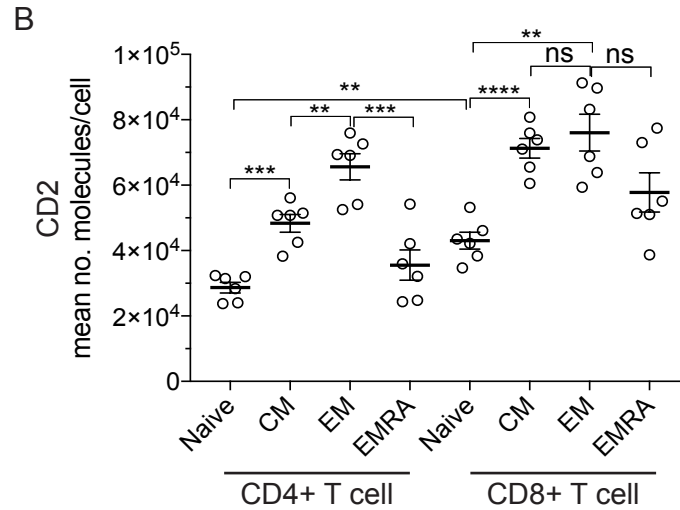
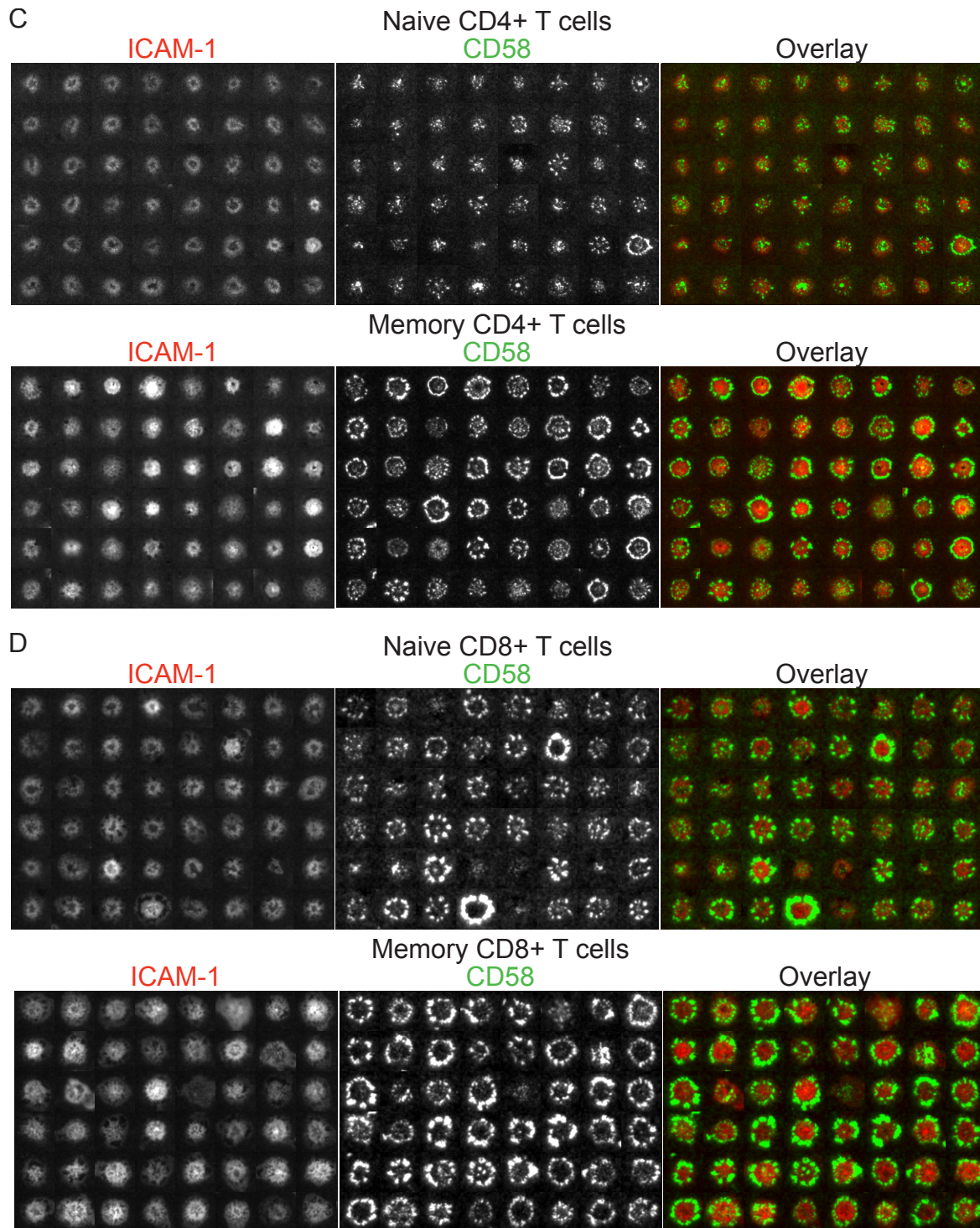


Figure 3.6 Naïve and Memory T cell subsets differ in their organisation of a CD2 corolla

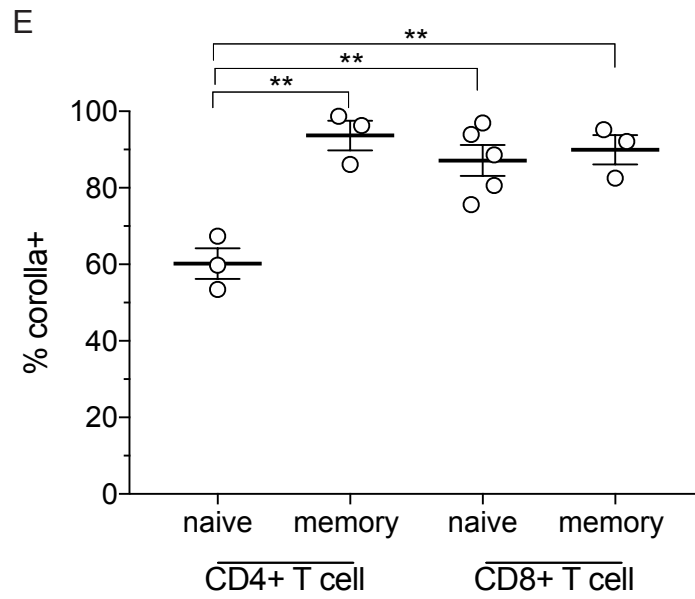
A) The gating strategy, in PBMCs, to quantify with flow cytometry the levels of CD2 in T cell subsets is shown. After gating on single and live cells, CD4+CD3+ or CD8+CD3+ cells were selected and divided in i) naïve CD4+ (CD62L+CD45RA+), ii) CD4+ central memory (CD62L+CD45RA-) iii) CD4+ effector memory (CD62L-CD45RA-) and iv) CD4+ effector memory that re-express CD45RA (CD62L-CD45RA+) and v)-viii) represent the corresponding CD8+ T cell subsets; finally the CD2 levels are shown in histograms in the bottom panel; left, CD4 T cell subsets and right, CD8+ T cell subsets, using the same roman numerals.



B) The expression levels, mean fluorescence intensity, MFI, of CD2, in each of the T cell subsets in (A), were translated into mean number of molecules/cell using antibody-capture calibration beads. The CD2 expression levels of 6 donors and their mean are shown. The mean number of CD2 molecules/cell for each subsets is 2.9×10^4 , naïve, 4.8×10^4 , CM, 6.6×10^4 , EM, 3.6×10^4 EMRA CD4+ T cells and 4.3×10^4 , naïve, 7.1×10^4 , CM, 7.6×10^4 , EM and 5.8×10^4 for CD8+ T cell subsets. ns, non-significant, **, p value < 0.007 , ***, p < 0.007 ****, p value < 0.0001 , with parametric Welch's *U*-test. Error bars represent SEM.



C) Naïve (CD45RA+CD62L+) CD4+ or CD8+ T cells and memory (CD45RA-CD62L+/-) CD4+ or CD8+ T cells were magnetically sorted, with >95% purity, from their respective total T cell populations previously isolated from whole blood. Whenever possible naïve and memory subsets from the same donor were prepared. Isolated T cells were incubated on ICAM-1 (200/ μm^2), anti-CD3 Fab (30/ μm^2) and CD58 (200/ μm^2) containing SLBs, for 15 mins followed by fixation. The ICAM-1 and CD58 signal in the IS of a random selection of naïve and memory CD4+ T cells from the same donor is shown in (C) and similarly for naïve and memory CD8+ T cells from the same donor in (D) (different to donor in (C)).



E) The percentage of T cells forming an immunological synapse with a CD2 corolla in experiments such as in (C-D), was determined for each naïve and memory T cell subset. At least 3 donors were tested for each T cell subset. **,p value <0.006, with parametric Welch's *U*-test. Error bars represent SEM.

3.3.7 The contribution of CD2 levels in driving CD2 corolla formation

AND+ T cells were used as an 'empty' T cell host to transfect different levels of hCD2FL. The mean number of hCD2FL molecules in transfected AND+ T cells was also quantified (Figure 3.7A) and compared to human CD2 levels that ranged from 2.9×10^4 - 7.6×10^4 molecules/cell. AND+ T cells expressing different levels of hCD2FL, ranging from 4.9×10^3 - 4.4×10^4 molecules per cell, were incubated on ICAM-1, pMHC, CD58 reconstituted SLBs (Figure 3.6B-E). The number of cells forming a CD2 corolla was determined and expressed as a percentage of the cells forming a synapse and expressing hCD2; hCD2FL was tagged with a fluorescent protein to allow its tracking when imaging. There was an increasing percentage of corolla positive cells (% corolla+) with increasing levels of transfected hCD2FL and this positively correlated with the mean number of hCD2FL molecules per cell (Figure 3.6F). Increasing numbers of hCD2FL molecules on the T cell membrane could determine the number of hCD2FL receptors that ultimately result in productive CD2-CD58 interactions hence cluster formation. This will, subsequently, determine the levels of downstream CD2 signalling which is also a factor influencing the efficiency of CD2 corolla formation (see section 3.3.5).

A

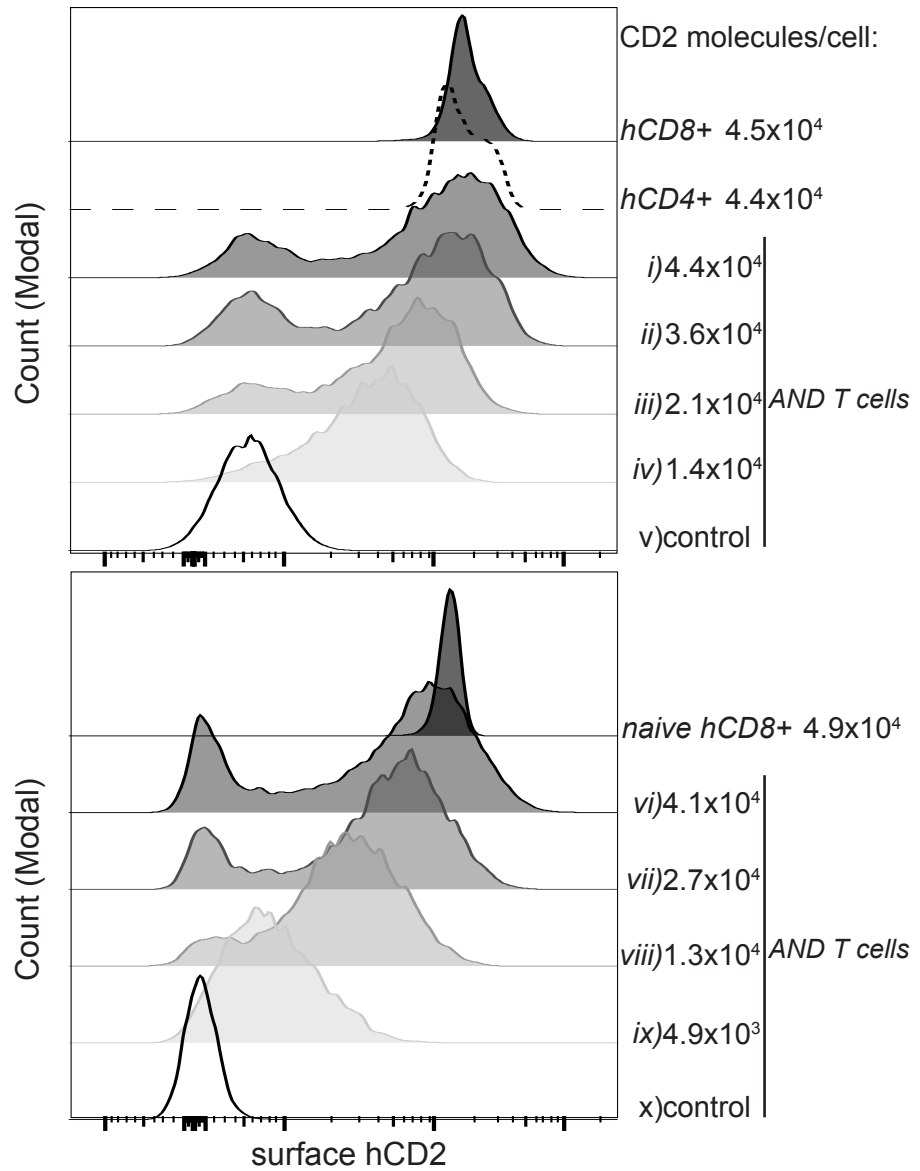
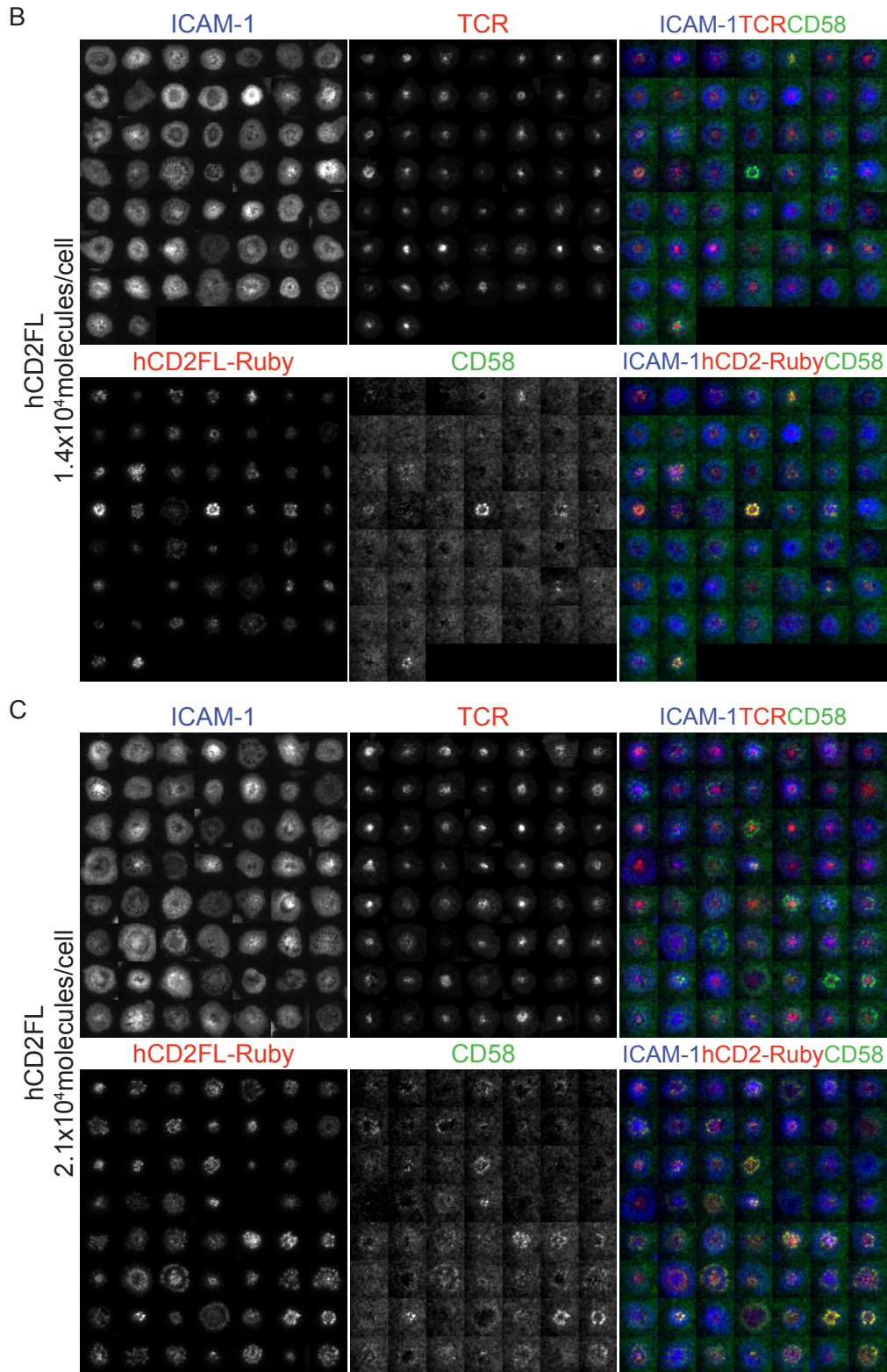
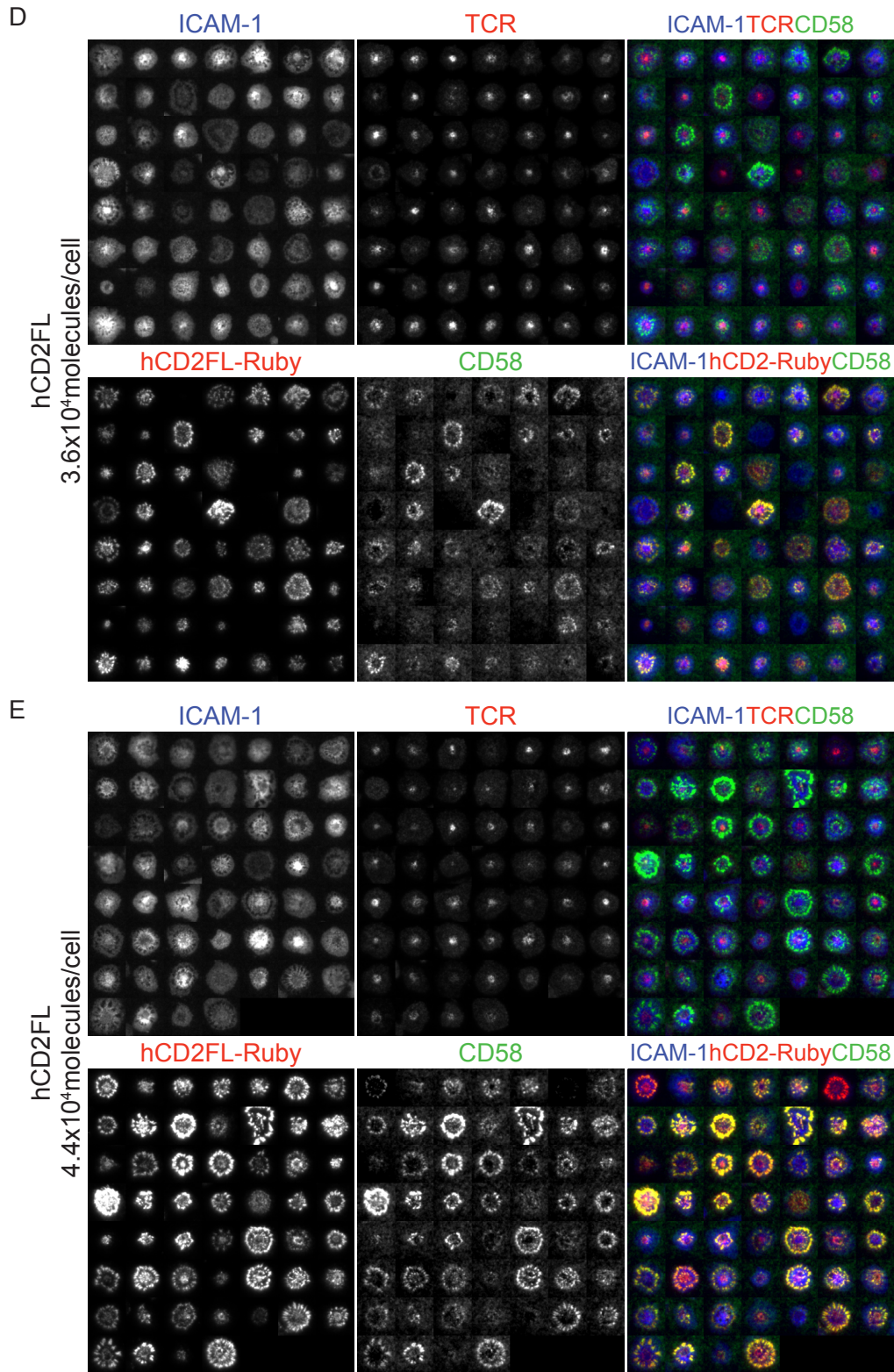


Figure 3.7 CD2 surface levels contribute to the efficiency of CD2 corolla formation.

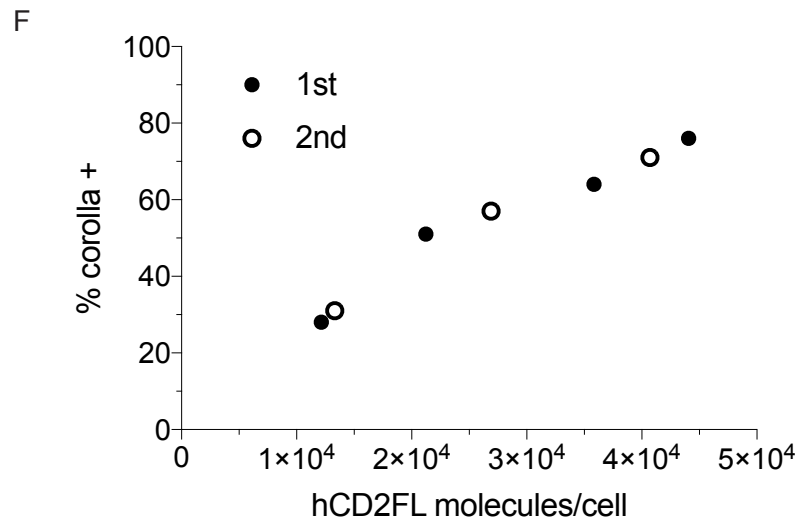
A) AND T cells (previously frozen) were transfected with different levels of human CD2 (hCD2; full length protein), ranging from 4.9×10^3 – 4.4×10^4 molecules/cell shown on the right of the plots, and compared to CD2 levels found in human peripheral blood T cells. Two experiments were performed and shown here. Control cells were stained untransfected AND T cells (empty solid line histogram).



Transfected AND T cells, with increased levels of surface hCD2, (B-E), were stained with fluorescently labelled anti-mouse TCR β Fab and were incubated on ICAM-1 (200/ μm^2), MCC-I-E^k (30/ μm^2) and CD58 (200/ μm^2) containing SLBs for 15 min followed by fixation and imaged with TIRFM. The ICAM-1, TCR, CD2 and CD58 signals in the IS of a random selection of AND T cells are shown for one of the experiments shown in A)i)-iv) corresponding to (B-E).



Transfected AND T cells, with increased levels of surface hCD2, (B-E), were stained with fluorescently labelled anti-mouse TCR β Fab and were incubated on ICAM-1 (200/ μm^2), MCC-I-E^k (30/ μm^2) and CD58 (200/ μm^2) containing SLBs for 15 min followed by fixation and imaged with TIRFM. The ICAM-1, TCR, CD2 and CD58 signals in the IS of a random selection of AND T cells are shown for one of the experiments shown in A)i)-iv) corresponding to (B-E).



F) The graph shows the percentage of transfected T cells that formed a CD2 corolla (% corolla+) in the aforementioned two experiments compared to the mean number of CD2 molecules expressed by each AND T cell population. 1st experiment corresponds to (A, top graph) and images shown in (B-E) and 2nd experiment corresponds to (A, bottom graph).

3.4 Discussion

Engagement of CD2 during the formation of a classical bull's eye immunological synapse has revealed a unique CD2 adhesion ring around the LFA-1-ICAM-1 enriched pSMAC effectively segregating CD2 clusters and the TCR-enriched cSMAC. Kaizuka *et al* previously reported segregation of CD2 and TCR upon engagement of both receptors by laterally mobile ligands on SLBs in the absence of a LFA-1-ICAM-1 adhesion. Our report of the CD2 corolla in T cells interacting with ligand reconstituted SLBs or antigen expressing APCs, is thus a new unique finding describing the localisation of an adhesion/costimulatory molecule in an unexpected area of the IS. In agreement with previous reports ^{20,64,66,82,94}, CD2 clusters found in a corolla arrangement, were also enriched in activated forms of classically linked TCR-signalling molecules such as pLAT, pPLC γ 1 and pSrc kinases. We propose that CD2 corolla effectively enhances TCR signaling by sustaining CD2 clusters in the periphery of the IS where signalling takes place as previously reported¹⁹ in contrast to terminating signaling by translocation to the TCR-enriched SMAC. In addition, we propose that these peripheral CD2 clusters act as close contact areas where CD45 is excluded, hence favoring a shift in the equilibrium of kinases and phosphatases towards kinase, and ultimately favouring productive TCR engagement, that takes place in the periphery¹⁹, and downstream signalling. Interestingly, the inverse interaction i.e. engagement of non signaling form of CD48 in the human T cell Jurkat line incubated on SLBs containing rat CD2 (similar affinity to human CD2:CD58 interaction) results in CD2 cluster accumulation in the interface that exclude CD45¹⁹⁰. Such an experimental set up would be useful to address the question whether a CD2 corolla forms in the context of 'bull's eye' IS by engaging the glycosylphosphatidylinositol(GPI)-anchored CD58 on activated T cells.

The CD2 corolla is a dynamic adhesion ring formed by both human CD4⁺ and CD8⁺ T cells and mouse CD4⁺ T cells. We tested corolla formation in both peripheral T cells and T cells isolated from human tonsils; both T cell populations arranged their ligated CD2 in the peripheral ring we have termed CD2 corolla. Peripheral human naïve CD4⁺ T cells had a lower percentage of corolla positive T cells when tested on ligand reconstituted SLBs. We, thus, investigated, whether this could be attributed to the lower CD2 expression of naïve CD4⁺ T cells. hCD2FL titration experiments in AND T cells showed that the number of CD2 molecules on the T cell membrane is influencing the percentage of T cells that form a corolla; the higher the CD2 levels, the higher the percentage of T cells that form a CD2 corolla upon IS formation. This provided evidence to support the hypothesis that the lower percentage of corolla positive T cells in naïve CD4⁺ T cells could be explained by the lower CD2 expression in naïve CD4⁺ T cells.

Increasing the number of surface CD2 molecules results in a higher percentage of T cells forming a CD2 corolla. Higher levels of CD2 molecules will result in stronger CD2 signalling, this has a positive feedback loop in CD2 clustering that ultimately influences CD2 corolla efficiency; previously described in^{91,92}. In fact, we are suggesting, here, that TCR signalling is needed for CD2 corolla formation and CD2 corolla itself is boosting TCR signalling, then we could propose the following model. Increasing number of CD2 molecules, on the cell membrane, would increase CD2 signalling upon CD2 engagement, and this would result in a stronger enhancement of TCR signalling and in turn enhanced TCR signalling would further favour CD2 corolla formation; in other words CD2 and TCR signalling and corolla formation are inter-linked. The question then becomes whether TCR interaction of different affinities impacts in any way this peripheral CD2 localisation. Finally, we show that a CD2 construct lacking the cytoplasmic

tail, hCD2TM, is potentially less efficient in forming a CD2 corolla when expressed at lower levels to those found normally in peripheral human T cells. Titration experiments of hCD2TM, similar to the ones performed with hCD2FL, should provide further evidence to support our current findings. We only investigated how the complete loss of all the cytoplasmic domains of CD2 or how blockade of the C-terminal proline-rich motifs that recruit adaptors coupling CD2 to actin cytoskeleton are contributing to CD2 corolla formation. Potentially one or more of the domains of the cytoplasmic tail might be playing a role in CD2 corolla formation. For example, it has been reported previously that two different domains of CD2 are responsible for the TCR-mediated increase in avidity of CD2 for its ligand and the downstream signalling initiated by CD2 ligation resulting in enhanced IL-2 production upon T cell activation^{71,72,77,91}.

4 Chapter 4. CD2 corolla and its effect on other T cell costimulatory/inhibitory receptors.

4.1 Introduction

The biochemical pathways downstream of CD2, CD28, ICOS and PD-1 signalling have been investigated extensively^{108,121,143,157}. However, new significant functions of these receptors are still being revealed¹⁹¹, and extensive interest in these molecules as therapeutic targets requires deeper investigation. One such unappreciated area of research is how costimulatory receptors might influence each other at the very early stages of T cell signal integration that is taking place during immunological synapse formation. For example, no study, to our knowledge, has looked at the dynamics of the immune synapse when both CD28 and CD2, or ICOS and CD2 costimulatory receptors are engaged at the same time and whether one influences the other in any way. In light of our discovery of the CD2 corolla as a new structure in the IS, we seek to investigate how other molecules are arranged relative to the CD2 corolla. A comment by Ron Vale after his study with Douglass A⁹⁴, nicely summarises our aim; “Many people in the signaling field think about which molecules interact with one another,” he says, “but the issue of how the molecules are organized spatially and how this affects their function is often not addressed.”

4.2 Hypothesis

The CD2 corolla influences the localisation of other co-stimulatory/inhibitory receptors in the IS, acting as a ‘second layer of regulation’ in addition to TCR signalling, to optimise signal integration during the formation of IS.

4.3 Results

4.3.1 The effect of CD2 corolla on CD28-CD80 interaction during T cell synapse formation

Current studies investigating the localisation of CD28 upon engagement by CD80/CD86 have shown that it generates peripheral microclusters (1-2mins) that form an annular at later stages when a TCR-enriched cSMAC is formed⁵⁰. Studies have also looked into the signalling and biochemical pathways triggered by ligating CD2 and CD28 separately or together^{105,106,108,112}. However, there have been no studies looking at their localisation when both are ligated by freely diffusing CD58 and CD80 during T cell synapse formation.

Having in mind the above studies and our initial observation of the unique distribution of CD2-CD58, we sought to visualise the CD28-CD80 interactions during the formation of CD2 corolla in the IS. We tracked ligated CD28 using fluorescently tagged CD80 on the SLBs; the limitation of this approach for tracking activated CD28 is discussed below in section 4.4. During the initial stages (1-2min) of synapse formation, CD2-CD58 and CD28-CD80 microclusters seem to co-localise and so where the CD3 and CD28 microclusters but not enough analysis was performed at this timepoint (data not shown). Upon CD2 corolla formation i.e. 5-15 min of synapse formation, there was significant enrichment of CD28-CD80 clusters outside the LFA-1-ICAM-1 enriched pSMAC; this signal colocalised with the CD2 corolla (Figure 4.1A, bottom panel). In the absence of CD2 corolla, CD28-CD80 formed an annular ring around the cSMAC (Figure 4.1A, top panel) and/or accumulated inside and around the cSMAC (data now shown). The above type of experiment was performed in a polyclonal activation SLB system using either CD4⁺ and CD8⁺ T cells blasts, freshly isolated resting total CD4⁺ and CD8⁺ T cell populations (Figure 4.1B, top panel) or naïve and memory CD8⁺ T cell populations.

All populations showed the same phenomenon of CD28-CD80 cluster localisation in the periphery of the IS, within the CD2 corolla area. This phenotype was reproduced using an antigen-specific SLB system, using transfected 1G4 TCR CD8+ T cells, (1G4+ CD8+ T cells) (Figure 4.1B, bottom panel).

We sought to investigate whether CD2 signalling is required for relocating CD28-CD80 clusters to the CD2 corolla. The fluorescently tagged tailless form of human CD2 (hCD2TM-Ruby) was transfected in AND+ T cells at similar levels to human T cells. Transfected AND+ T cells were incubated for 12-15 min on ICAM-1, pMHC, CD58 and CD80 reconstituted SLBs. hCD2TM+ AND+ T cells that formed a CD2 corolla, showed sustained formation of CD28-CD80 clusters in the periphery of the synapse outside the LFA-1-ICAM-1 enriched pSMAC; these clusters co-localised with hCD2TM-Ruby clusters i.e the CD2 corolla (Figure 4.1C). Within the transfected AND+ T cells, some T cells were not successfully transfected hence no hCD2TM-Ruby signal was detected in the synapse. In these cells, CD28-CD80 clusters formed the annular ring around the TCR-enriched cSMAC.

Engagement of CD28 results in enhanced and sustained recruitment of adaptor and signalling molecules such as PKC- θ and PI3K, forming a complex at the cytoplasmic tail of CD28^{50,51,106,111,143,147}. Ligated CD28 boosts and structures PKC- θ signal at the synapse in defined clusters colocalising with CD28-CD80 clusters. We, thus, looked into the PKC- θ recruitment at CD28-CD80 clusters in the presence and absence of CD2 corolla. In the absence of CD2 corolla, CD28 formed an annular ring around the cSMAC that colocalised with PKC- θ clusters (Figure 4.1D, top panel). Upon corolla formation, CD28-CD80 clusters were mainly detected in the periphery of the pSMAC as well as within the pSMAC. The majority of the PKC- θ clusters were detected within the CD2 corolla and colocalised with

the peripherally sustained CD28-CD80 clusters (Figure 4.1D, bottom panel). This was reproduced in antigen-specific SLB system by incubating transfected 1G4+ CD8+ T cells on ICAM-1, pMHC, CD58 and CD80 reconstituted SLBs (Figure 4.1E). These results are suggesting that the CD28-CD80 clusters retained in the periphery of the IS because of the CD2 corolla are actively signaling hence the presence and colocalisation of PKC- θ signal with these clusters.

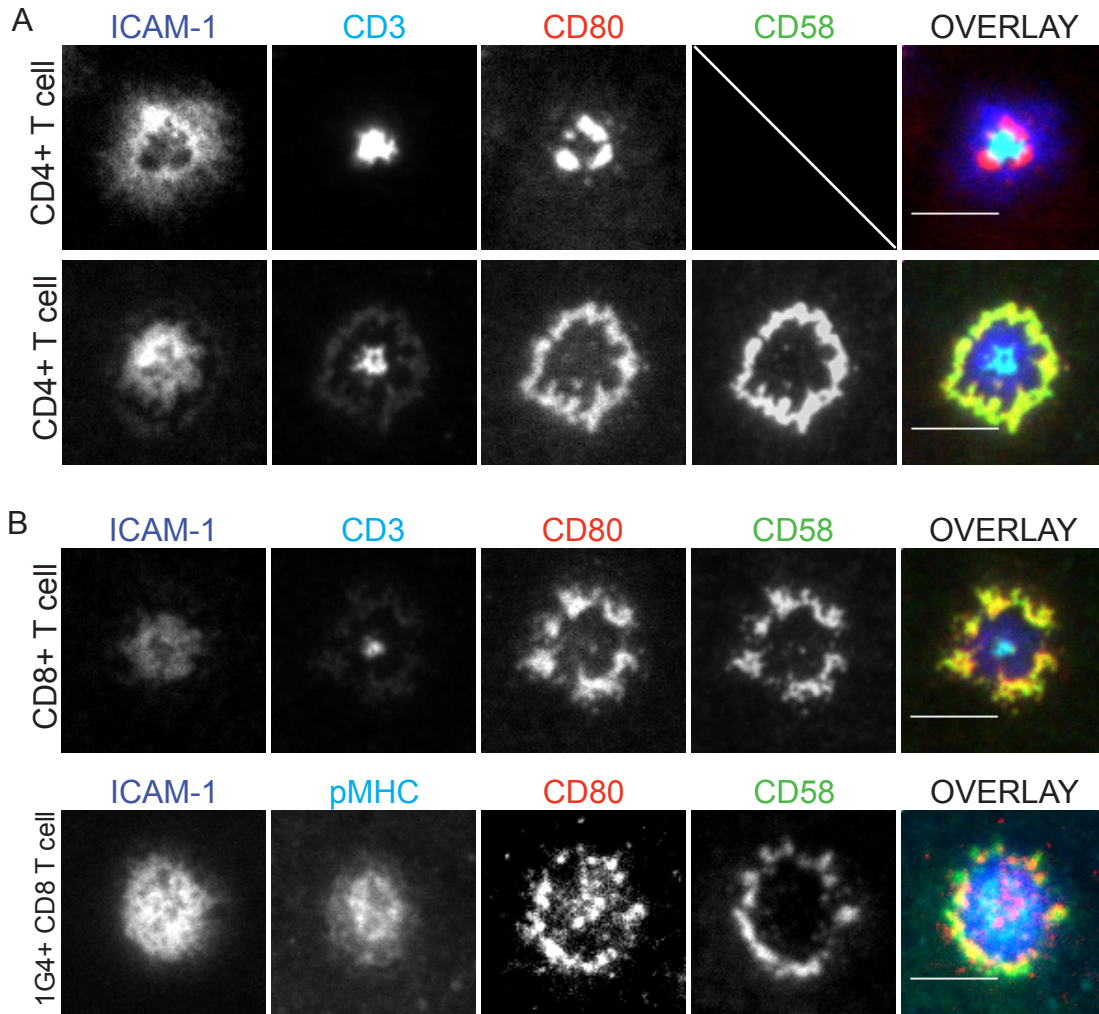
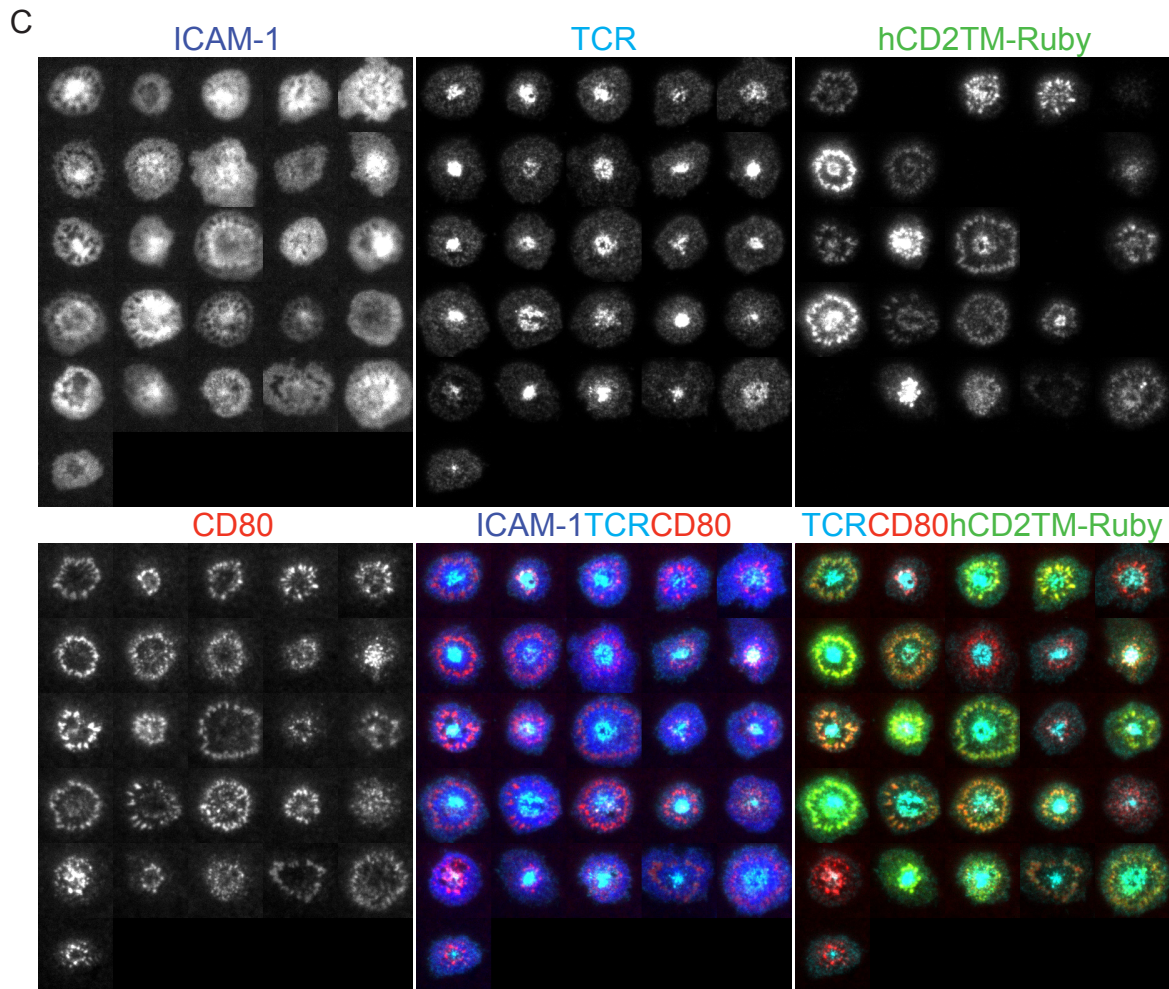
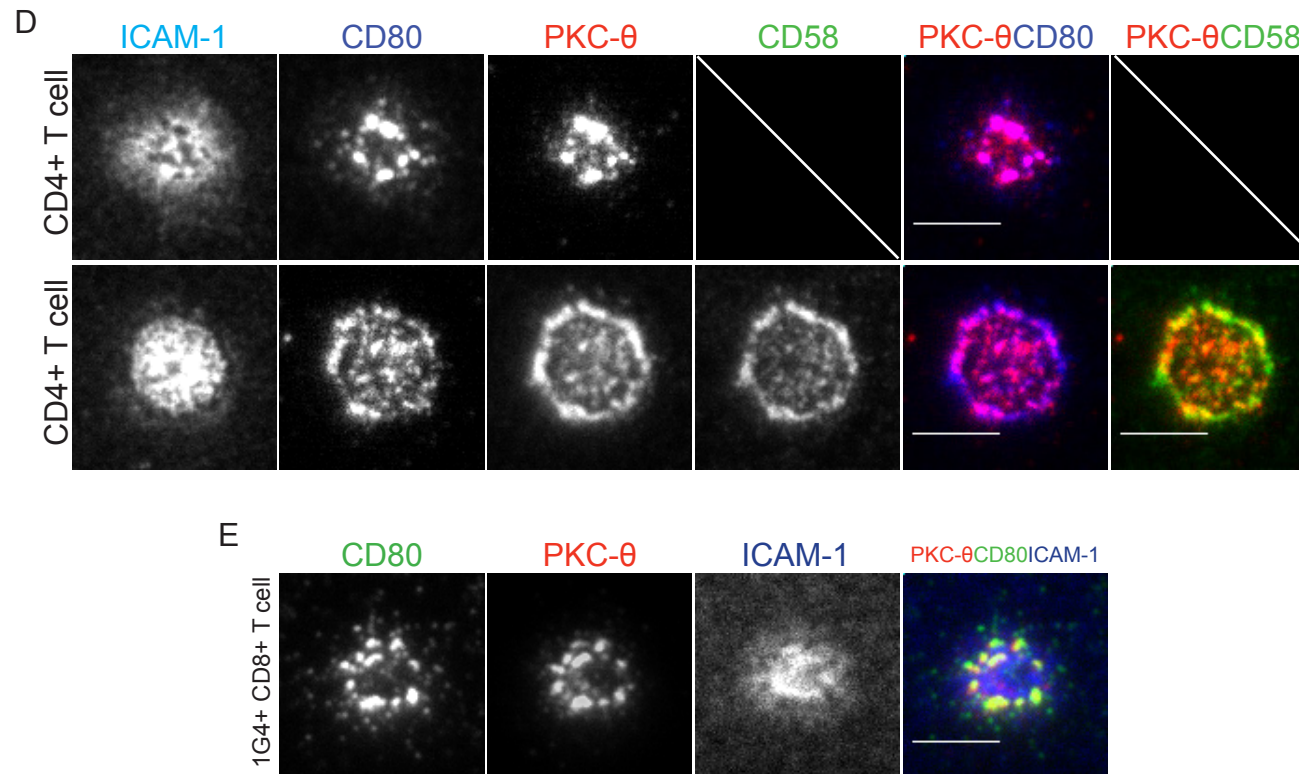


Figure 4.1 CD2 corolla sustains CD28-CD80 clusters in the periphery of the T cell synapse.

A) Resting human CD4+ T cells were incubated on SLB containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) without CD58 (top) and with CD58 ($200/\mu\text{m}^2$), (bottom) and real-time imaged with TIRFM after 10-15 min of contact with SLB. A representative of three independent experiments is shown. B) (top) Resting human CD8+ T cells were incubated on SLBs containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) with CD58 ($200/\mu\text{m}^2$), for 15 min before fixation. Cells were imaged with TIRFM. A representative of at least three independent experiments is shown. (bottom) Resting human CD8+ T cells were transfected with 1G4 TCR and incubated on SLB containing ICAM-1 ($200/\mu\text{m}^2$), NY-ESO-9V peptide loaded HLA-A2 ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) with CD58 ($200/\mu\text{m}^2$) and real-time imaged with TIRFM at 10-15 min after contact and/or fixed at 15 min of incubation. A representative of two independent experiments is shown. Scale bar, 5µm. C) (see next page)



C) AND T cells were transfected with human CD2 lacking its cytoplasmic tail (hCD2TM) at comparable CD2 levels found in human peripheral blood T cells. Transfected T cells were stained with fluorescently labelled anti-mouse TCR β Fab and were incubated on ICAM-1 ($200/\mu\text{m}^2$), MCC-I-E^k ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) and CD58 ($200/\mu\text{m}^2$) containing SLBs for 15 min before fixation. Cells were imaged with TIRFM or a wide field microscope. The ICAM-1, TCR, CD2TM, CD80 signals of the IS of a random selection of AND T cells are shown in panels. A representative of three independent experiments is shown. Images taken with TIRFM are shown here.



D) Human CD4+ T cells were incubated on SLB containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) without CD58 (top) and with CD58 ($200/\mu\text{m}^2$) (bottom), fixed at 15 min incubation, permeabilised, blocked and stained for total PKC- θ . Cells were imaged with TIRFM. A representative of three independent experiments is shown. E) 1G4 TCR transfected resting CD8+ T cells were incubated on SLBs containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) and CD58-unlabelled ($200/\mu\text{m}^2$). A representative of two experiments is shown. Scale bar, $5\mu\text{m}$.

4.3.2 The effect of CD2 corolla on ICOS-ICOS-ligand interaction during T cell synapse formation

We examined whether the effect of CD2 corolla on CD28 clusters during synapse formation was unique to CD28 or if it also applied to other costimulatory receptors interactions such as ICOS-ICOS-L. In these experiments, we have used either CD4⁺ T cell blasts that were enriched in ICOS⁺ T cells or two Influenza H3 hemagglutinin(HA)-specific, HLA-DRB1*0901-restricted, CD4⁺ T cells clones, that are selected for being ICOS⁺ (Figure 4.2B); these were kindly provided by Dr Yanchun Peng (WIMM, University of Oxford). In both, CD4⁺ T cells blasts (Figure 4.2A, bottom panel) and HA-specific CD4⁺ T cell clones, CD2 corolla sustained ICOS-ICOS-L clusters in the periphery of the LFA-1-ICAM-1 enriched pSMAC (Figure 4.2C). In the absence of CD2 corolla, ICOS-ICOS-L clusters were instead enriched within and around the TCR-enriched cSMAC (Figure 4.2A, top panel). The antigen-specificity of the SLB was confirmed by the use of CLIP-loaded MHC instead of the cognate-antigen loaded MHC (data not shown).

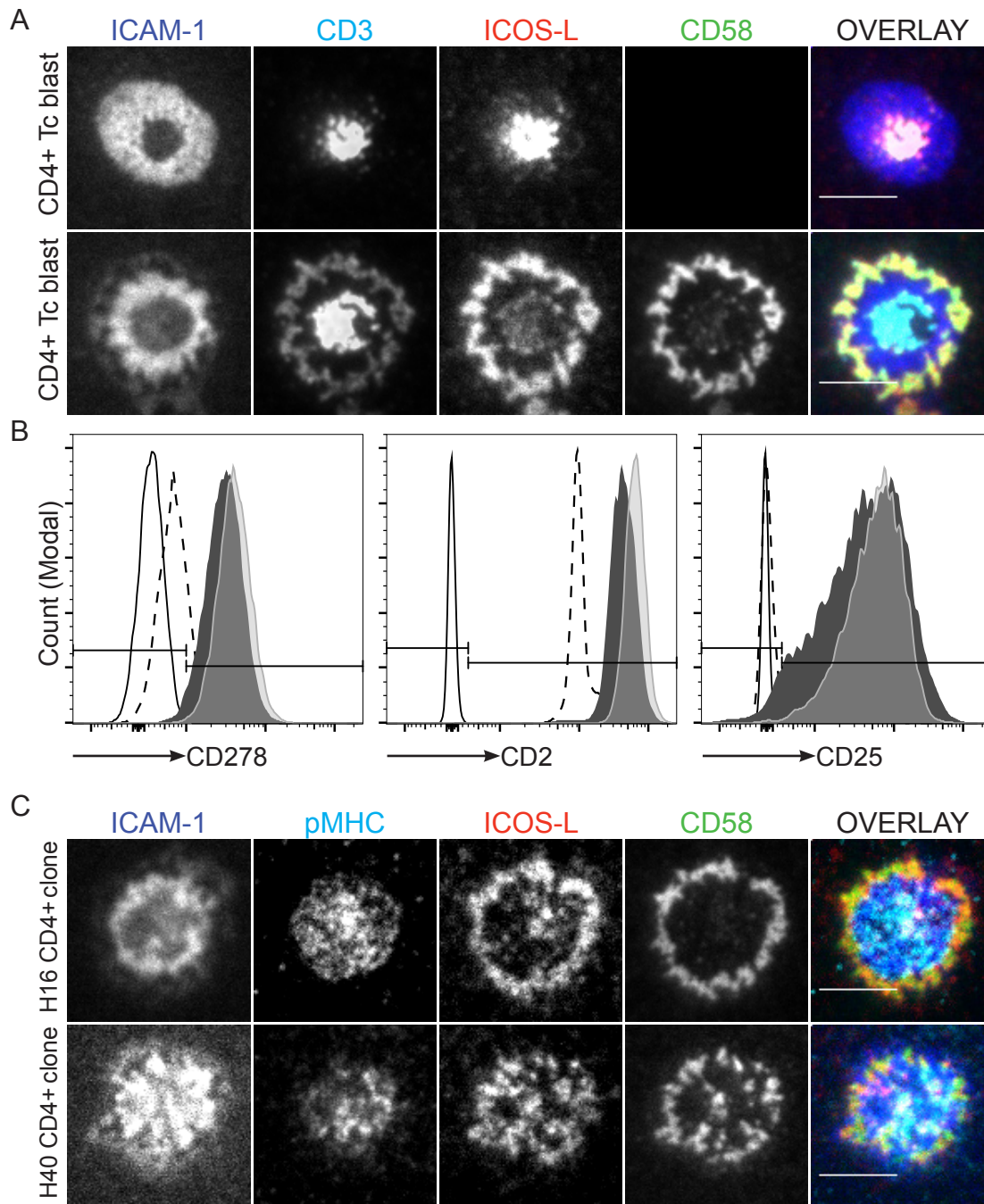


Figure 4.2 CD2 corolla sustains ICOS-ICOS-L clusters in the periphery of the T cell synapse

A) Activated human CD4⁺ T cells, T cell blasts (Day 4-5 post removal from stimulation; Tc blasts) were incubated on SLB containing ICAM-1 (200/ μm^2), anti-CD3 Fab' (30/ μm^2), ICOS-ligand (ICOS-L) (200/ μm^2) without CD58 (top) and with CD58 (200/ μm^2) (bottom), fixed at 15 min incubation and imaged with TIRFM. B) The levels of ICOS, CD2 and CD25 (activation marker) of freshly isolated peripheral blood CD4⁺ T cells (empty dashed histogram) compared to H16 (solid light grey) and H40 (solid dark grey) Influenza H3 HA-specific CD4⁺ T cell clones, selected for being ICOS positive. C) H16 and H40 CD4⁺ T cells were incubated on SLBs containing ICAM-1 (200/ μm^2), HA-loaded-HLA-DRB1*09 (30/ μm^2), ICOS-ligand (ICOS-L) (200/ μm^2) and CD58 (200/ μm^2) for 15 min before fixation and imaged with TIRFM. Scale bar, 5 μm .

4.3.3 The effect of CD2 corolla on PD-1-PD-L1 interaction during T cell synapse formation

PD-1 expression levels vary between donors and so is the percentage of PD-1+ T cells found in freshly isolated T cells from PBMCs (Figure 4.3A, top panel). In order to address our questions, human Day 5-7 CD4+ T cell blasts were used since those were enriched for PD-1+ T cells (Figure 4.3A, bottom panel); this can also vary between donors. CD4+ T cell blasts were incubated on ICAM-1, anti-CD3 Fab, reconstituted SLBs, with or without CD58. Despite using a sample enriched in PD-1+ T cells, there were still only a small percentage of cells that accumulated PD-L1 in the IS. Amongst those cells, PD-L1 was localised around and inside the cSMAC (Figure 4.3B,i). This agrees with previous studies on PD-1 dynamics in the T cell synapse¹⁴³. In the presence of CD58 on SLB and formation of a CD2 corolla, the majority of the cells continued to accumulate PD-L1 around and inside the cSMAC, too (Figure 4.3B, ii, top and middle panel). Only very few cells showed a weak enrichment of PD-L1 within the CD2 corolla (Figure 4.3B,ii,bottom panel). Once again, these experiments were not very efficient in determining the localisation of ligated PD-1 in the context of CD2 corolla formation. Exhausted T cells are also known to have high levels of PD-1 (discussed in Chapter 1 Introduction). *In vitro*, exhausted T cells were generated (Figure 4.3C) and tested on polyclonal activation SLBs. However, this was again not efficient enough as only a percentage of T cells was responsive to polyclonal activation on SLBs. This is, potentially, because our *in vitro* exhaustion protocol requires continuous stimulation with activation beads and IL-2; it is known, from our practice, that T cells require a 24hr rest from activation beads and IL-2 prior to incubation on SLBs.

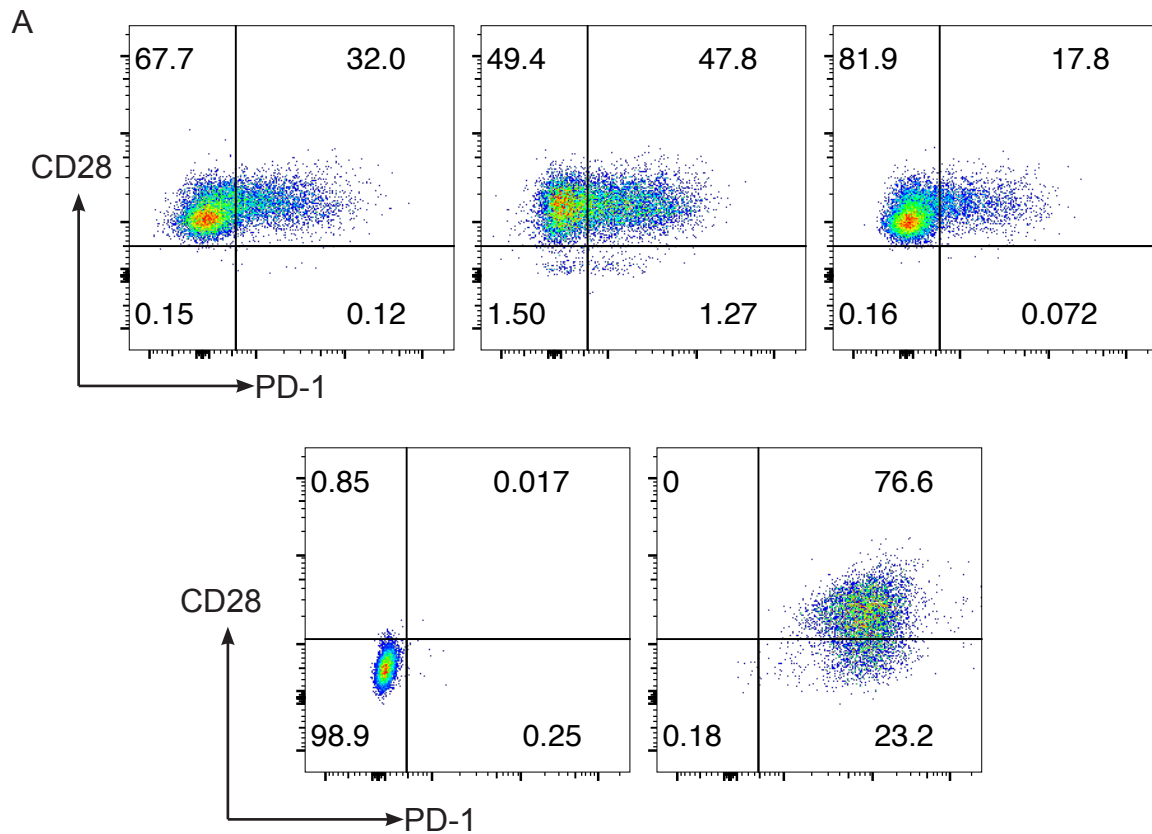
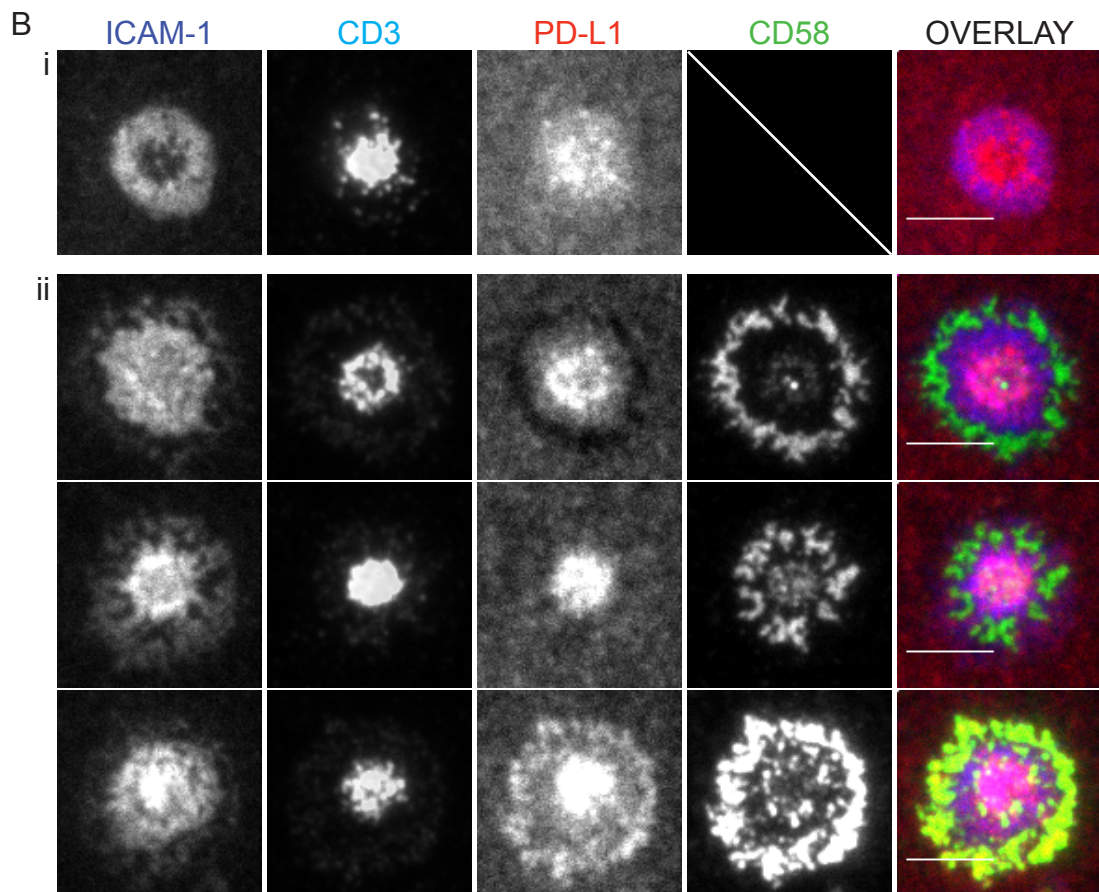
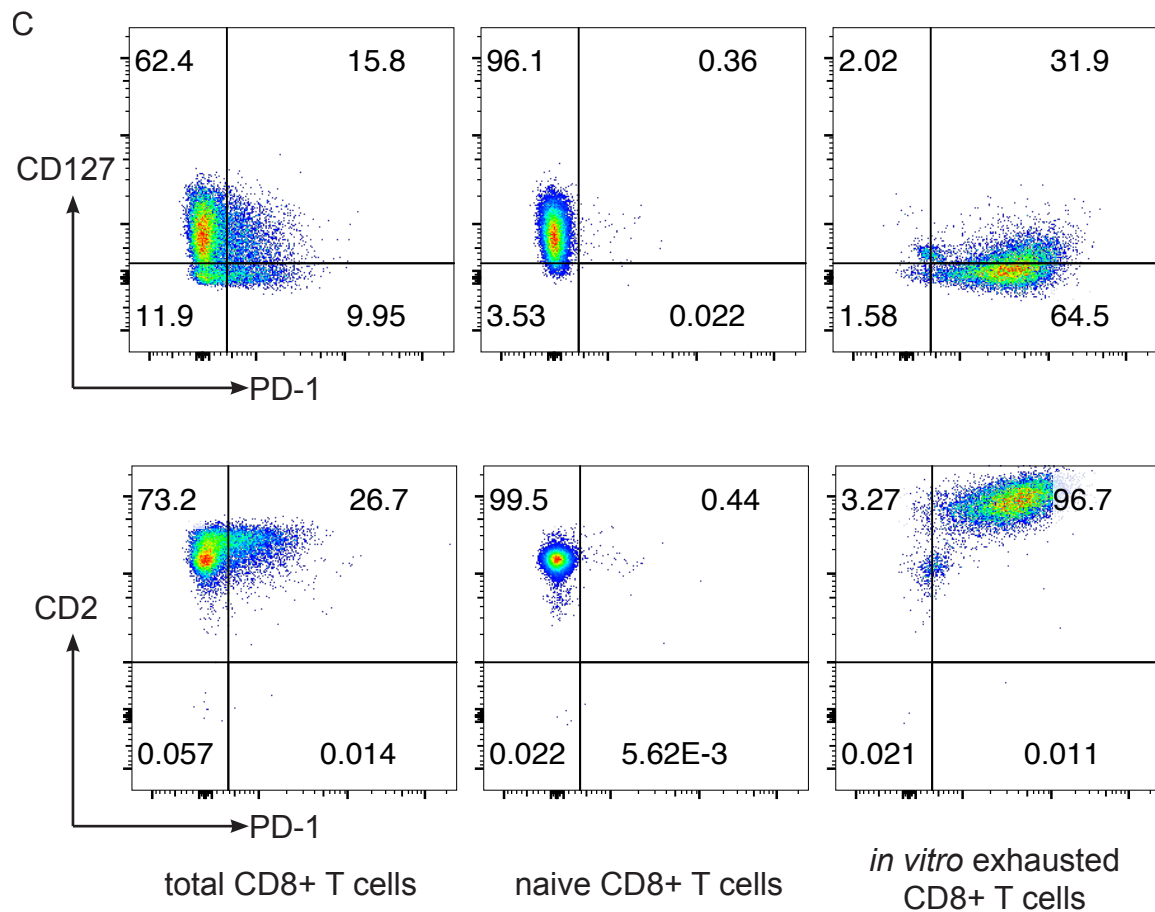


Figure 4.3 PD-1 engagement in the IS in the presence and absence of CD28 corolla.

A) The plots show the percentage of PD1⁺ CD4⁺ T cells amongst the CD4⁺ T cell population of representative three (left to right) out of five donors tested along with their CD28 expression profile (top); CD4⁺ T cells were isolated from human peripheral blood. The percentage of PD1⁺ CD4⁺ T cell in activated CD4⁺ T cells, T cell blasts (Day 4-5 post stimulation removal; Tc blasts) along with their CD28 expression profile is shown in the bottom panel, right, and unstained control, left.



B) Activated human CD4⁺ T cells, T cell blasts (Day 3-4 post stimulation removal; Tc blasts) were incubated on SLBs containing ICAM-1 (200/ μm^2), anti-CD3 Fab (30/ μm^2), PD-L1 (100/ μm^2) (i) without CD58 and (ii) with CD58 (200/ μm^2) and real-time imaged with TIRFM or fixed 15 min after initial contact with SLB and then imaged with TIRFM. Three different examples of PD-1-PD-L1 accumulation in the IS are shown; the majority of T cells accumulated PD-1-PD-L1 as in example images shown in ii) top and middle panel. A representative of three experiments is shown. Scale bar, 5 μm .



C) (top) The plots show a representative example of the CD127-PD1 expression profile of total CD8+ T cells, left, isolated from peripheral blood, followed by isolation of naïve CD8+ (same donor), middle, and *in vitro* 'exhausted' CD8+ T cells, right. (bottom) The plots show the CD2-PD-1 expression profiles of the T cell populations from the top panel, respectively. These data are representative of four donors tested.

The RNA transfection method offered as a way to obtain a sample with titrated levels of transfected PD-1 and a more uniform expression of PD-1. A PD-1 construct, (kindly provided by Anton van der Merwe lab), was coupled to a fluorescent protein to allow tracking of the PD-1 receptor when imaging. It is known, that PD-1 levels vary amongst T cell subsets and it is thought that the highest levels are detected in exhausted T cells and T_{FH} cells. It was important to us to quantify the physiological levels in the various T cell subsets and try to replicate those levels using the RNA transfection approach. We were very interested to find out what are the highest PD-1 levels T cells can express *in vivo*, for example in exhausted T cells or T_{FH}.

The mean number of PD-1 molecules was determined in human in PD-1+ CD4⁺ T cells (2.5×10^4 PD-1 molecules/cell), found in human tonsils, in CXCR5^{HI}CD278^{HI} CD4⁺ T_{FH} cells (7.1×10^4 PD-1 molecules/cell), (gating strategy shown in Figure 4.4A) and finally in tumour infiltrating T cells (TILs) in colorectal cancer (CRC) patient tumours (gating strategy shown in Figure 4.4B); the latter often have an exhaustion phenotype described as CD127^{LOW}PD-1^{HI}. The PD-1 levels in the above subsets were compared to the mean number of PD-1 molecules detected in peripheral blood T cells and in T cells found in the normal adjacent tissue of a CRC tumour (Figure 4.4C-D). This data provided us with a range of the physiological expression of PD-1, *in vivo*. Both CD4⁺ and CD8⁺ TILs expressed three times more PD-1 molecules ($9.0\text{--}10.0 \times 10^3$ molecules/cell) when compared to PD-1 positive peripheral blood CD4⁺ and CD8⁺ T cells (3.0×10^3 molecules/cell). Figure 4.4C. Overall, T cells found in tonsils or peripheral tissue like the colon had higher levels compared to peripheral blood T cells. Of note, is that the *in vitro* exhausted T cells obtained in the lab also expressed similar levels

to TILs. CXCR5^{HI}CD278^{HI} CD4⁺ T_{FH} cells expressed the highest levels of PD-1 found in the T cell subsets investigated in our study. Finally, in five out of eight CRC patients, there was an enrichment of a subset of CD4⁺ T cells in the normal adjacent tissue that had even higher levels of PD-1 than CD4⁺ and CD8⁺ TILs (Figure 4.4E).

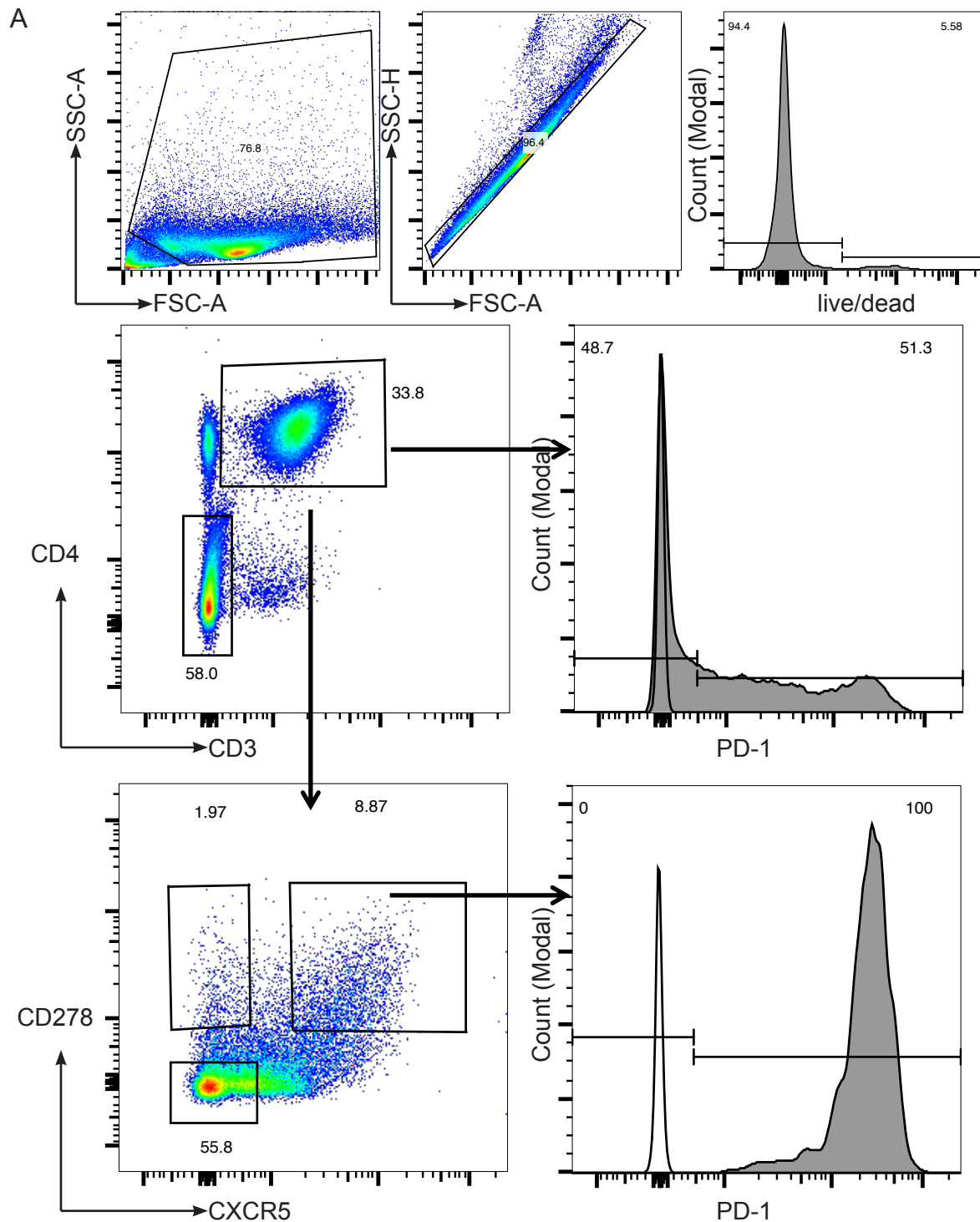
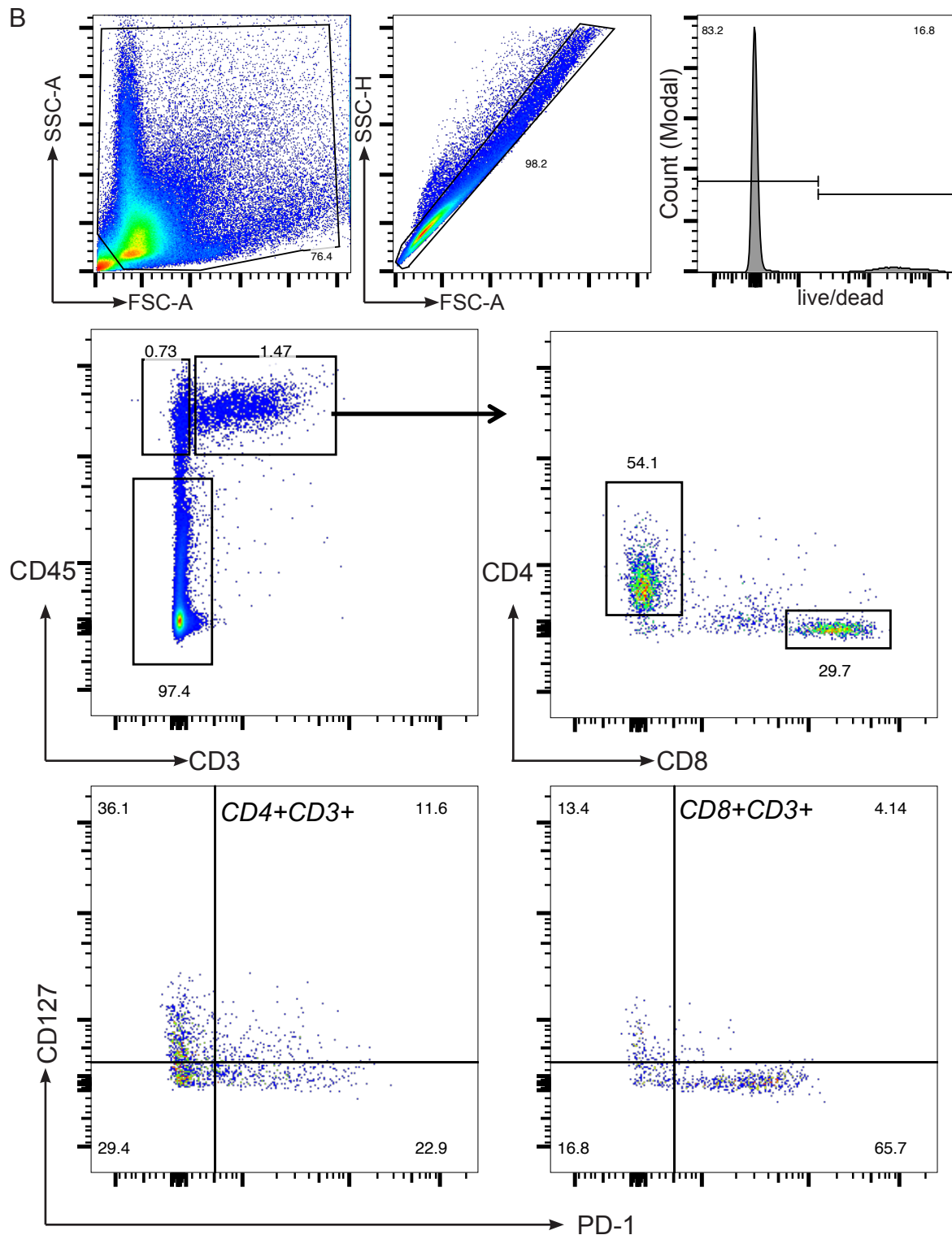
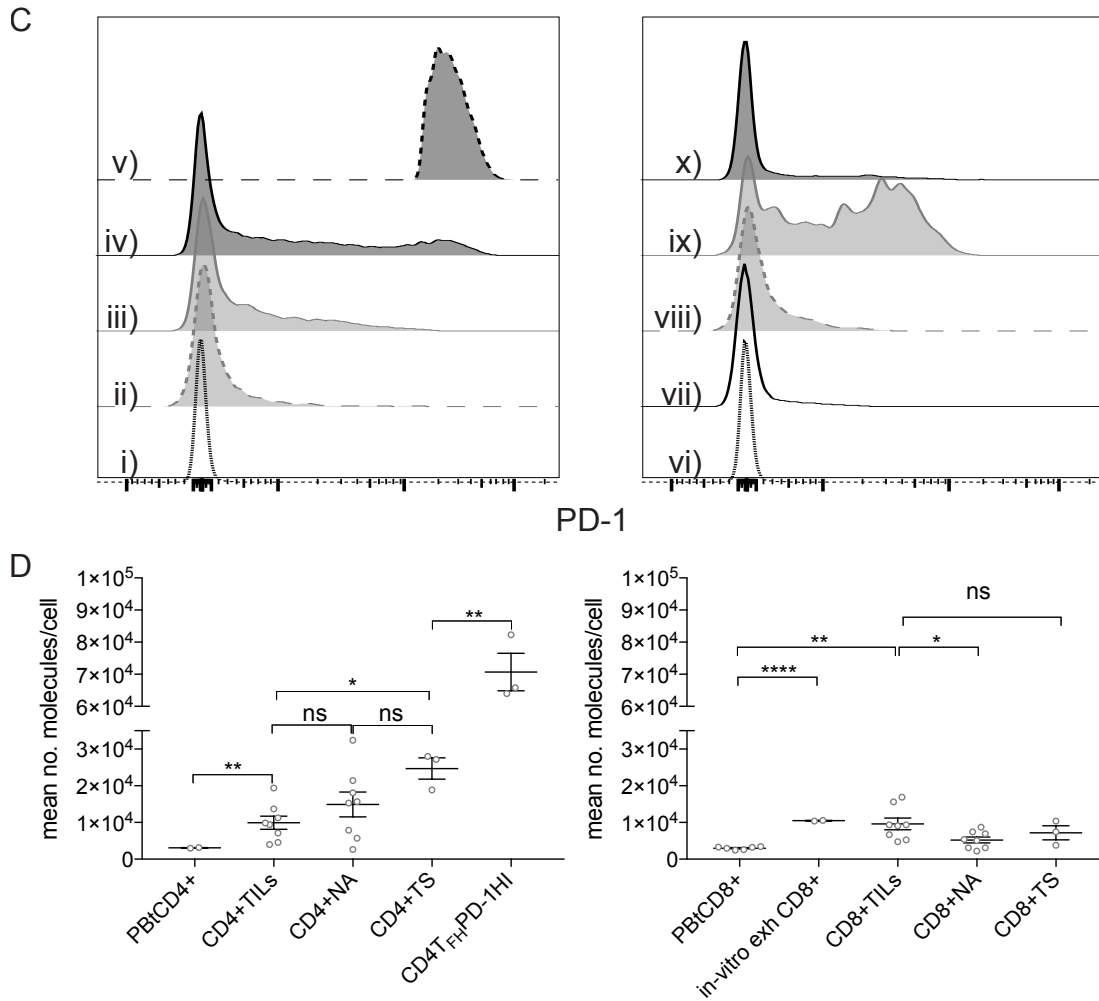


Figure 4.4 The PD-1 levels in various human T cell subsets, *ex vivo* analysis.

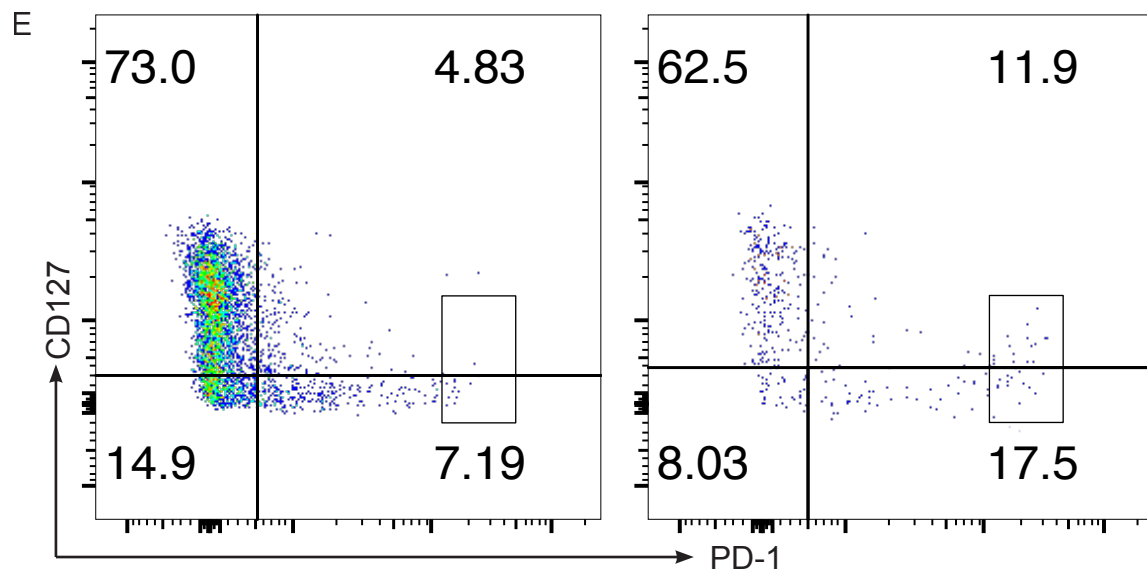
A) An example of the gating strategy to investigate the levels of PD-1 in total CD4⁺ and CD8⁺ T cells found in human tonsils. The levels of PD-1 in CXCR5^{HI}CD278^{HI} CD4⁺ T cells were also investigated (bottom). Empty solid histogram represents isotype stained control.



B) An example of the gating strategy to investigate the levels of PD-1 in total CD4⁺ and CD8⁺ T cells found in human colorectal cancer samples compared to normal adjacent tissue. The exhaustion phenotype of CD4⁺ and CD8⁺ T cell subsets was also investigated based on CD127-PD-1 expression profile (bottom left and right, respectively). PD-1 gates were based on isotype stained control similar to (A)



C) Example of the levels of PD-1 on the various populations gated in (A-B) are shown here; i) CD4+ T cells from normal adjacent tissue from CRC patient (ii), tumour tissue from CRC patient (iii), human tonsils (iv), PD-1^{HI} T_{FH} from tonsils (v) and vi)-x) CD8+ T cells from peripheral blood(vii), normal adjacent tissue from CRC patient(viii), tumour tissue from CRC patient(ix), human tonsils(x). i) and vi) are isotype control stained CD4+ and CD8 + T cells, respectively. D) The PD-1 mean fluorescence intensity from flow cytometry was translated to PD-1 molecules/cell using antibody-capture calibration beads. CD4+ T cell, left, CD8+ T cells, right. PBt, peripheral blood total T cell population, TILs, tumour infiltrating lymphocytes, NA, normal adjacent tissue to CRC tissue, TS, tonsil, in-vitro exh, *in-vitro* exhausted. ns, non-significant, *, p value=0.015 for CD4+ T cells and *, p≤0.04 for CD8+ T cells, **, p<0.006, ****, p value <0.0001, with parametric Welch's *U*-test. Error bars represent SEM.



E) Two examples of the CD127-PD-1 expression profile of total CD4⁺ T cells from normal adjacent tissue to tumour from a CRC patient with a PD-1^{HI} CD4⁺ T cell (right), representative of 5 out of 8 CRC patients, and from a patient with PD-1^{low-int} CD4⁺ T cells (left), representative of 3 out of 8 CRC patients. PD-1 gates were based on isotype stained control similar to (A).

4.3.4 Investigating the effect of CD2 corolla on PD-1-PD-L1 interaction using titration of PD-1 levels in naïve CD8+ T cells.

Using RNA transfection of T cells, PD-1 levels were titrated in freshly isolated naïve CD8+ T cells and compared to peripheral blood CD8+ T cells whenever possible (Figure 4.5A). PD-1 transfected T cells, as well as controls i.e. naïve CD8+ T cells not transfected, were incubated on ICAM-1, α -CD3 Fab, CD58, PD-L1 reconstituted bilayers for 15 min before fixation. PD-1 was detected mainly around and within the TCR-enriched cSMAC with a weak PD-1 signal detected within the CD2 corolla at PD-1 levels above 8×10^3 molecules/cell and only in a fraction of cells. Representative images are shown in Figure 4.5B. In order to appreciate better the fraction of cells with a weak localisation of PD-1 within the CD2 corolla, with increasing levels of PD-1 in T cells, a random collection of cells for each condition is shown in Figure 4.5C-I. Due to the heterogeneity of the above response, we wished to capture the mean behaviour of the T cell population. For this, a z-projection of the average intensity of all the cells collected was obtained (Figure 4.5J), followed by a radial distribution analysis to capture the distribution of this mean intensity of PD-1, within the T cell synapse, as well of the rest of the receptors engaged in our SLB experiments (Figure 4.5K).

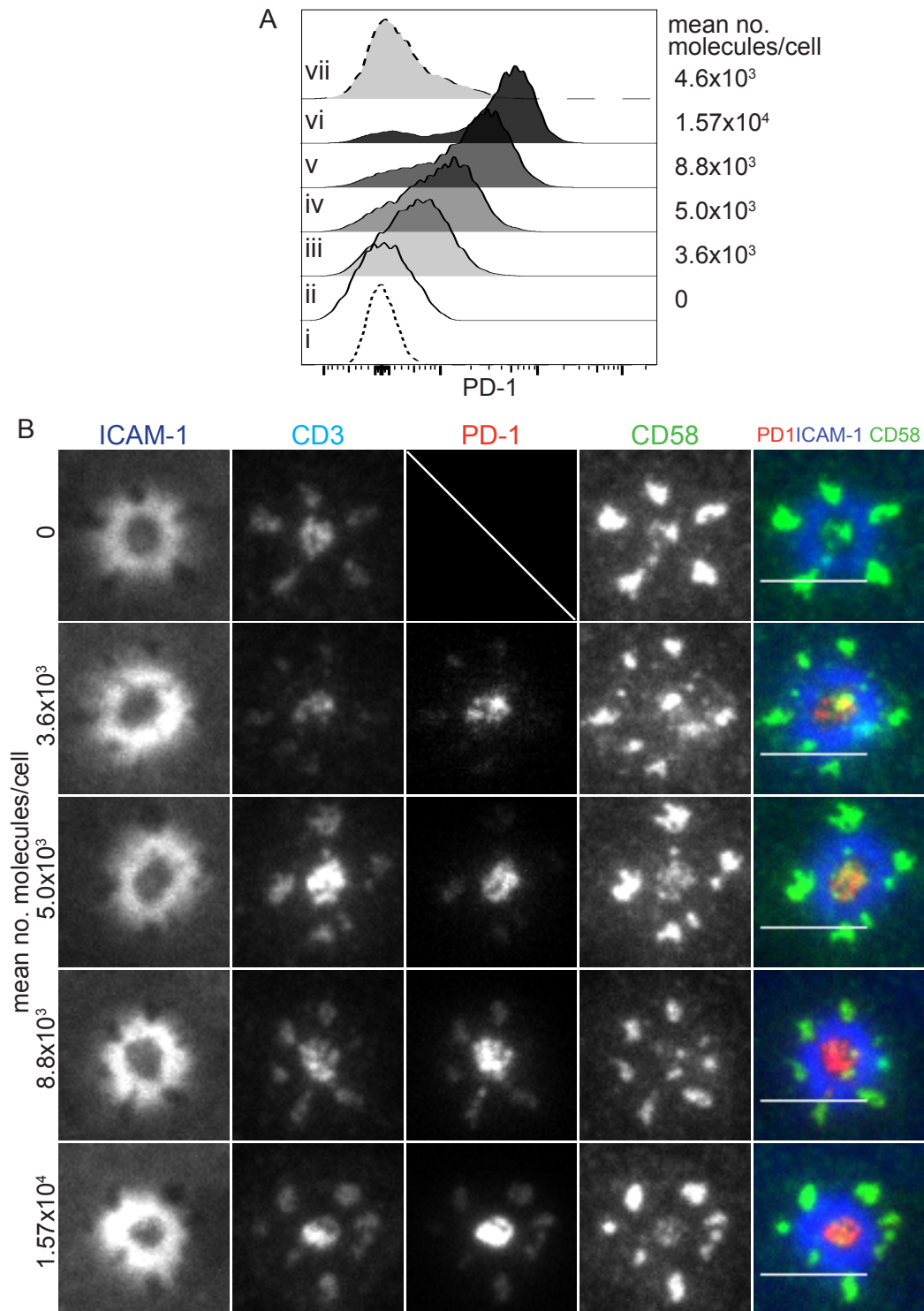
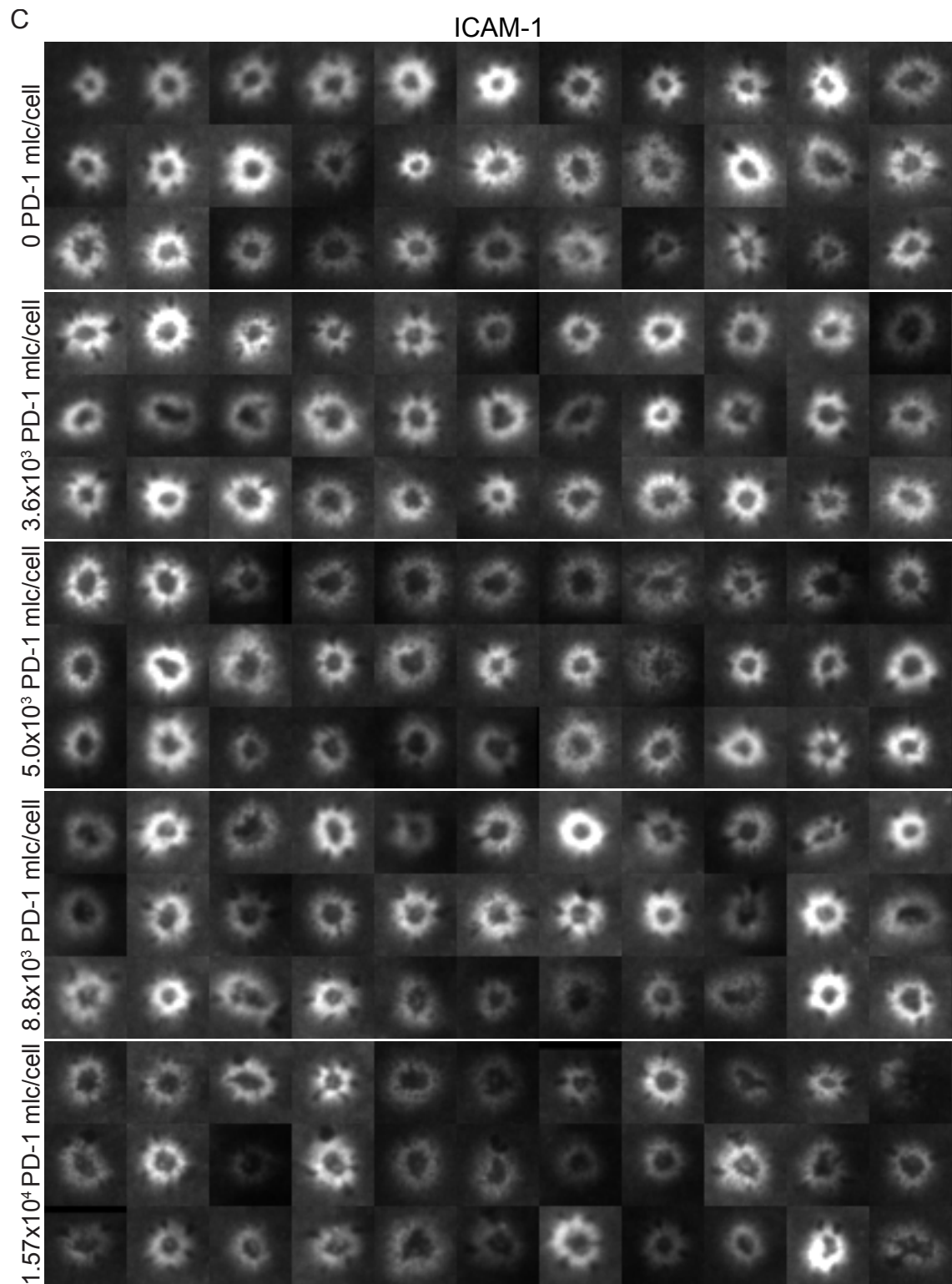
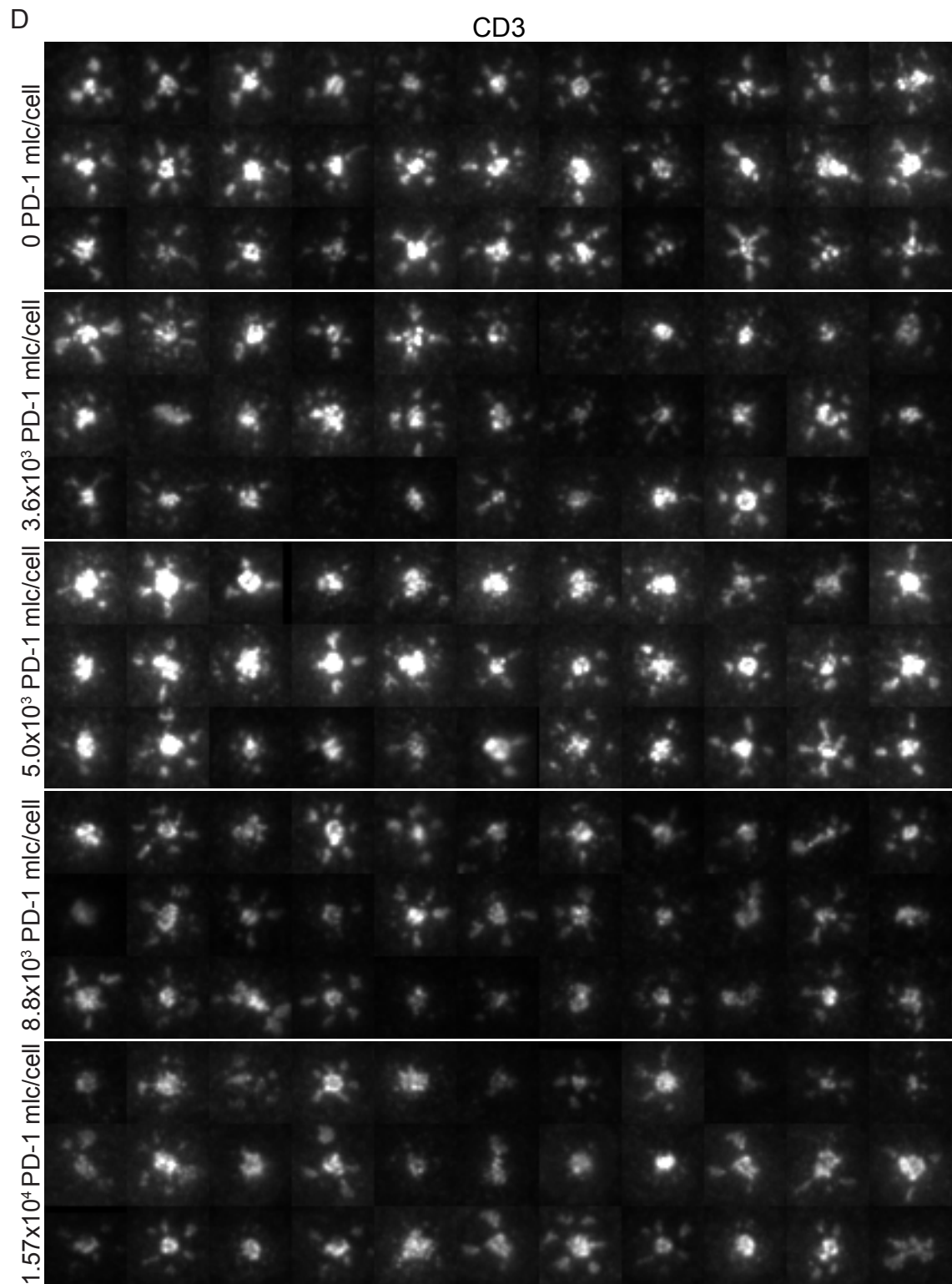


Figure 4.5 PD-1 titration in naïve CD8+ T cells and PD-1-PD-L1 localisation in the IS.

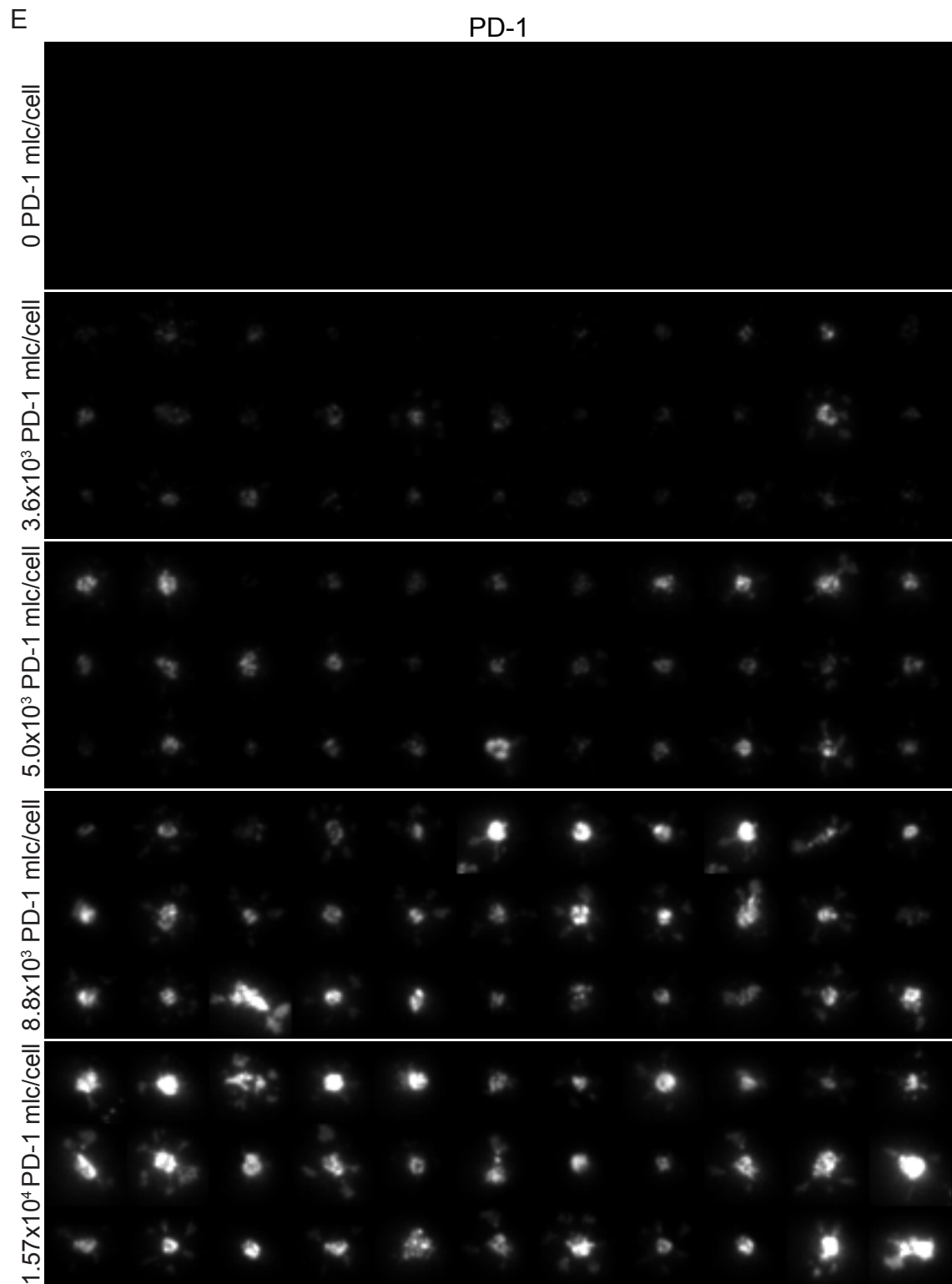
A) The histograms show an example of PD-1 titration in naïve CD8+ T with the corresponding PD-1 levels expressed in mean number of molecules/cell determined by flow cytometry (right). i) Fluorescence minus one (FMO) control, empty dotted histogram, ii) untransfected naïve T cells, iii)-vi) PD-1-transfected naïve T cells, vii) peripheral blood total CD8+ T cells. B) All naïve CD8+ T cells populations from (A, ii-vi) were incubated on SLBs containing ICAM-1 (200/µm²), anti-CD3 Fab (30/µm²), CD58 (200/µm²) without PD-L1 (top one) or with PD-L1 (1000/µm² *see note in discussion). A representative experiment of five is shown for (A-K). Scale bar, 5µm.



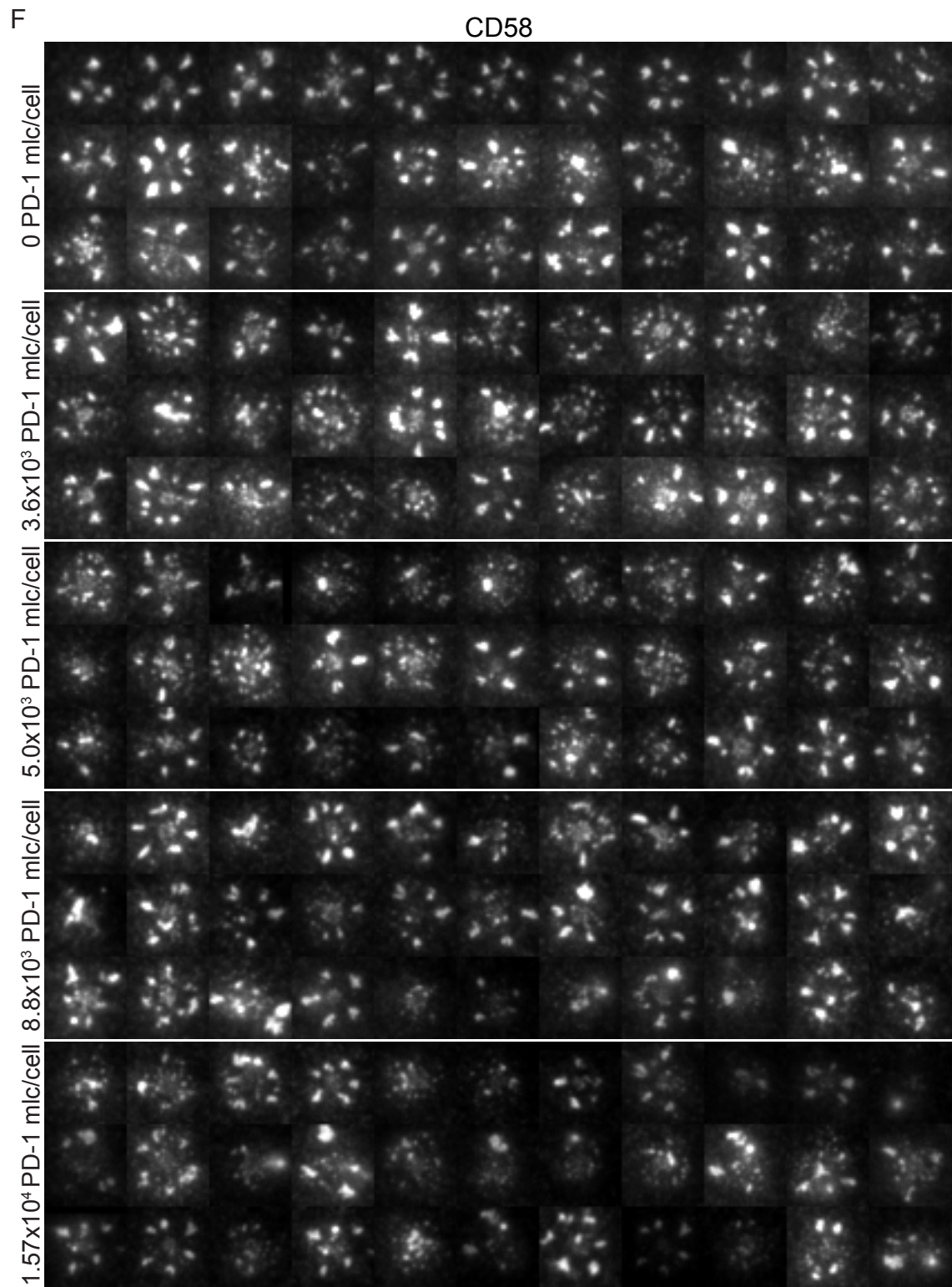
C) The ICAM-1 signal in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules/cell, mlc/cell) from top to bottom.



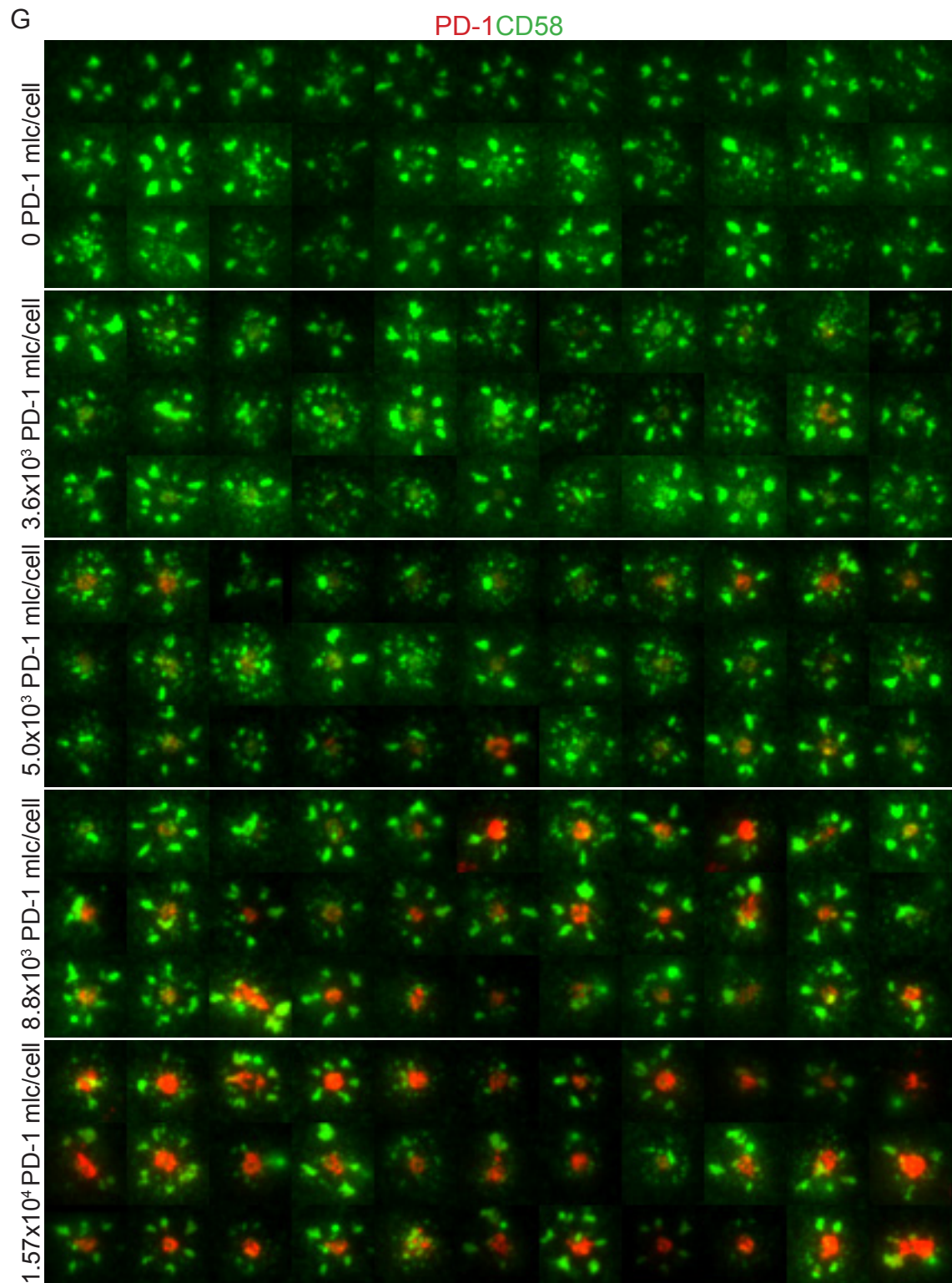
D) The CD3 signal in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules/cell, mlc/cell) from top to bottom.



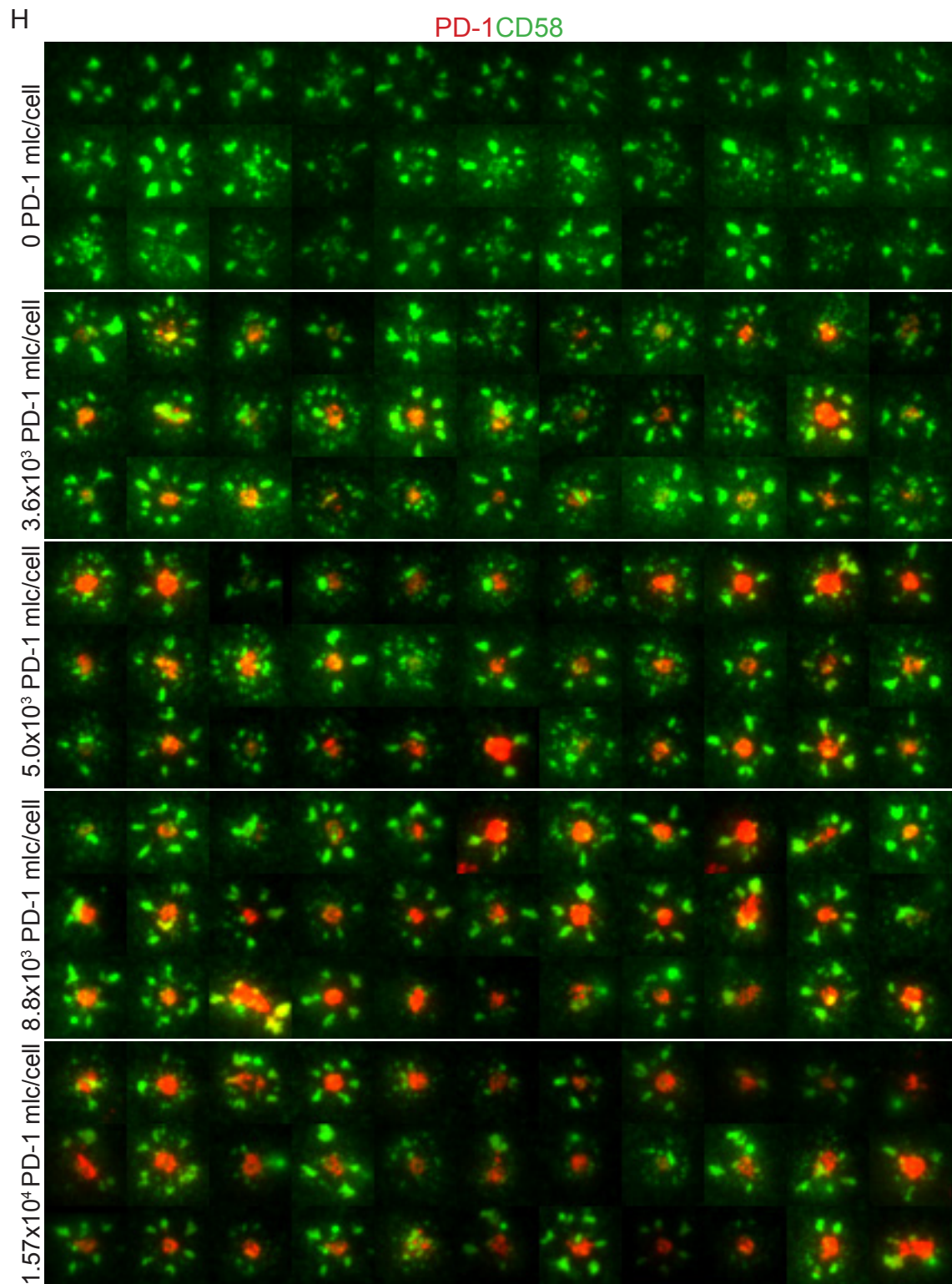
E) The PD-1 signal in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules/cell, mlc/cell) from top to bottom.



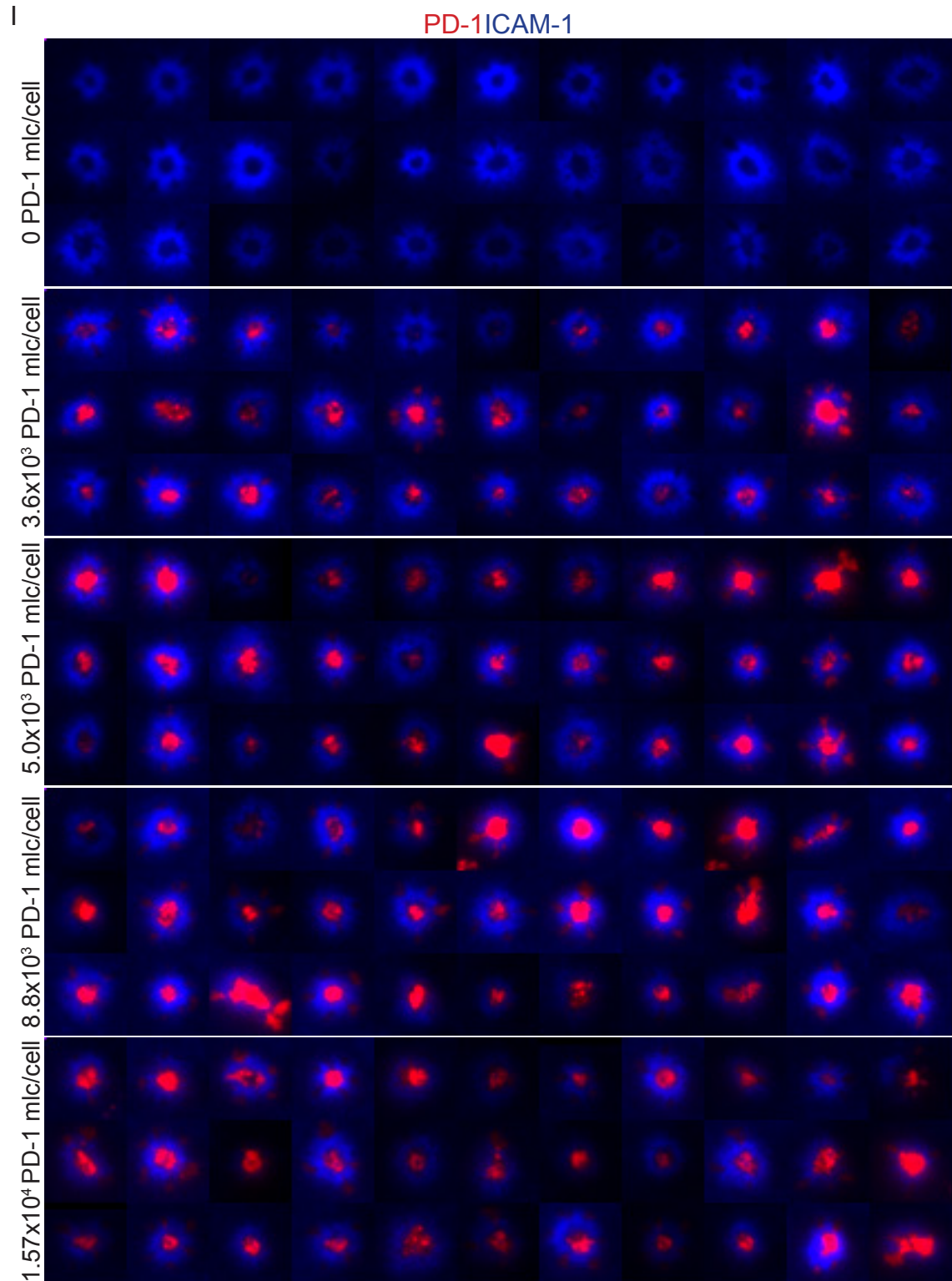
F) The CD58 signal in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules/cell, mlc/cell) from top to bottom.



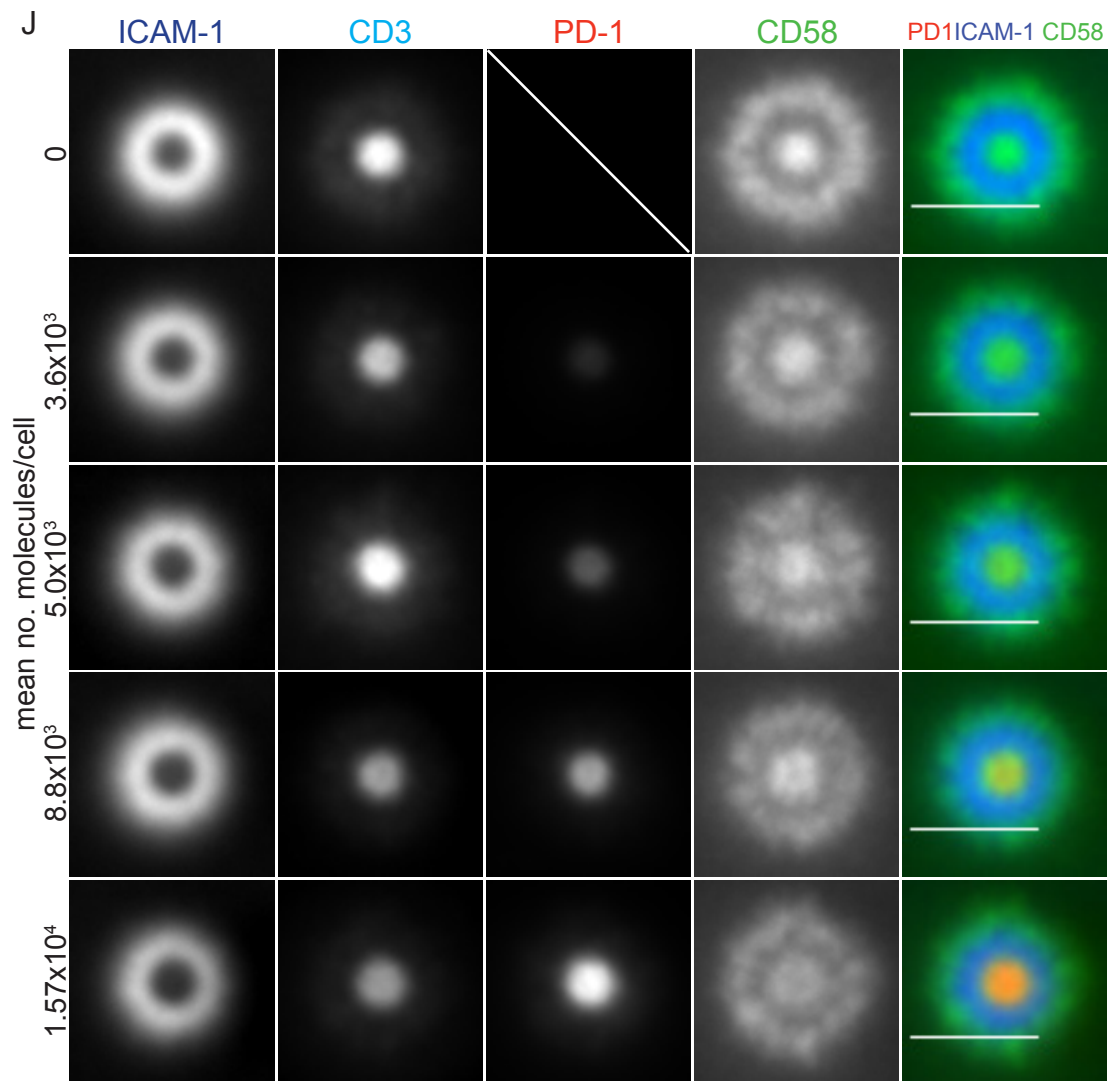
G) The overlay of PD-1 and CD58 signals in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules/cell, mlc/cell) from top to bottom



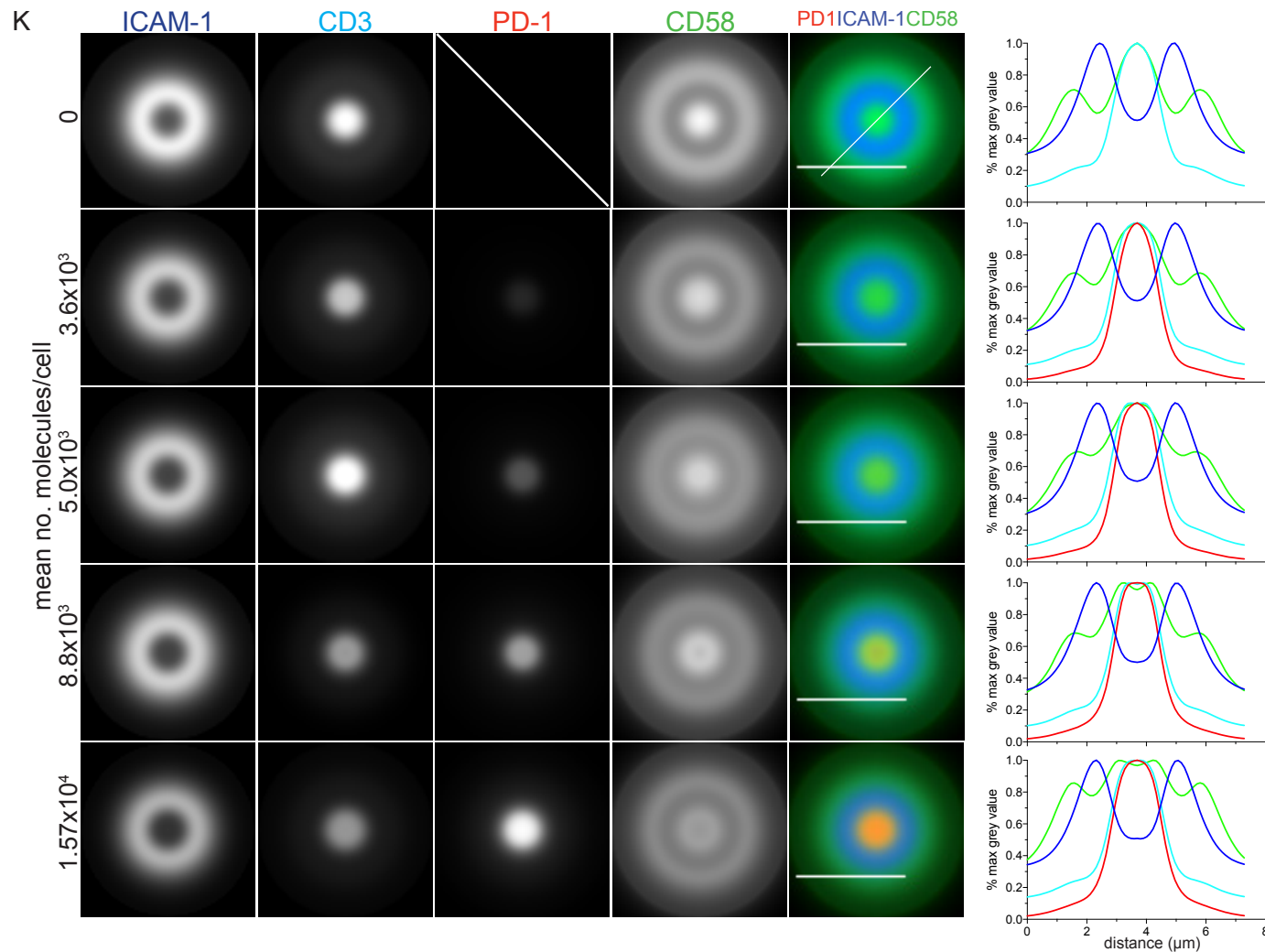
H) Same as (G) but the PD-1 intensity histograms settings were set to optimal for each condition (different PD-1 levels between conditions) to better appreciate the localization of PD-1.



I) The overlay of PD-1 and ICAM-1 signals in the IS of a random selection of T cells from all conditions in (B) with increase PD-1 levels (mean no of molecules.cell, mlc/cell) from top to bottom. The PD-1 intensity histograms settings were set to optimal for each condition (different PD-1 levels between conditions) to better appreciate the localization of PD-1 as in (H).



J) The panels show the z-projection average intensity of ICAM-1, CD3, PD-1 and CD58 in the IS of at least 100 aligned T cells (based on their cSMAC) from a random selection as shown in (C-F).



K) (left) The panels show the radial distribution of the z-projection average intensity of ICAM-1, blue, CD3, cyan, PD-1, red, and CD58, green, in the IS of at least 100 aligned T cells (based on their cSMAC) from a random selection as shown in (C-F). The histograms (right) depict the intensity profiles on the diagonal white line in the overlay images as shown in an example in the top panel. y-axis; raw pixel intensity signal normalized to maximum intensity pixel of each channel. Scale bar, $5\mu\text{m}$.

4.3.5 The orchestrating effect of CD2 corolla on colocalisation of PD-1 and CD28 in the T cell synapse.

Hui E. *et al* have reported the close colocalisation of PD-1 and CD28 and the dephosphorylation of ligated CD28 by PD-1-Shp2¹⁴⁷. In our study, we show that, from 5 min onwards of synapse formation, the CD2 corolla can sustain CD28 clusters in the periphery and the two signals were colocalised. In contrast, at the same time point, CD2 corolla and PD-1 were mainly segregated; PD-1 shows more of a TCR-like distribution in the T cell synapse rather than a peripheral ring like CD2 does. The distribution of CD28 and PD-1 was, thus investigated, together in the context of CD2 corolla. PD-1 was titrated in naïve CD8⁺ T cells on a range $\sim 3.0 \times 10^3$ - 1.0×10^4 molecules/cell (Figure 4.6A); on a couple of experiments up to 2.3×10^4 molecules/cell were achieved. In these experimental settings, CD28 clusters were sustained in the periphery and colocalised with the CD2 corolla whereas PD-1 was enriched around and within the cSMAC; with only weak enrichment in the CD2 corolla in a fraction of the cells (Figure 4.6B).

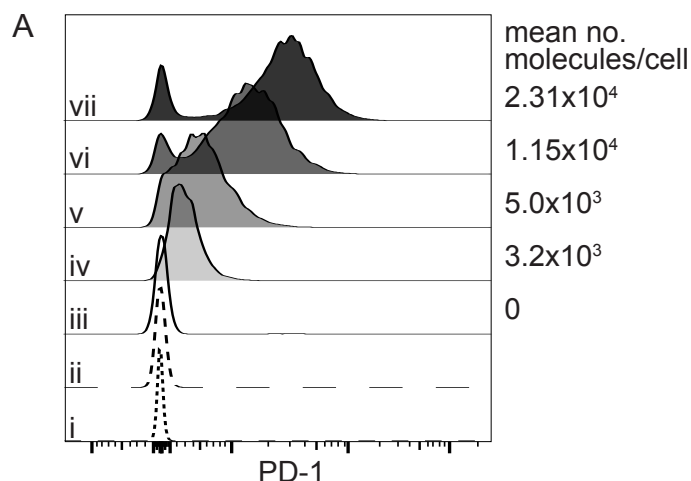
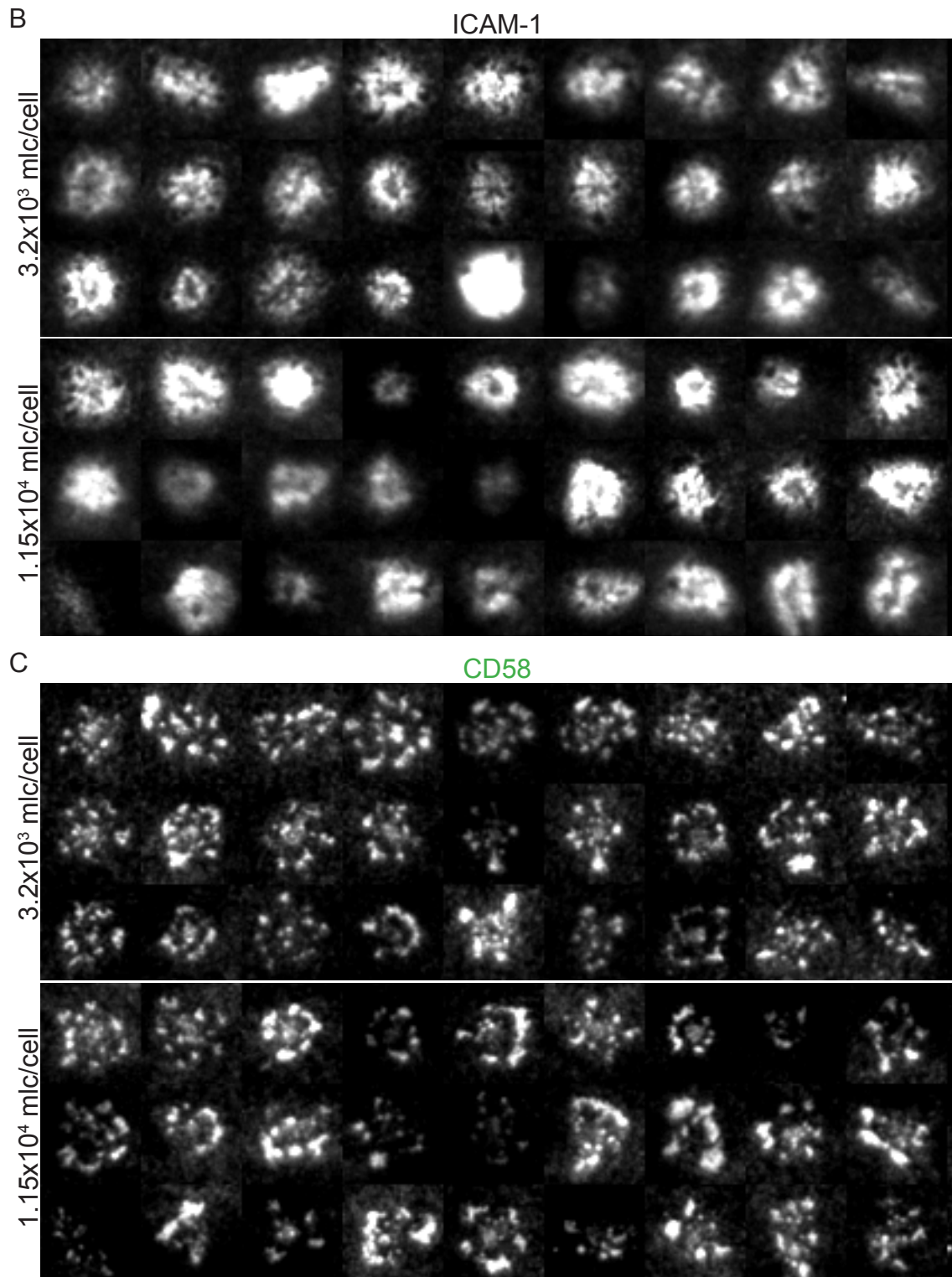


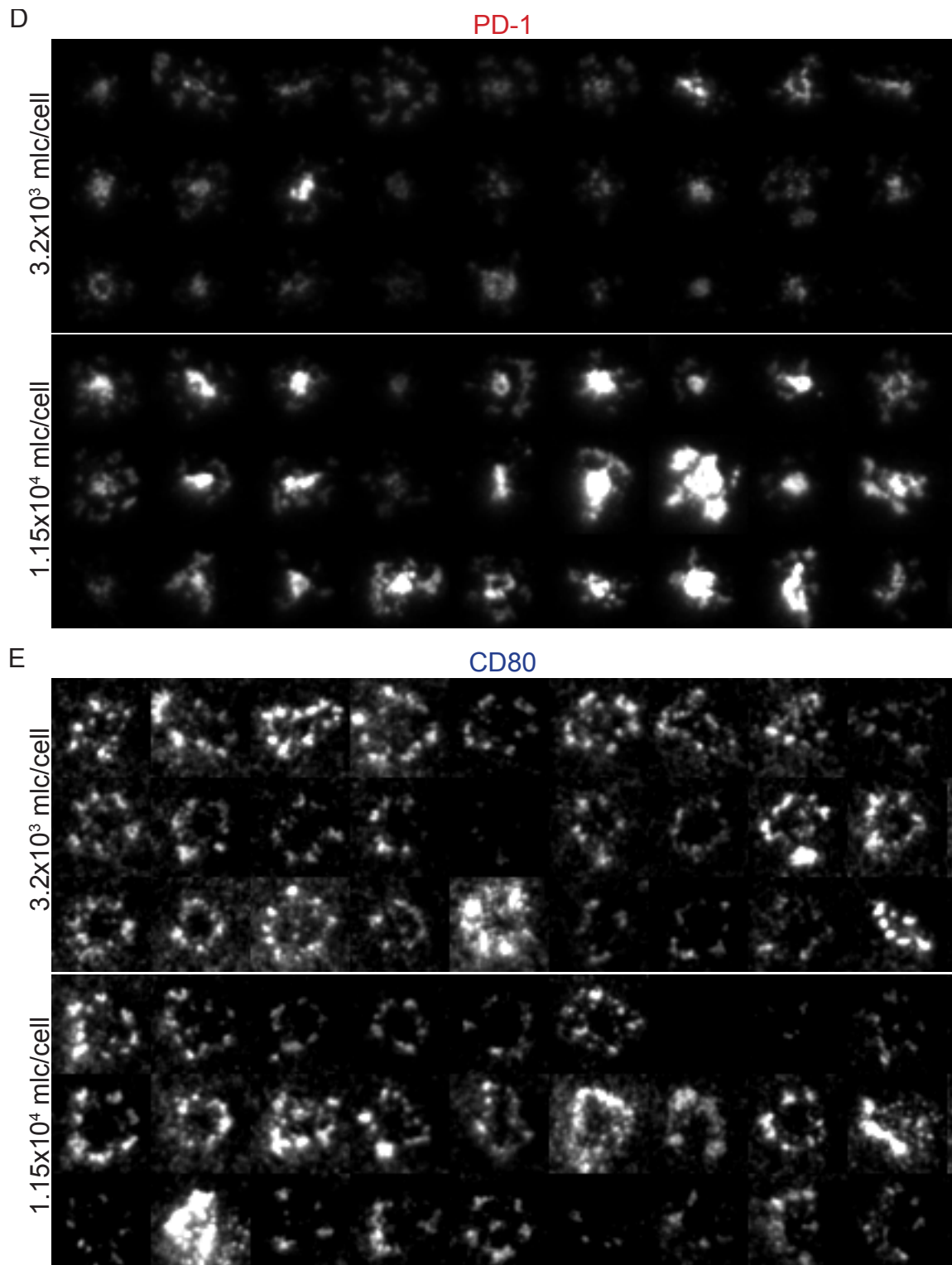
Figure 4.6 CD2 corolla segregates CD28 and PD1 clusters in the SMAC-stage of IS.

A) The histograms show an example of PD-1 titration in naïve CD8⁺ T with the corresponding PD-1 levels expressed in mean number of molecules/cell determined by flow cytometry. i) FMO control, empty dotted, ii) isotype stained control, empty dashed, iii) untransfected naïve T cells. iv)-vii) PD-1-transfected naïve T cells.

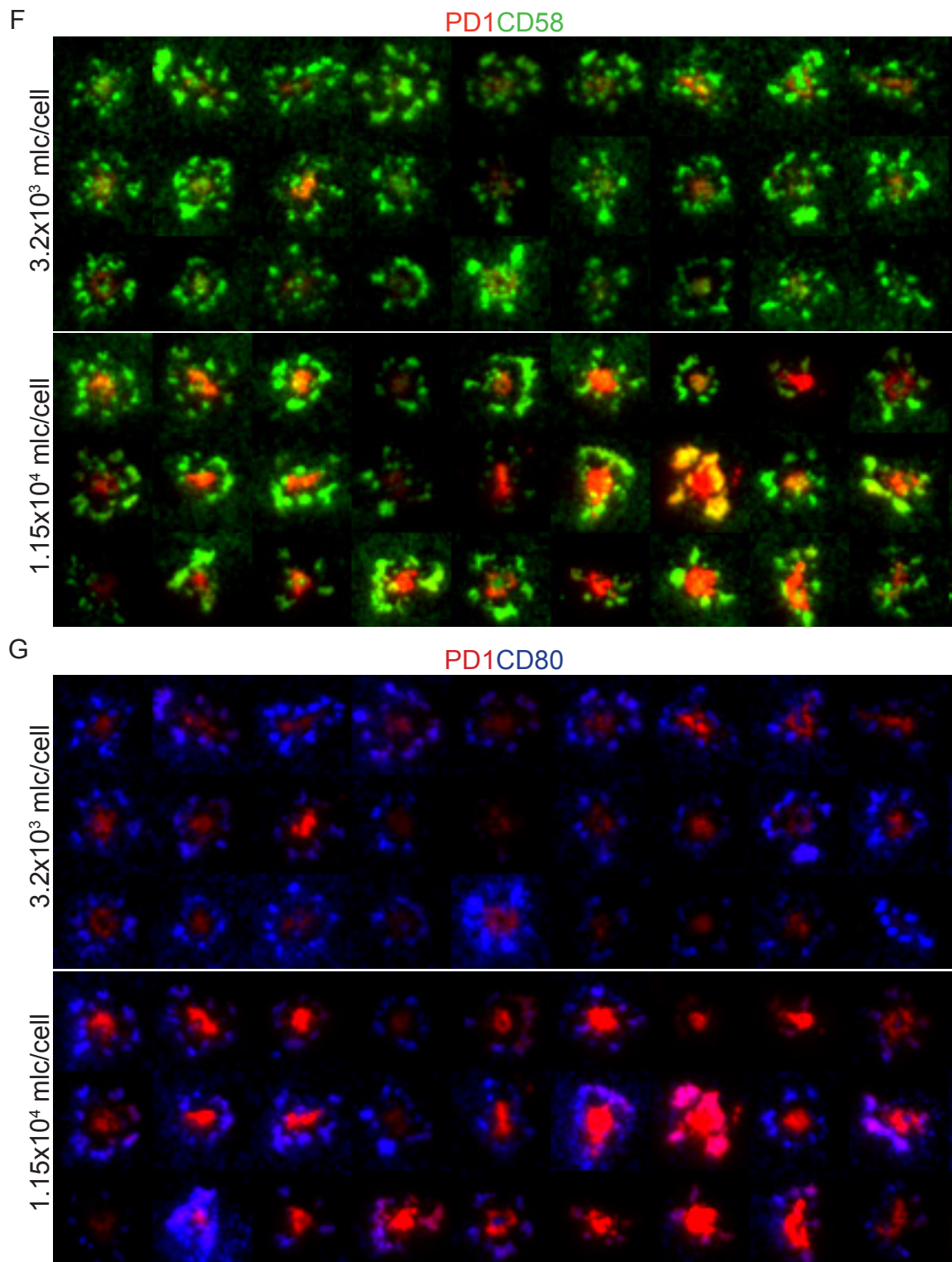
All naïve CD8⁺ T cells populations from (A, iii-vi) were incubated on SLBs containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$), CD58 ($200/\mu\text{m}^2$), CD80 ($100/\mu\text{m}^2$) without PD-L1 (for the untransfected cells condition) or with PD-L1 ($1000/\mu\text{m}^2$ *see note in discussion section). A representative experiment of four is shown for (A-H).



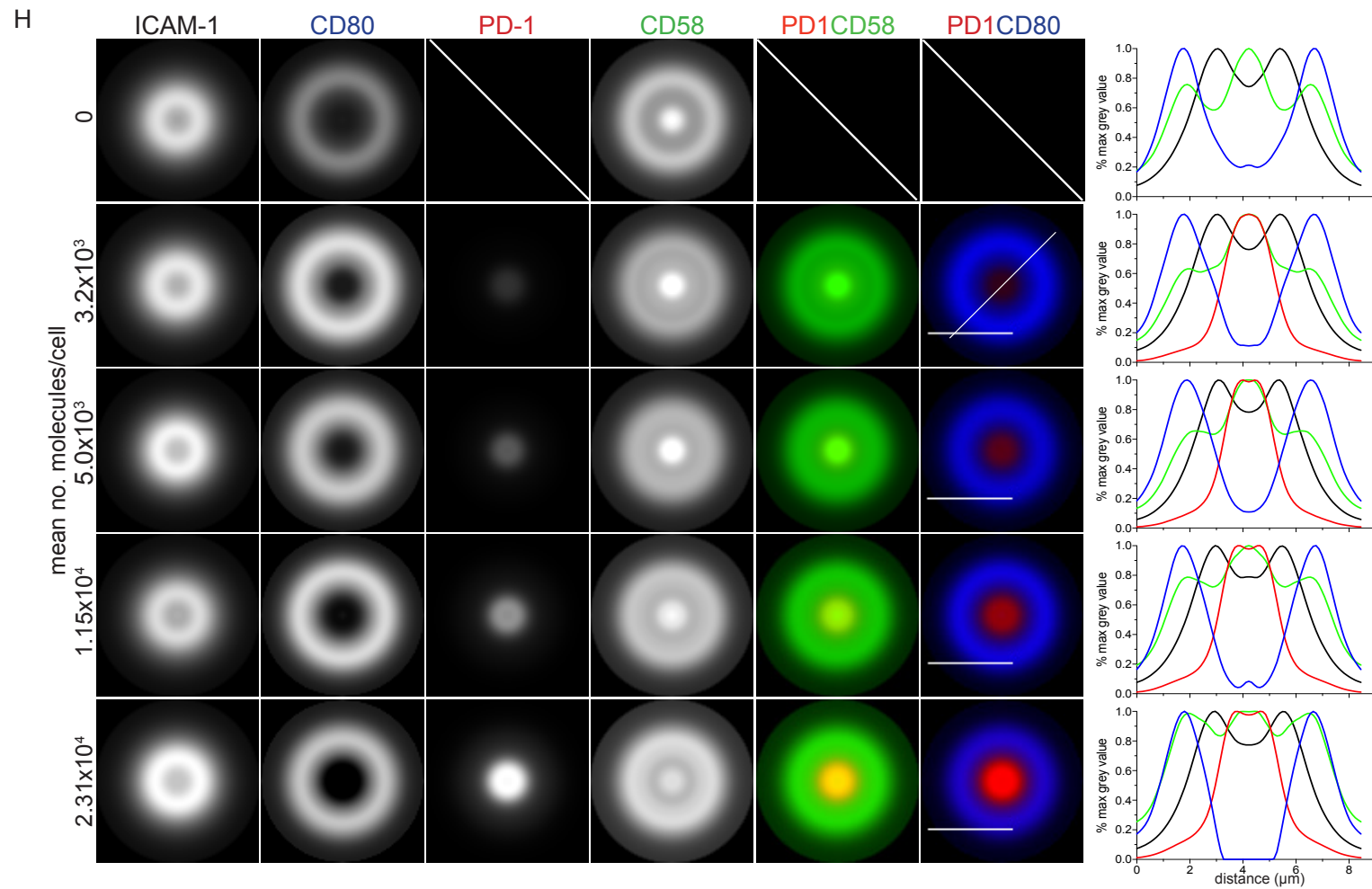
B-C) The ICAM-1 and CD58 signals, respectively, in the IS of a random selection of T cells from two example conditions, low PD1 levels (3.2×10^4 molecules/cell) and high PD-1 levels (1.15×10^4 molecules/cell) are shown. PD-1 levels shown as mean no of molecules/cell, mlc/cell), left.



D-E) The PD-1 and CD80 signals, respectively, in the IS of a random selection of T cells from two example conditions, low PD1 levels (3.2×10^4 molecules/cell) and high PD-1 levels (1.15×10^4 molecules/cell) are shown. PD-1 levels shown as mean no of molecules/cell, mlc/cell), left.



F-G) The overlay of PD-1-CD58 and PD-1-CD80 signals, respectively, in the IS of a random selection of T cells from two example conditions, low PD1 levels (3.2×10^4 molecules/cell) and high PD-1 levels (1.15×10^4 molecules/cell) are shown. PD-1 levels shown as mean no of molecules/cell, mlc/cell), left.



H) (left) The panels show the radial distribution of the z-projection average intensity of ICAM-1, CD80, PD-1 and CD58 signals in the IS of at least 100 aligned T cells for each condition; increasing PD-1 levels, mean no. molecules/cell shown top-bottom. (right) The histograms depict the intensity profiles on the diagonal white line in the overlay images as shown in one example. y-axis; raw pixel intensity signal normalized to maximum intensity pixel of each channel. Scale bar, 5μm.

4.3.6 Quantification of CD2, CD28, PD1, CD3, TCR, CD11a and CD18 in peripheral blood lymphocytes.

One of the hypotheses to explain why CD28 would be sustained in the periphery with the CD2 corolla while PD-1 is localised around and within the cSMAC is that the enrichment of a receptor within the CD2 corolla is dependent on the number of molecules expressed on the T cell membrane. For this reason, the mean number of molecules of each of the following receptors, CD2, CD28 and PD1 in peripheral blood T cell subsets cells and CD56intermediate (CD56^{int}) and CD56bright(CD56^{HI}) NK cells was determined along with other receptors used in our synapse experiments like CD3, TCR, CD11a and CD18. This hypothesis is only one of the potential ones to provide an explanation for the described effects of CD2 on CD28 and PD-1.

The gating strategy for looking at the different T cell subsets and NK cells is shown in Figure 4.7A-B.

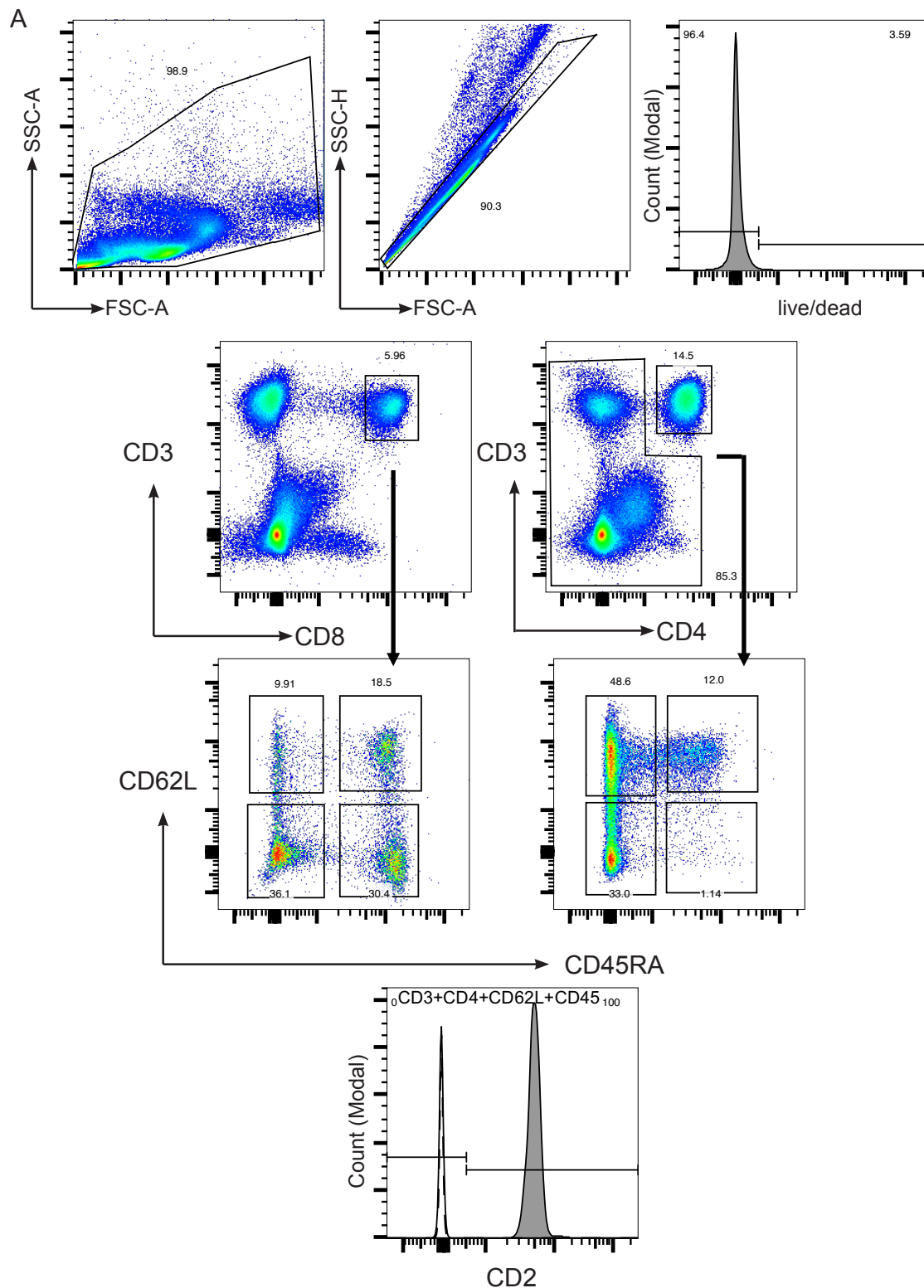
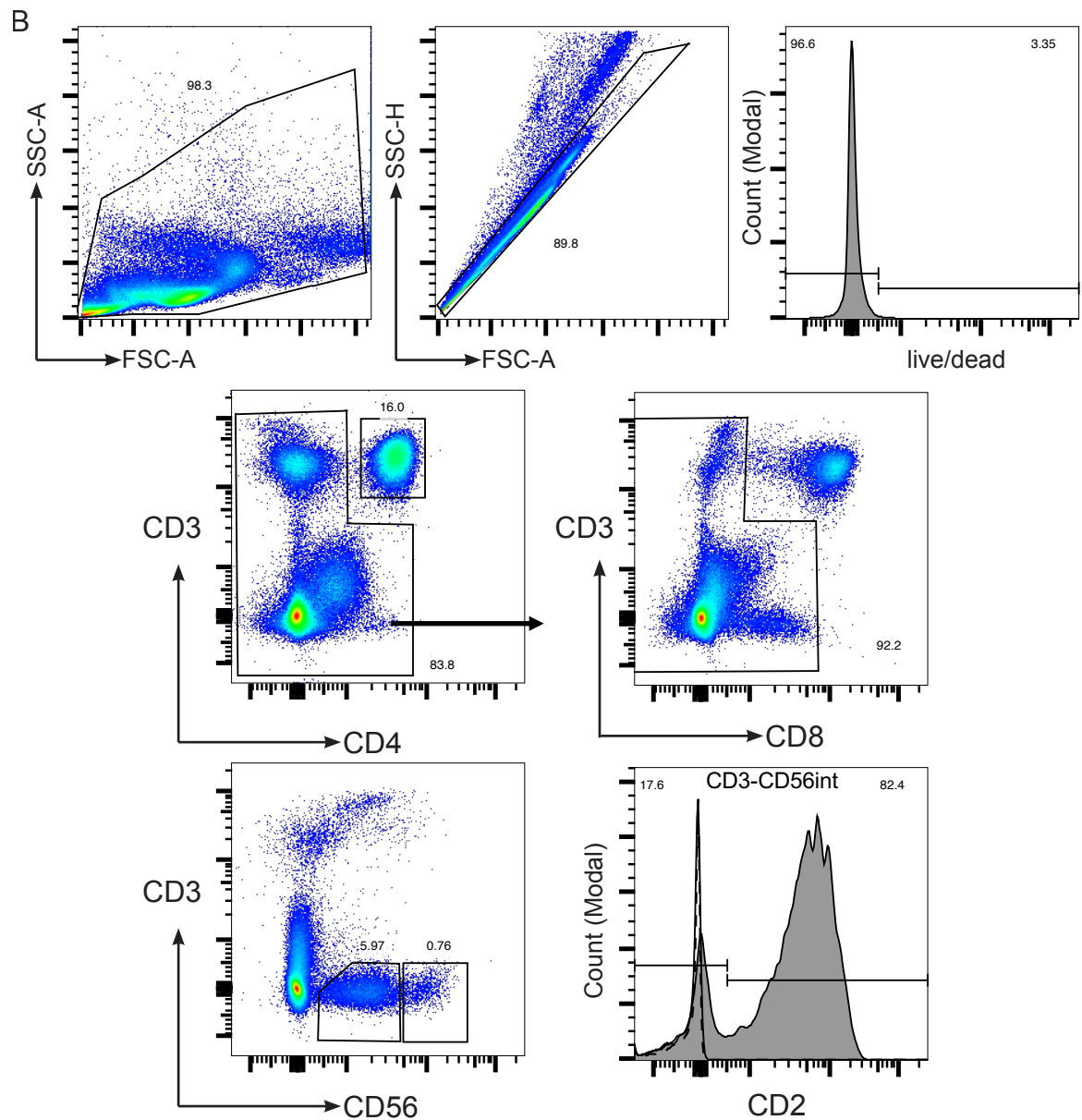
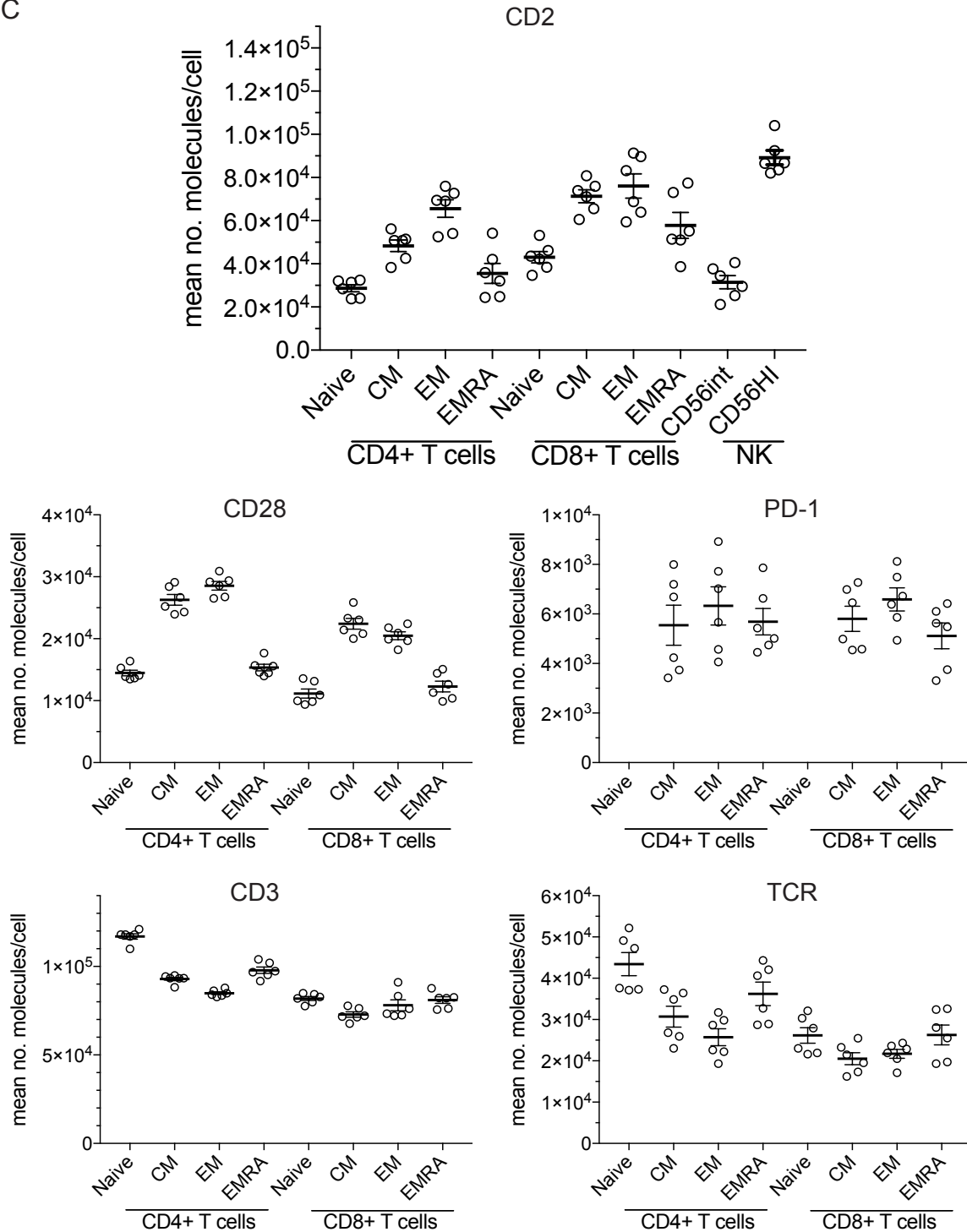


Figure 4.7 Determining the mean number of molecules/cell for CD2, CD28, PD1, TCR-CD3 and LFA-1 on the cell surface of human T (and NK) cells from peripheral blood.
 A) The gating strategy, is shown, for determining the receptor levels, for example CD2, of naïve and memory CD4+ and CD8+ T cell subsets, based on CD62L-CD45RA expression; naïve (CD62L+CD45RA+), central memory, CM, (CD62L+CD45RA-) effector memory, EM, (CD62L-CD45RA-) and effector memory that re-express CD45RA, EMRA (CD62L-CD45RA+). CD2 histogram; FMO, solid empty histogram, isotype control, dashed empty.

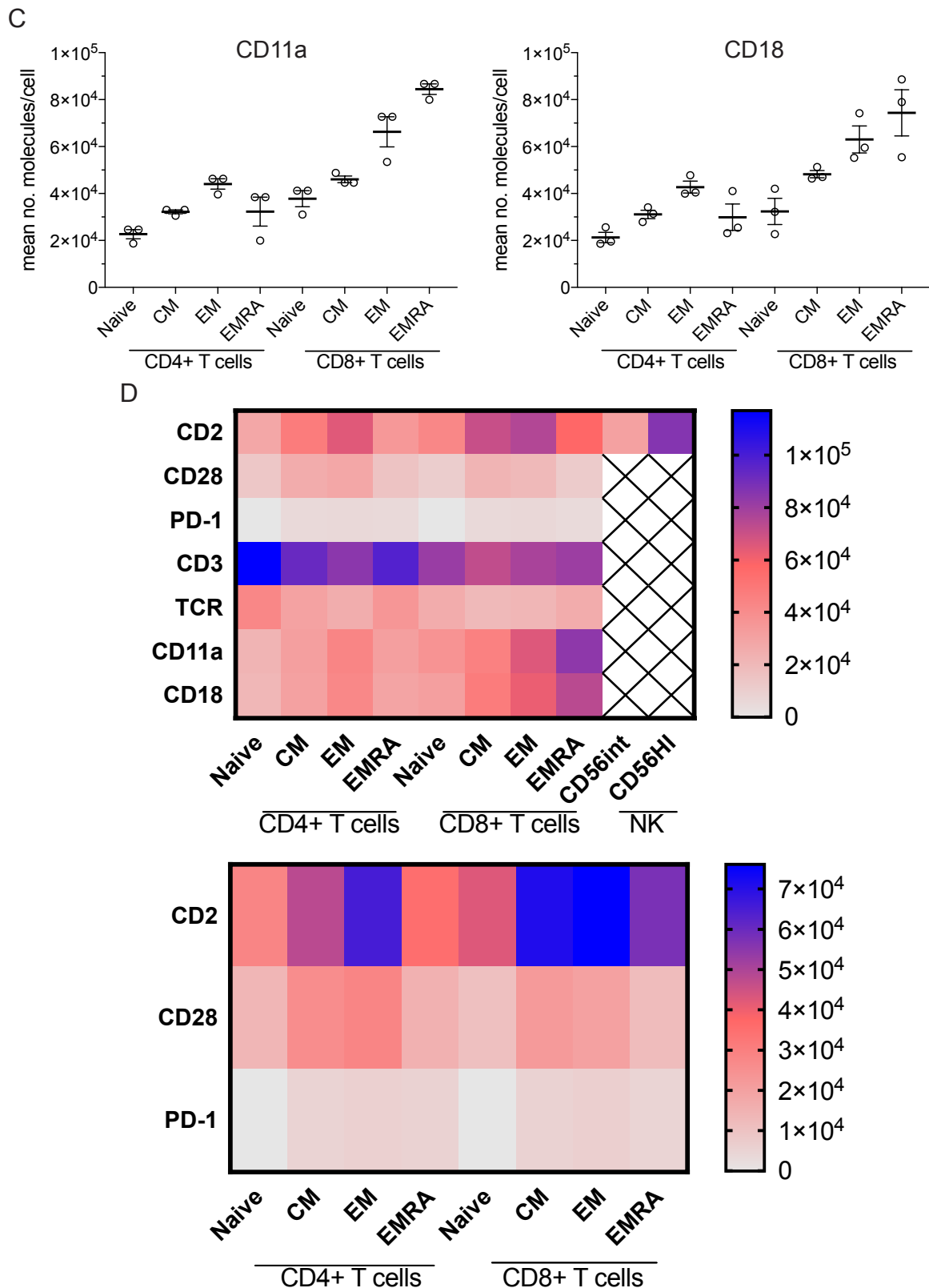


B) The gating strategy, is shown, for determining the CD2 levels, of two main NK cell subsets, based on CD56 expression; CD56 intermediate (CD56^{int}) and CD56bright (CD56^{hi}) NK cells. CD2 histogram: FMO, solid empty histogram, isotype control, dashed empty

C



C) (to) The graph shows the surface expression levels of CD2 in T and NK cell subsets of 6 donors, based on the gating strategies shown in (A-B). Following plots show the surface expression levels of CD28, PD-1, CD3(ϵ -chain), TCR(β -chain), in T cell subsets of 6 donors and CD11a and CD18, in T cells of 3 donors (see also next page) based on the gating strategy shown in (A). Mean fluorescence intensity expression levels were translated to mean number of molecules/cell using antibody-capture calibration beads. NK cells did not express CD28 or PD-1. Data for CD11a and CD18 expression in NK cells are not shown here. Error bars, SEM.



D) (top) A heat map summarizing the 'expression level' means from (C), for T and NK cell is shown. NK cells did not express CD28 or PD-1. Data for CD11a and CD18 expression in NK cells are not shown here. (bottom) A focused heat map of the 'expression level' means for CD2, CD28, PD-1 from (C) is shown.

Two-colour gradient scale, mean no. molecules/cell.

4.3.7 Could any other co-stimulatory/inhibitory receptor result in a corolla-structure of its own?

The levels of CD2 determine the efficiency of corolla formation. We questioned whether other costimulatory receptors like CD28 and PD-1 could be arranged in a corolla structure, too, in the absence of the CD2 corolla, when expressed at similar levels to CD2. CD28 is expressed at $\sim 2.5\text{-}3 \times 10^4$ in memory T cells. We have already shown that these levels result in an annular CD28 ring and not a CD28 corolla (Figure 4.1). We wished to investigate this in the context of PD-1 as we are able to use the already available PD-1 RNA transfection for PD-1 titration experiments. Using the RNA transfection method, we tried to express PD-1 in naïve CD8⁺ T cells, at similar levels of molecules as CD2 levels in human peripheral blood T cells (Figure 4.8A). At the same time, a PD-1 construct lacking the cytoplasmic/signalling domain of PD-1 (PD-1TM) was generated. This was done to address whether the signalling domain is imposing any localisation effects on PD-1 clusters when compared to the full length PD-1 (PD-1-FL). Incubation of PD-1-FL transfected cells, expressing a mean of 2.7×10^4 PD-1 molecules/cell, similar to CD2 levels in naïve CD4⁺ T cells, a mean of 4.4×10^4 PD-1 molecules/cell, similar to CD2 levels in naïve CD8⁺ T cells or a mean of 6.0×10^4 PD-1 molecules/cell, similar to CD2 levels in memory CD4⁺ T cells, on ICAM-1, α -CD3 Fab and PD-L1 resulted in no PD-1 corolla of its own. PD-1 signal was localised around and within the TCR-enriched cSMAC (Figure 4.8B-C). In fact, at really high levels of PD-1, the majority of T cells at 15 min of incubation had already broken the initial symmetrical bull's eye synapse formation, leaving behind tracks of fluorescent signal, enriched in both PD-1 (Figure 4.8C top and bottom panels). and TCR signal (data not shown, here).

Transfection of PD-1TM in naïve CD8⁺ T cells, in a similar way described above for PD-1-FL (Figure 4.8A) and incubation of these T cells on ICAM-1, α -

CD3 Fab and PD-L1 resulted in no PD-1 corolla of its own for ~96-99% of PD-1TM transfected T cells forming a synapse (Figure 4.8D-E). A 1-4% of PD-1TM transfected T cells found in a synapse formation showed some signs of PD-1TM clusters in the periphery, around the pSMAC, resembling weakly the corolla formed by ligated CD2 (Figure 4.8E).

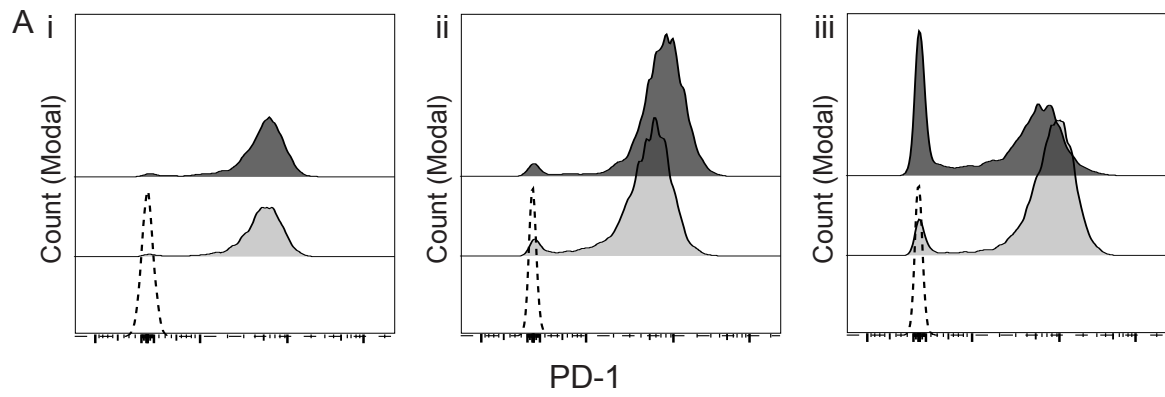
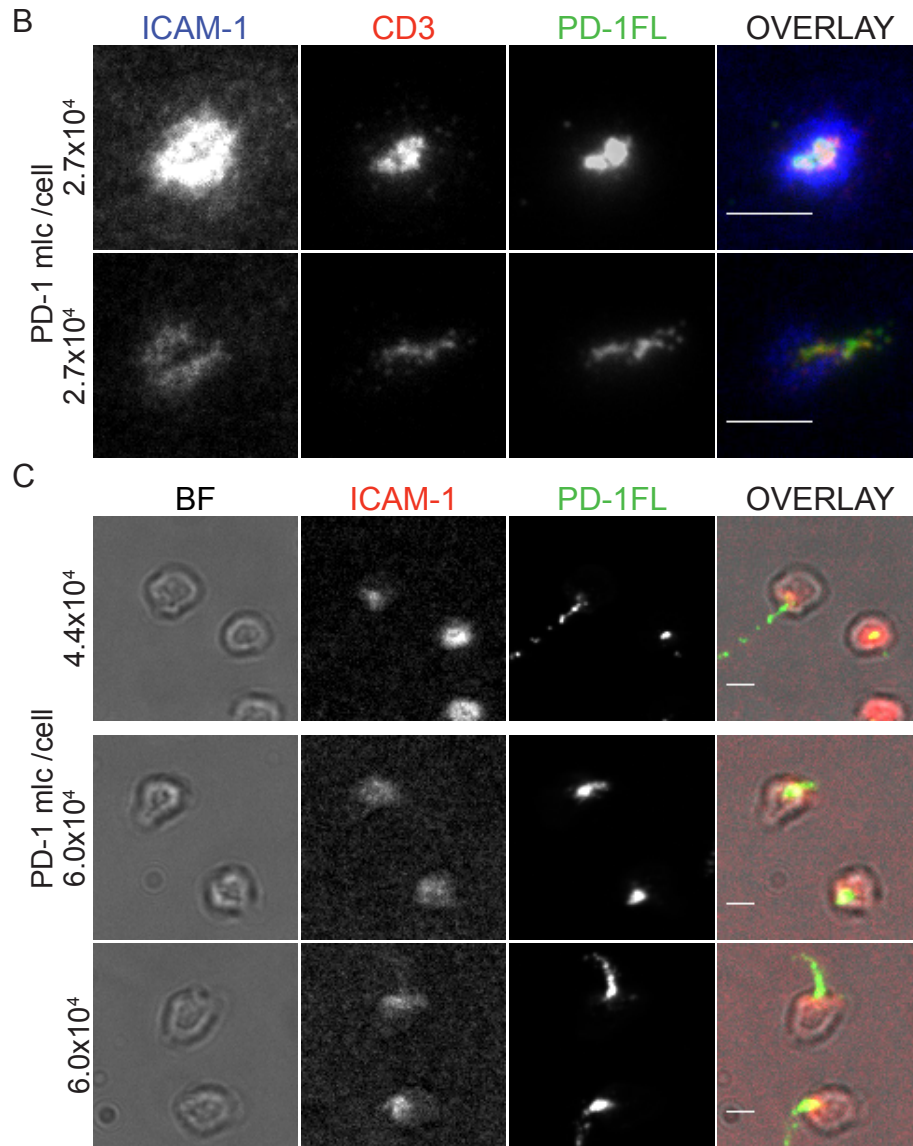
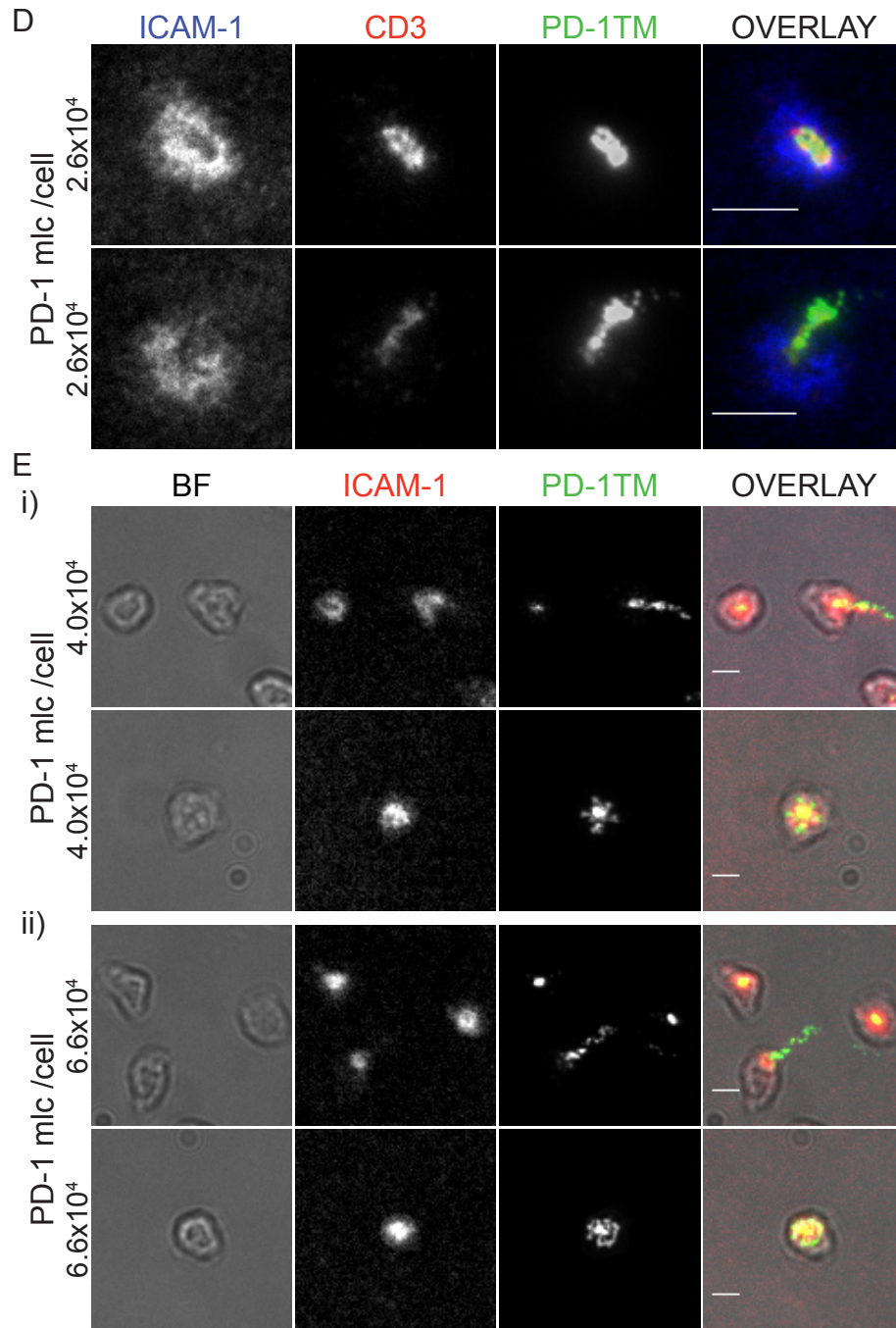


Figure 4.8 The localisation of PD-1 when expressed at similar levels to CD2.

A) Three trials (3 different donors) to overexpress PD-1 full length, PD-1FL (dark grey) or PD-1, lacking its cytoplasmic tail, PD-1TM (light) in naïve CD8⁺ T cells to levels comparable to CD2 levels found in T cells, are shown; isotype control stained naïve CD8⁺ T cells, dashed empty histogram. PD-1 mean number of molecule/cell in i) FL, 2.7×10^4 , TM, 2.6×10^4 , ii) FL, 6.0×10^4 , TM, 4.0×10^4 iii) FL, 4.4×10^4 , TM, 6.6×10^4 .



B-C) Naïve CD8⁺ T cells expressing high levels of PD-1FL from (A, i-iii) were incubated on SLBs containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$) and PD-L1 ($1000/\mu\text{m}^2$ *see note in discussion section) for 15 min before fixation and imaged with TIRFM (B) or wide field microscopy (C). Representative images of the two main IS phases T cells were observed in; symmetrical immune synapse and broken symmetry kinapse. Experiments from 3 donors are shown. Scale bar, $5\mu\text{m}$.



D-E) Naïve CD8⁺ T cells expressing high levels of PD-1TM from (A, i-iii) were incubated on SLBs containing ICAM-1 ($200/\mu\text{m}^2$), anti-CD3 Fab ($30/\mu\text{m}^2$) and PD-L1 ($1000/\mu\text{m}^2$ *see note in discussion section) for 15 min before fixation and imaged with TIRFM (D) or wide field microscopy (E). Representative images of the two main IS phases T cells were observed in; symmetrical immune synapse and broken symmetry kinapse. Example of a rare PD-1 signal distribution (1-4% of T cells) in the IS are shown in (E,i) and (E,ii) bottom panels. Experiments from 3 donors shown here. Scale bar, 5 μm .

4.3.8 Does CD28-corolla-induced segregation of CD28 and PD-1 reduce the effect of PD-1-Shp2 on phosphorylated CD28.

Hui E. *et al* showed that the colocalisation of PD-1 and CD28 also resulted in dephosphorylation of CD28 cytoplasmic tail and subsequent decrease in p85 recruited to CD28 cytoplasmic domain¹⁴⁷. A similar study by, Yokosuka T. *et al*, showed the same effect by looking at the recruitment of PKC- θ at the cytoplasmic tail of ligated CD28. Both studies used co-immunoprecipitation (co-IP) to show the above effects. We sought to investigate the same effect in the context of the CD28 corolla, CD28 and PD-1 ligation using our SLB system and the same co-IP readouts. We tried to set up the co-IP system using three main experimental conditions prior to introducing PD-1 transfected T cells. T cells were incubated for 15 min on i) ICAM-1, anti-CD3 Fab, ii) ICAM-1, anti-CD3 Fab, CD80 or iii) ICAM-1, anti-CD3 Fab, CD80 and CD58 reconstituted SLBs. T cells were lifted off the SLBs, washed and lysed. There was successful immunoprecipitation of CD28 (Figure 4.9A) but only weak co-immunoprecipitation of PKC- θ ; there was no signal detected for p85 (Figure 4.9A). Positive controls for p85 signal were run to make sure the antibody is working (data not shown). The co-immunoprecipitation of PKC- θ was not reproducible thought and it was always very weak, whenever, there was any signal at all. In the same experiments, phosphorylation of immunoprecipitated CD28 was investigated using an antibody against general phosphorylation of tyrosine residues. This readout showed not difference between CD28 ligation and no CD28 ligation. In order to show that CD28 was, indeed, activated in our SLB system, we detected the levels of phosphorylated Akt (pAKT) in the whole cell lysate of the aforementioned experiments. This was also used as a potential second readout for CD28 signalling in addition to CD28-p85/PKC- θ . This experiment showed that, indeed CD28, is activated and signals in our SLB system, as shown by the increased levels of pAKT when compared to ICAM-1, a-

CD3 Fab alone reconstituted SLBs (Figure 4.8C). CD2 ligation also induced a higher increase in pAKT when compared to ICAM-1, anti-CD3 Fab alone condition. Ligation of both CD2 and CD28, resulted in even higher levels of pAKT than any of the other 3 conditions. However, this experiment showed the pAKT cannot be used as a readout for CD28 signalling when CD2 is also ligated as CD2 is also inducing an increase in pAKT levels. This is in agreement with a previous study reported the activation of these pathways by antibody ligated CD2 and CD28¹⁰⁸.

Along with the above SLB-co-IP experiment, a cell conjugate experiment was performed, too. 1G4 TCR transfected CD8⁺ T cells were conjugated to NY-ESO-9V-peptide loaded monocyte derived-DCs or a B cell line for 15-20 min at 37°C before washing and cell lysis. This was used as another positive control for CD28 activation but also as an alternative to using the SLB system. This proved to be a more robust way of immunoprecipitating good amounts CD28 and pulling down also p85 and PKC- θ (Figure 4.9B). Of note, though, it was later found out that 20% of the B cells of that B cell line were also positive for CD28 expression. Since then, CD28 negative B cells of that line were sorted and cultured.

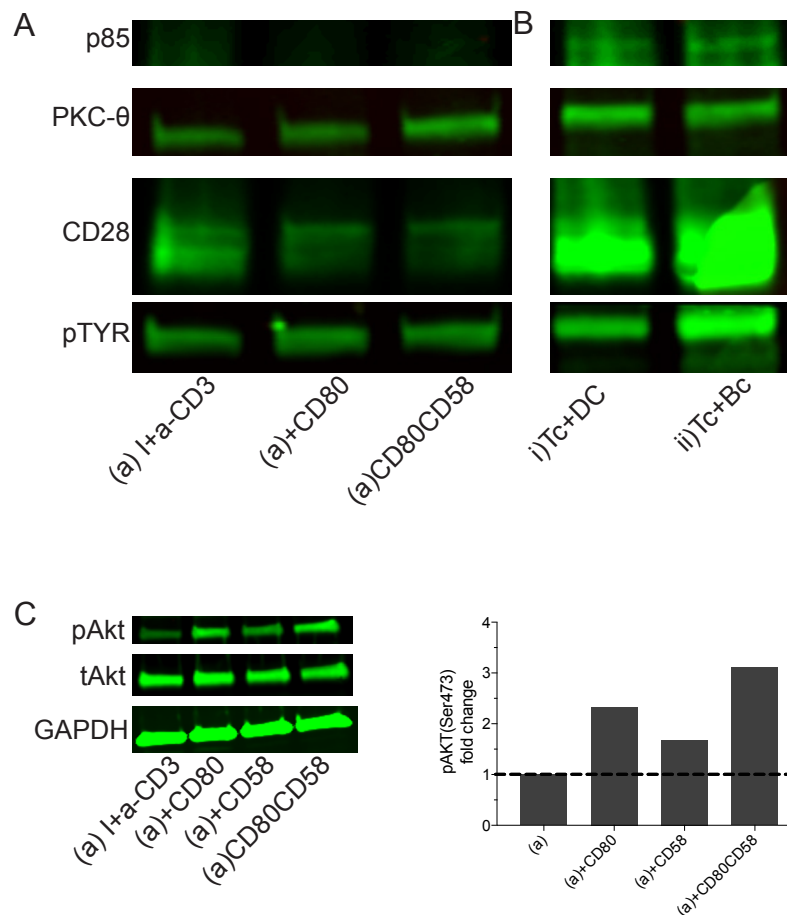


Figure 4.9 Co-immunoprecipitation of CD28-p85-PKC-θ.

A) Memory CD4+/CD8+ T cells were incubated on SLBs containing (a) ICAM-1 (200/μm²), anti-CD3 Fab (30/μm²), (a) plus CD80 (200/μm²) or (a) plus CD80 (200/μm²) and CD58 (200/μm²) for 15 min. Cells were detached from SLBs with a 15-min incubation in ice-cold PBS/2%FBS/2mMEDTA. Cells were recovered, washed twice and then lysed, immunoprecipitated with anti-CD28 (9.3 clone) and blotted for CD28, p85, followed by a second reblotting for total phosphoTYR (pTYR) and PKC-θ. pTYR-CD28, bottom panel. Four such experiments were performed.

B) 1G4 TCR transfected CD8+ T cells were conjugated with either i) NY-ESO-9V loaded monocyte-derived Dendritic cells (Tc+DC) or ii) NY-ESO-9V loaded EBV-transformed B cell line, for 15 min, then cells were washed, lysed, immunoprecipitated with anti-CD28 (9.3 clone) and blotted for CD28, p85, followed by a second reblotting for total phosphoTYR (pTYR) and PKC-θ. pTYR-CD28, bottom panel. This was done in parallel to experiment shown in (A).

C) Memory CD8+ T cells were incubated on SLBs containing (a) ICAM-1 (200/μm²), anti-CD3 Fab (30/μm²), (a) plus CD80 (200/μm²), (a) plus CD58 (200/μm²) or plus CD80 (200/μm²) and CD58 (200/μm²) for 15 min. Cells were detached from SLBs with a 15-min incubation in ice-cold PBS/2%FBS/2mMEDTA. Cells were recovered, washed twice and then lysed. Whole cell lysate was blotted for phosphoAktSer473 (pAkt) and GAPDH and then reblotted for total Akt (tAkt) (left). The pAkt signal was normalized to condition ICAM-1 and anti-CD3 Fab alone, (a), dashed line (right); y-axis, fold change over condition (a). This complete experiment was a one-off experiment. This experiment, though, is representative of three similar experiments where the (a)+CD58 condition was missing.

4.3.9 Is CD2 ligation able to counteract PD-1 mediated effect on T cell activation?

PD-1 is a coinhibitory receptor acting on reducing T cell activation. Studies have shown that PD-1-Shp2 dephosphorylate TCR- ζ chain^{143,145}. This also results in reduction in intracellular calcium flux, reduced expression of activation markers and production of IL-2¹⁴⁰. In our study, we have shown that CD2 ligation results in increased levels of classically associated TCR-signalling molecules such as pLAT, pPLC- γ 1 and these molecules are enriched within the CD2 corolla. We, also, showed, that CD2 and PD-1 segregate as CD2 forms this peripheral ring, CD2 corolla while PD-1 is mainly localised around and within the TCR-enriched cSMAC. We, thus, hypothesized, that based on the above effects, CD2 could indirectly counteract some of the PD-1 effects on TCR signalling, allow stronger overall TCR signalling and hence stronger T cell activation despite PD-1 inhibition when compared to the absence of CD2 ligation.

In order to investigate the above hypothesis, we monitored the changes in intracellular calcium levels, calcium flux, during T cell synapse formation. So based on our hypothesis, CD2 ligation would allow a stronger calcium flux upon synapse formation and also a higher number of cells to respond to stimulation. Transfected memory CD8⁺ T cells with two different levels of PD-1 were used; ones with PD-1 levels similar to what is expressed in peripheral blood T cells and ones with PD-1 levels similar to what is expressed in TILs (Figure 4.10A). Transfected T cells were incubated on SLBs reconstituted with ICAM-1, anti-CD3 Fab +/- PD-L1 and/or +/-CD58.

Looking at the intracellular calcium flux profiles of T cells in our experiments, we could detect three main categories of response profiles. The first is a category of activated/triggered cells and a second one is the category of cells that were not activated/triggered i.e. there were no significant detectable changes

in their intracellular calcium levels. The first category was also divided in profiles with a single main peak of calcium change prior to decrease over time, termed as single peak profile response (Figure 4.10B left) and profiles with a more than two main peaks of calcium change prior to decrease over time, termed the oscillating peak profile response (Figure 4.10B, right). Finally, an average of aligned single peak profiles was obtained to compare between different stimulation conditions. An example of average calcium flux profile over time along with all the profiles used in the averaging process is shown in Figure 4.10C.

In the absence of any PD-1 signalling, CD2 ligation resulted in increased sustained intracellular calcium levels when compared to ICAM-1, anti-CD3 Fab alone stimulation (Figure 4.10D and E, i)) and this was observed in two donors tested. The CD2 ligation effect on calcium levels was also detectable when PD-1 ligation was introduced at either PD-1 levels tested (Figure 4.10D and E, ii) and iii). Moreover, in PD-1 ligation conditions, CD2 ligation, allowed an initial higher calcium flux by T cells as shown by the higher single maximum peak at the early stages of synapse formation; this CD2 contribution in a higher initial calcium flux was not obvious, in the absence of PD-1 signalling. Of note is that the increase to higher sustained calcium levels induced by CD2 ligation starts around 3-5 min of synapse formation and correlates with the time that the CD2 corolla starts to form in the IS. In fact, the increased sustained calcium levels time frame also correlates with the presence of CD2 corolla in the synapse i.e. ~5-20 min of synapse formation. Finally the number of cells analysed, assigned in each category is shown in Figure 4.10F and the percentage of activated/triggered T cells and the percentage of single peak profile T cell responses are shown in Figure 4.10G-H. The results of two donors are presented and both donors showed a weak trend towards increased % of triggered T cells upon CD2 ligation. We, also, looked into

whether the ratio of oscillating peak and single peak profiles changed in the different stimulation settings. In the presence of CD2 ligation there was a trend towards a higher percentage of single peaks, too.

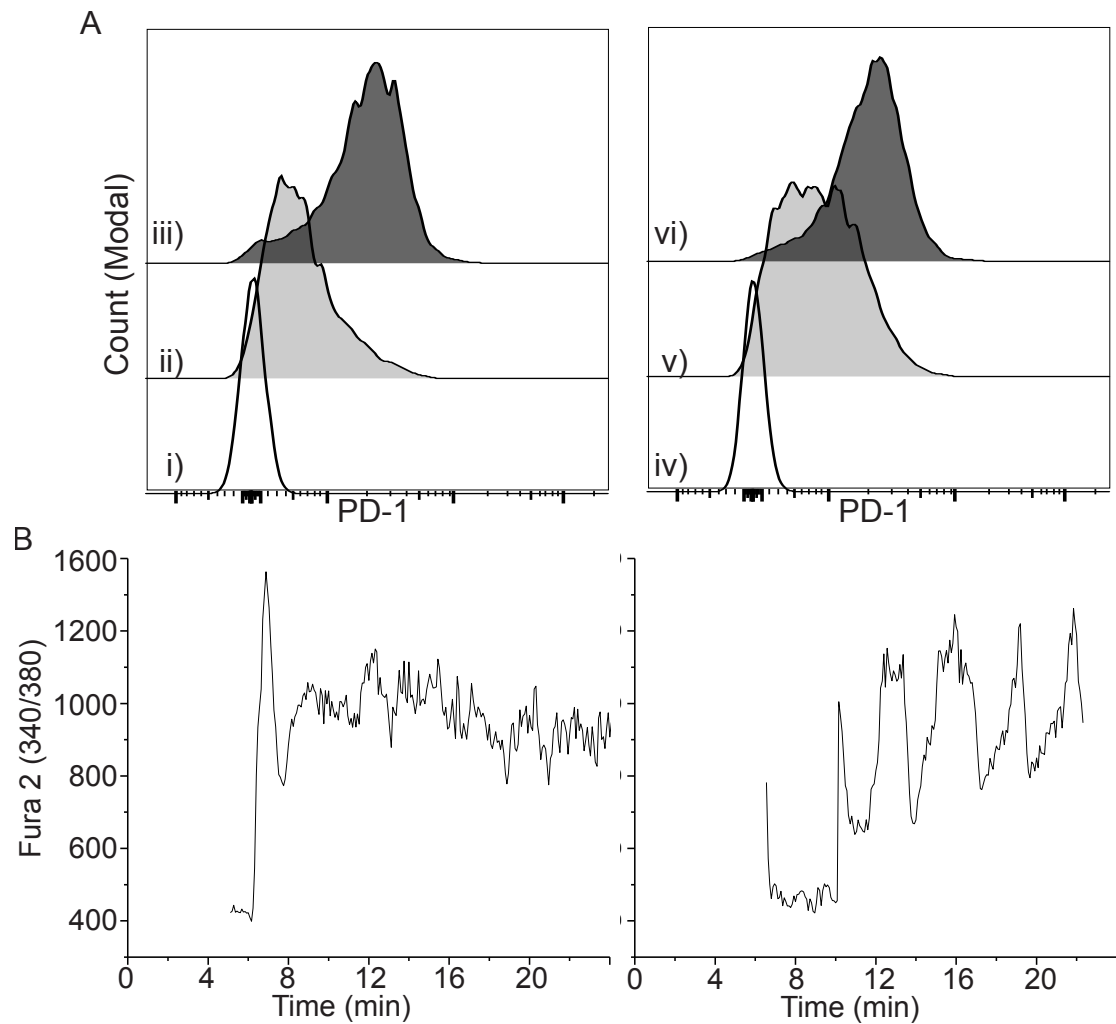
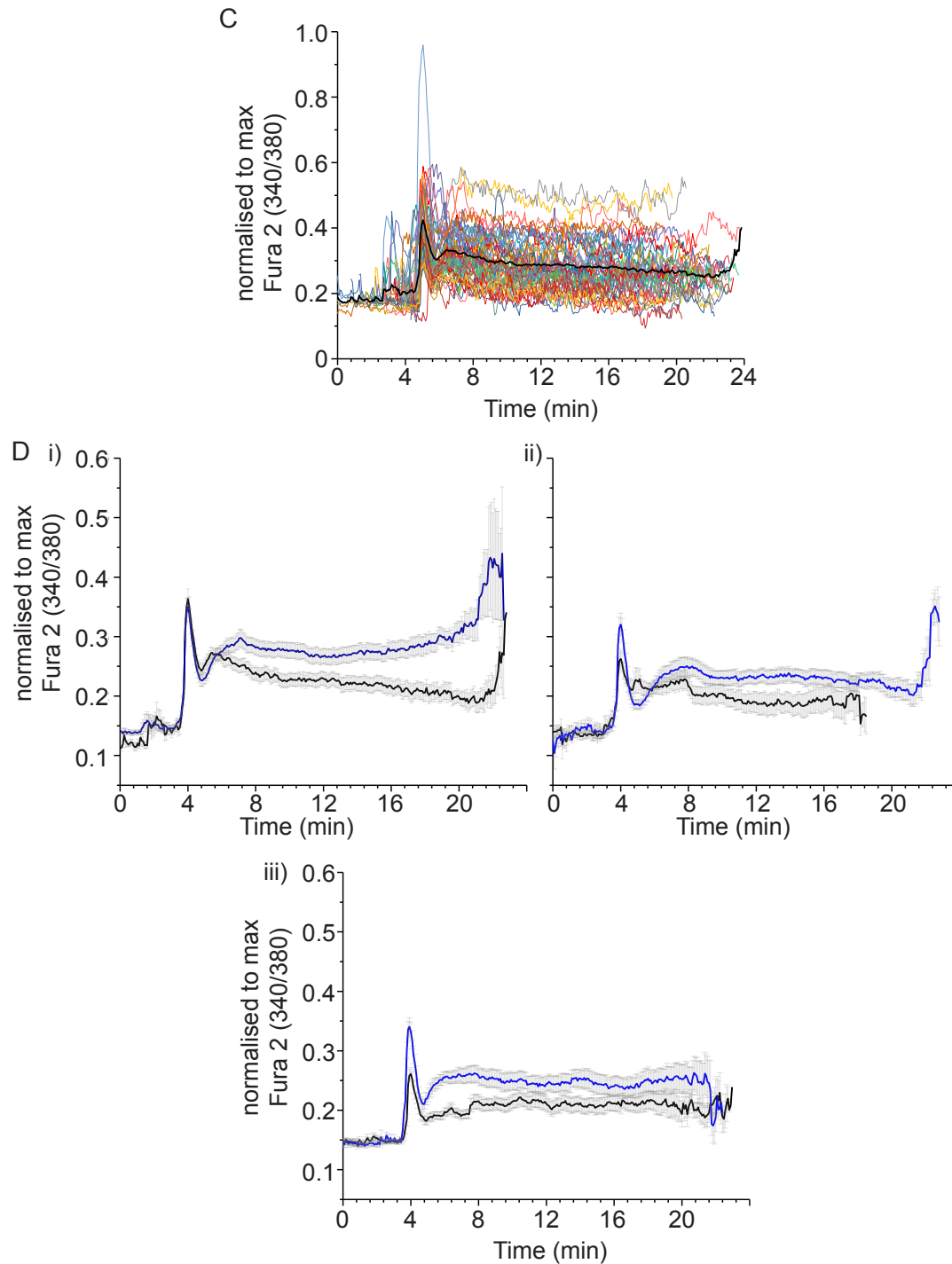
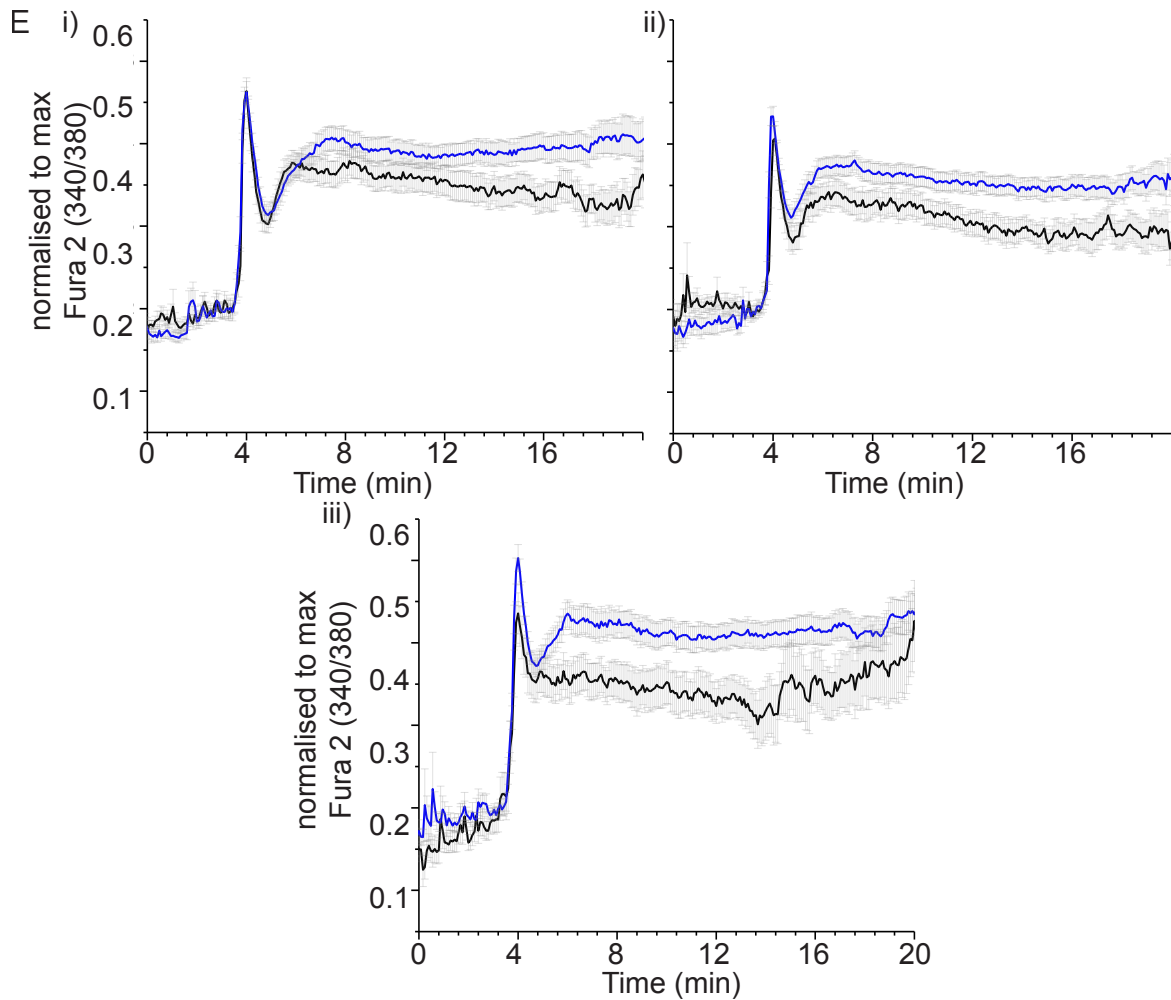


Figure 4.10 CD2 ligation could counteract PD-1 inhibitory effects on T cell activation
A) Memory CD8⁺ T cells from two donors, Donor 1 left panel and Donor 2 right panel, were transfected to express low PD-1 levels ii) and v) and high PD-1 levels iii) and vi); i and iv) are FMO of each donor, ii) 6.1×10^3 molecules/cell (PD-1^{LOW}) iii) 1.23×10^4 molecules/cell (PD-1^H) v) 7.3×10^3 molecules/cell (PD-1^{LOW}) vi) 1.23×10^4 molecules/cell (PD-1^H). B) Left: An example of intracellular calcium changes detected using the ratio of Fura-2 dye at 340/380nm that is assigned in the category of single peak profile response. Right: An example of intracellular calcium changes detected using the ratio of Fura-2 dye at 340/380nm that is assigned in the category of oscillating peak profile.



C) An example of the total number of triggered cells with single peak profiles, shown in various colours, aligned and analyzed for Donor 1 for one of the experimental conditions tested. The solid black line represents the mean of all aligned single peak profiles. D) i) The mean single peak profile of T cells incubated on ICAM-1, anti-CD3, without CD58 (black) reconstituted SLBs and with CD58 (blue) ii) The mean single peak profile of PD-1^{LOW} T cells (6.1×10^3 molecules/cell) incubated on ICAM-1, anti-CD3, PD-L1, without CD58 (black) reconstituted SLBs and with CD58 (blue), iii) The mean single peak profile of PD-1^{HI} T cells (1.23×10^4 molecules/cell) incubated on ICAM-1, anti-CD3, PD-L1 without CD58 (black) reconstituted SLBs and with CD58 (blue). y-axis; Fura-2 ratio (340/380nm) normalized to the maximum ratio value amongst all experimental conditions of the Donor1, error bars; $\pm$ -SEM.

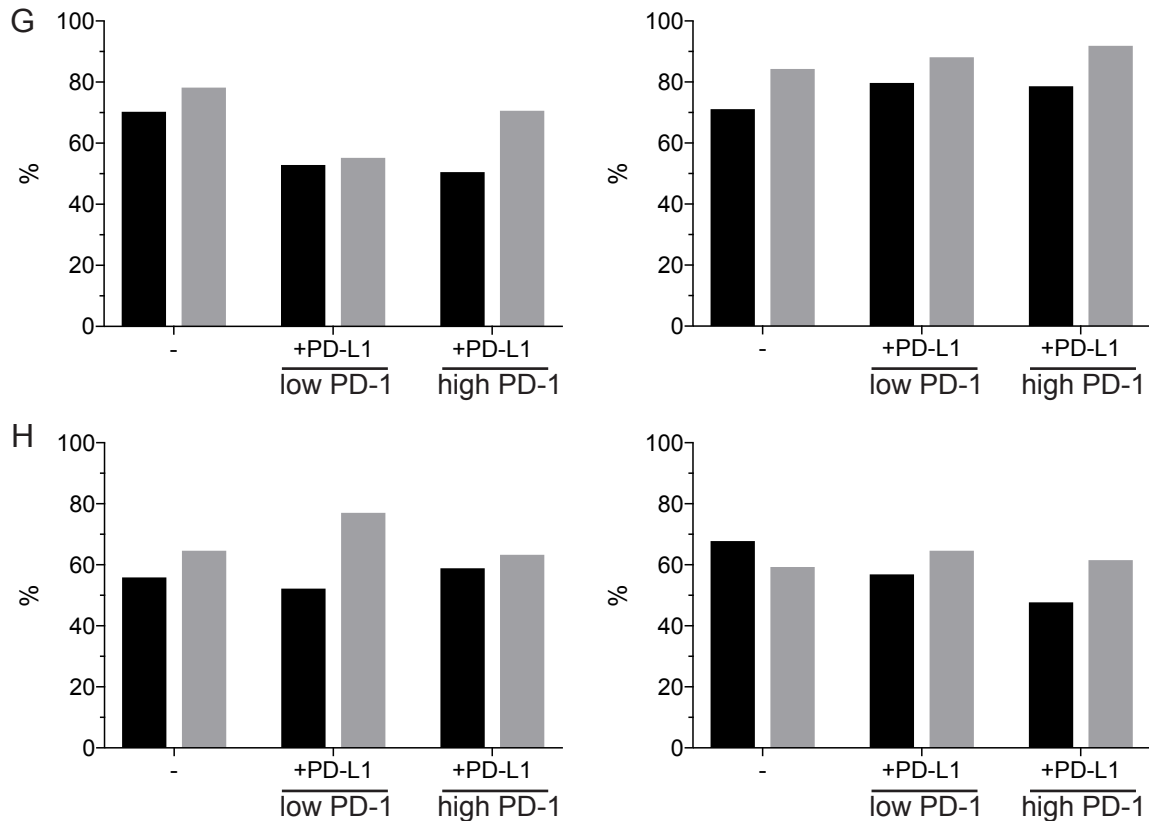


E) i) The mean single peak profile of T cells incubated on ICAM-1, anti-CD3, without CD58 (black) reconstituted SLBs and with CD58 (blue) ii) The mean single peak profile of PD-1^{LOW} T cells (6.1×10^3 molecules/cell) incubated on ICAM-1, anti-CD3, PD-L1, without CD58 (black) reconstituted SLBs and with CD58 (blue), iii) The mean single peak profile of PD-1^{Hl} T cells (1.23×10^4 molecules/cell) incubated on ICAM-1, anti-CD3, PD-L1 without CD58 (black) reconstituted SLBs and with CD58 (blue). y-axis; Fura-2 ratio (340/380nm) normalized to the maximum ratio value amongst all experimental conditions of the Donor2, error bars; +/-SEM.

F

Category of response	-	+CD58	low PD-1		high PD-1	
			+PD-L1	+PD-L1+CD58	+PD-L1	+PD-L1+CD58
single peak	57	51	24	57	33	38
oscillating peak	45	28	22	17	23	22
triggered cells	102	79	46	74	56	60
non-triggered cell	43	22	41	60	55	25
total No. cells	145	101	87	134	111	85

single peak	40	51	29	62	21	56
oscillating peak	19	35	22	34	23	35
triggered cells	59	86	51	96	44	91
non-triggered cell	24	16	13	13	12	8
total No. cells	83	102	64	109	56	99



F) Details of the number of cells analyzed in each experimental condition and the number of cells assigned in each of the three main categories; single peak profile, oscillating peak profile and non-triggered peak intracellular calcium Fura-2 profiles for Donor 1, top table and Donor 2, bottom table. G) The percentage of cells that were triggered (includes T cells with single and oscillating peak profiles) is shown in black for ICAM-1, anti-CD3 stimulation conditions and with the addition of CD58 in grey, Donor 1, left and Donor 2, right. H) The number of T cells that had a single peak profile is shown as percentage of the total number of triggered T cells; in black for ICAM-1, anti-CD3 stimulation conditions and with the addition of CD58 in grey, Donor 1, left and Donor 2, right. Only T cells that were detected and tracked for more than 8.3 min were considered in the above analysis. Single peak profiles were aligned at their maximum peak at time 4 min on x-axis.

4.4 Discussion

We have shown, in this study, that the CD2 corolla, as a structure, can sustain CD28, ICOS clusters in the periphery of the IS. The periphery of the IS is the site of active signalling hence we propose that the CD2 corolla can enhance CD28 and ICOS signalling by retaining the microclusters in the periphery of the IS. One unmet question is how CTLA-4 contributes to CD28-CD80 clusters sustained in the periphery of the IS as CTLA-4 compete for the same ligands as CD28. A CTLA-4-GFP construct is now available to us and in the future an RNA titration approach of this would reveal the CTLA-4 localisation dynamics and effect on CD28-CD80 clusters. In our experiments, we have mostly used resting CD4⁺ and CD8⁺ T cells, including naïve or memory T cells, that express no CTLA-4 or very low levels of CTLA-4, respectively. In fact, human CD8⁺ T cells were shown to express low to null CTLA-4 when compared to human CD4⁺ T cells that express significantly higher levels¹⁹². We have performed experiments with CD8⁺ T cells and observed accumulation of CD80 in the periphery colocalising with CD2 corolla hence we can be confident that this CD80 signal accumulation is mainly CD28 driven. This is also supported by our experiments showing colocalisation of PKC- θ with this peripheral CD28-CD80 signal in the CD2 corolla.

In contrast, CD2 corolla does not readily retain PD-1 microclusters in the periphery of the IS. We detected some peripheral PD-1 microclusters within the CD2 corolla but this was only obvious only in a small fraction of the cells at the highest levels of PD-1 tested, here. A potential factor contributing to the observed differences between CD2 corolla-mediated effect on localization of CD28/ICOS and PD-1 could be the different expression levels of CD28 and PD-1. PD-1 is always at a lower range to CD28, about half the magnitude. We could use the same PD-1 overexpression we used for determining if PD-1 can form a corolla of

its own to determine if these higher levels could result in a peripheral ring of PD-1. We found that T_{FH} cells express the highest levels of PD-1 from all the T cell subsets we have tested; these PD-1 levels are similar to the levels of CD2 expressed in T cells. Preliminary experiments with T_{FH} cells on SLB suggest that PD-1 microclusters can be retained in the periphery of the IS within the CD2 corolla, suggesting that indeed the PD-1 expression level can determine the localisation of PD-1 relative to the CD2 corolla. Only recently we were able to achieve high enough PD-1 levels (see Figure 4.8) similar to the levels expressed in T_{FH} cells. So we should repeat this transfection experiment and look at the localization of the fluorescently coupled PD-1 at these high levels in the presence of CD2 corolla. This should be a way to reproduce the aforementioned preliminary experiments with T_{FH} cells.

The question then becomes what is the significance of the CD28, PD-1 differential localisation relative to the CD2 corolla. An obvious consequence is that the CD2 corolla can segregate CD28 and PD-1; this induced segregation could effectively spare CD28 from PD-1 mediated inhibition since PD-1 mediated inhibition depends on the close localization of the two receptors. We have tried to prove that co-immunoprecipitation of CD28-p85-PKC- θ in our SLB system but this was not efficient due to experimental limitations. A promising alternative seems to be the use of T:APC cell conjugates but this has its own limitations, as it is not a stimulation platform that allows flexible control over the ligands and densities of ligands presented to the T cells.

We have explored also the hypothesis that CD2, due to its TCR signaling enhancing effect, could counteract some of the inhibitory effects of PD-1 on T cell activation. Indeed, CD2 can sustain augmented intracellular calcium levels upon IS formation even in the presence of PD-1 ligation. There was also a trend towards

an increased percentage of activated T cells in the presence of CD2 ligation even in the presence of PD-1 inhibition. However, due to our low number of T cells and the small number of donors tested we cannot make firm conclusions on the above. We wish to improve on our method and analysis of intracellular calcium flux of T cells upon IS formation to investigate this more thoroughly and reach solid conclusions.

* In the case of PD-L1 unlabelled used in experiments presented in Figures 4.5, 4.6, 4.8 and 4.10, it was find out later (after submission of copy to examiners) that this form of PD-L1 was actually at 1000 molecules/ μm^2 when used on SLB instead of 100 molecules/ μm^2 as intended and as initially calculated. This was probably due to a combination of errors: the overestimating error of the commercially available calibration beads (discussed in Materials Section) and a wrongly calculated fluorescent to protein ratio of an in-house labelled anti-human PD-L1 mAb.

However, the experiments have been repeated with 3 more donors with the corrected density for unlabeled PD-L1 with similar results.

5 Chapter 5. Functional consequences downstream of CD2 ligation and CD2 profiling of T cells in the tumour microenvironment.

1.1 Introduction

In this study we are proposing that a key player enhancing T cell activation by orchestrating co-stimulatory signalling in the T cell synapse is the formation of the CD2 corolla. We wished to support this with further evidence with downstream assays where T cell activation also results in effector functions like cytokine production or T cell cytotoxicity. We, thus, set up a cytotoxicity assay to test the contribution of CD2 in this setting. We have no way, yet, to perturb the formation of CD2 corolla without reducing the number of CD2 receptors on the T cell membrane so until a way is designed to do so, we tested our hypothesis in the presence and absence of CD2 ligation. It is our working model that the CD2 corolla is directly linked to how ligated CD2 behaves, and hence it will be difficult to uncouple the corolla formation from the function of CD2.

It was shown previously, in mouse, *in vivo* experiments that CD2 lowers T cell activation threshold and allows T cells to respond to lower levels of antigen⁸⁴. We sought to investigate this further in humans while at the same time looking into what is the effect of CD2 ligation in the response of T cells to a lower affinity antigens compared to their WT counterparts; this question has not been addressed before to our knowledge. We, thus, took advantage of the alter peptide ligand library (APL) of the NY-ESO-9V peptide¹⁹³ and our system of 1G4 TCR transfected CD8 T cells to look at activation of T cells in response to the APLs in the presence and absence of CD2 ligation. We hypothesized that CD2-CD58

blockade will reduce T cell activation and this effect will be more pronounced for low levels of antigen and for low affinity peptides.

Finally, we are proposing that the expression levels of CD2 play a role in CD2 corolla formation and subsequently the degree by which CD2 can enhance T cell activation. We thus, hypothesized, that the CD2 pathway will be overactive or down-regulated in a disease setting such as in autoimmunity or cancer, respectively. Higher levels of CD2 would enhance T cell activation and potentially contribute to autoimmune responses whereas in the tumour setting, exhausted T cells, especially CD8⁺ T cells that are key in T cell cytotoxicity against the tumour, will be impaired in their CD2 pathway due to a reduction in CD2 expression levels.

5.1 Results

5.1.1 The role of CD2 in CD8⁺ T cell cytotoxicity against cancer

We tested the killing capacity of a 1G4⁺ CTL clone against the Trombelli cell line¹⁸⁹, a melanoma tumour cell line, in the presence and absence of CD2 ligation. CD2-CD58 interactions were blocked using an anti-CD58 blocking antibody (TS2/9). The 1G4 cytotoxic lymphocyte (CTL) clone generated by Li Yi *et al*, was kindly provided by Dr Jili Chen (WIMM, University of Oxford) and so was the Trombelli melanoma cell line. We, initially, determined the time point at which we can detect the effect of CTL activity against the tumour cell line i.e. cell death of melanoma cells using a colormetric assay quantifying the levels of lactate dehydrogenase released by dying cells previously used in¹⁸⁹. There was no detectable cytotoxicity at 4hrs of co-culture of the 1G4 T cell with melanoma cell line¹⁸⁹, while we were able to detect significant CTL cytotoxicity effects at 24hrs of co-culture (Figure 5.1A). Blocking CD2-CD58 interactions reduced the detectable cytotoxicity by ~10-fold (68% to 7%). Following this initial experiment, we titrated the ratio of 1G4 CTLs, effector cells, to melanoma cells, target cells (E:T ratio) in an effort to see the contribution of CD2 ligation and its significance at limiting number of CTLs, which is often the case in the tumour microenvironment. The effect of CD58 blockade was more pronounced, ~8-fold; (47% to 6%) at 1:1, E:T ratio, when compared to 3:1 E:T ratio in the first titration experiment (Figure 3.1B, left); similarly to the time course experiment (Figure 5.1A). However, even at the ratio of 3:1 CD58 blockade resulted in a significant 3-fold reduction in CTL cytotoxicity (74% to 27%). In the 2nd and 3rd experiments, there was only significantly detectable cytotoxicity at 3:1 ratios; it is thought that this is potentially due to signs of exhaustion phenotype of CTL clone (CD127^{LOW}PD-1^{HI} as well as CD28^{LOW}) observed at the time of those experiments induced by the extensive

propagation of the clone in culture (data not shown). Even in those cases, CD58 blockade reduced CTL killing capacity; (2nd experiment; 38% to 3% and 3rd experiment 23% to 1%). In summary, the overall effect of CD58 blockade was a significant impairment of CTL cytotoxicity against tumour cells.

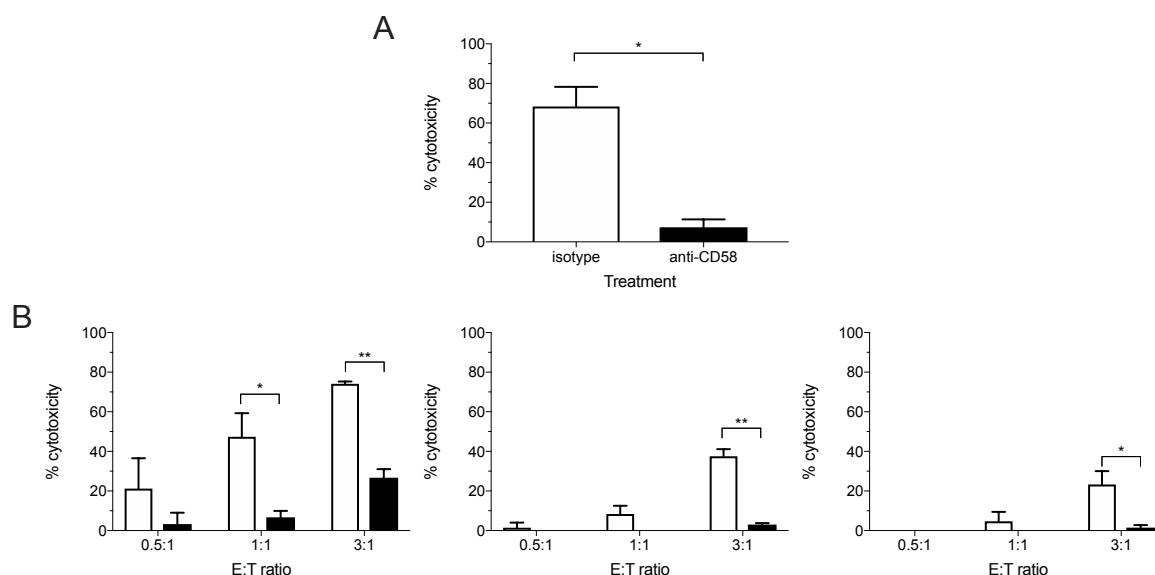


Figure 5.1 Blocking CD2-CD58 impairs CTL cytotoxicity against tumour cells

A) Trombelli cell line melanoma cells were co-cultured with 1G4 CTL clone at a 3:1 Effector:Target (E:T) ratio for either 4hr or 24hr in the presence of blocking anti-CD58 mAb, TS2/9 clone, (black) or isotype control (empty). Cell-free culture supernatants were harvested and the percentage of cytotoxicity (% cytotoxicity) was determined using the lactate dehydrogenase cytotoxicity assay and their method of calculating % cytotoxicity i.e. calibration of the degree of cytotoxicity using two conditions, untreated targets cells alone and targets cells alone treated with a 1% Triton-X100 (final concentration); the mean of triplicates per condition is shown. There was detectable cytotoxicity above background only at 24hr. *, p value<0.05 with parametric Welch's *U*-test, Error bars represented SD. One such a experiment was performed

B) Trombelli cell line melanoma cells were co-cultured with 1G4 CTL clone at titrating E:T ratios, 0.5:1, 1:1 and 3:1 Effector:Target (E:T) ratio for 24hr in the presence of blocking anti-CD58 mAb (black) or isotype control (empty). The percentage of cytotoxicity (% cytotoxicity) was determined as in (A); the mean of triplicates per condition is shown. Three independent experiments were performed and shown, here, from first to last, left to right. *, p value<0.03, **, p<0.003 with parametric Welch's *U*-test, Error bars represented SD.

5.1.2 The effect of CD2 on the T cell response to different levels of cognate antigen and different affinity cognate antigens.

1G4 TCR transfected CD8⁺ T cells were co-cultured for 24hrs with an EBV-transformed B cell line, CF996, loaded with varying levels of the NY-ESO-9V peptide cognate antigen and/or NY-ESO 9V-APLs of varying affinities¹⁹³. T cell activation was evaluated by determining the percentage and levels of activation markers CD25 and CD69 as well as of PD-1 and CD2 receptors that are known to be up-regulated upon T cell activation. An example of the gating strategy is shown in Figure 5.2A. As controls, 1G4 TCR⁺ T cells were either co-cultured with no peptide loaded B cells or alone and 1G4 TCR⁻ T cells were co-cultured with no peptide loaded B cells (Figure 5.2B-C). The APLs used and their respective K_D (μ M) are the following: ESO-9V, 7.2, ESO-3M, 9.2, ESO-3I, 17, ESO-3Y, 30, ESO-9L, 56, ESO-6T, 101, ESO-4D, 252.

The response of T cells to decreasing levels of 9V-peptide antigen is shown in Figure 3.2D and F while the T cell response to same conditions with additional CD2-CD58 blockade is shown in Figure 3.2E and G. As expected, there was a gradual reduction in the percentage of activated T cells defined as CD25⁺CD69⁺ or CD2^{HI}PD-1^{HI} with decreasing levels of loaded 9V peptide. CD2-CD58 blockade resulted in a lower percentage of activated T cells (CD25⁺CD69⁺) compared to control. If we take into account the “background” activation detected when co-culturing 1G4 TCR⁺ T cells with no peptide loaded APCs, this reduction was only 5% for peptide loading concentrations 40-100nM and 0% for 8nM. This reduction was slightly more pronounced, 12%, at lower peptide loading concentrations 0.32-1.6nM. A similar trend was observed for CD25 mean fluorescence intensity, CD25 MFI; there was a decreasing CD25 MFI with decreasing 9V peptide concentration. CD2-CD58 blockade reduced further CD25 MFI and the reduction was more profound at 0.3-8nM 9V peptide loaded concentrations. However, when comparing

the CD2-CD58 blockade-induced reduction to 'background activation' levels it seems that CD2-CD58 blockade is only blocking this 'background activation' and it is clearly obvious at 0.3-8nM 9V peptide loaded concentrations. This reduction is not clear at higher peptide concentrations. This is potentially shadowed by the increased CD25 MFI of the other 60-75% of CD25+CD69+ T cells, beyond the 'background activation' percentage. A similar phenotype was observed for the 9V-APLs used here (Figure 5.3). Indeed, the 'background activation' makes CD2-CD58 blockade effects difficult to evaluate and interpret confidently. Interestingly, thought, it is clear that CD2-CD58 blockade can abrogate this 'background activation'.

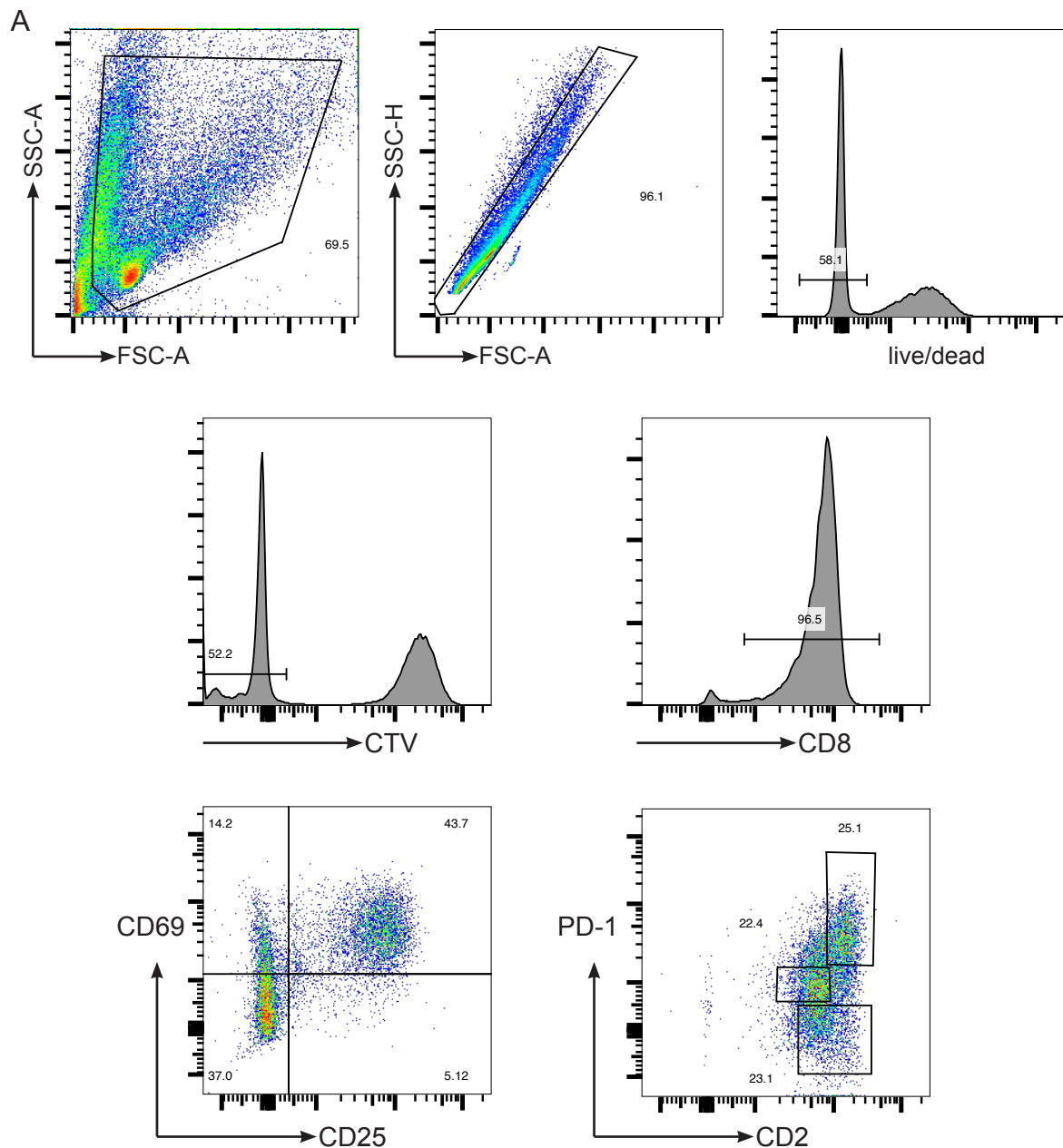
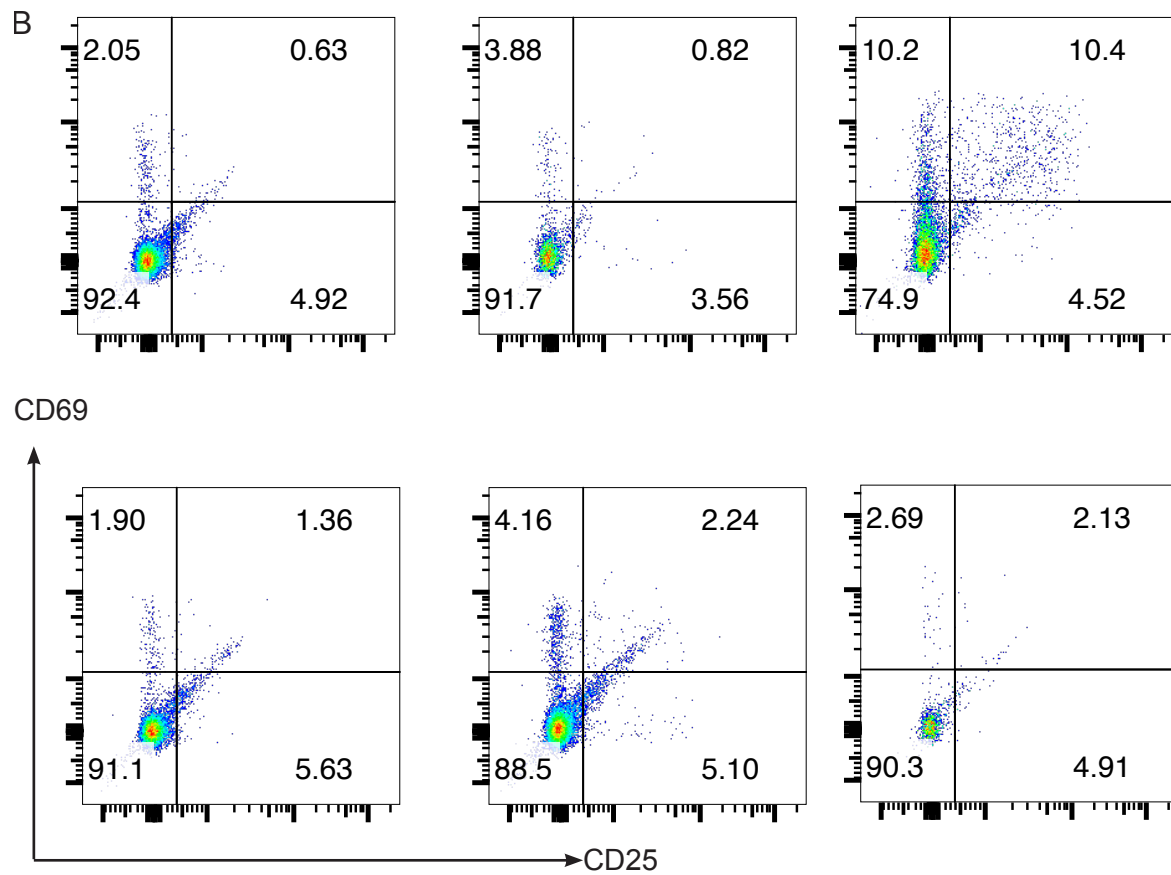
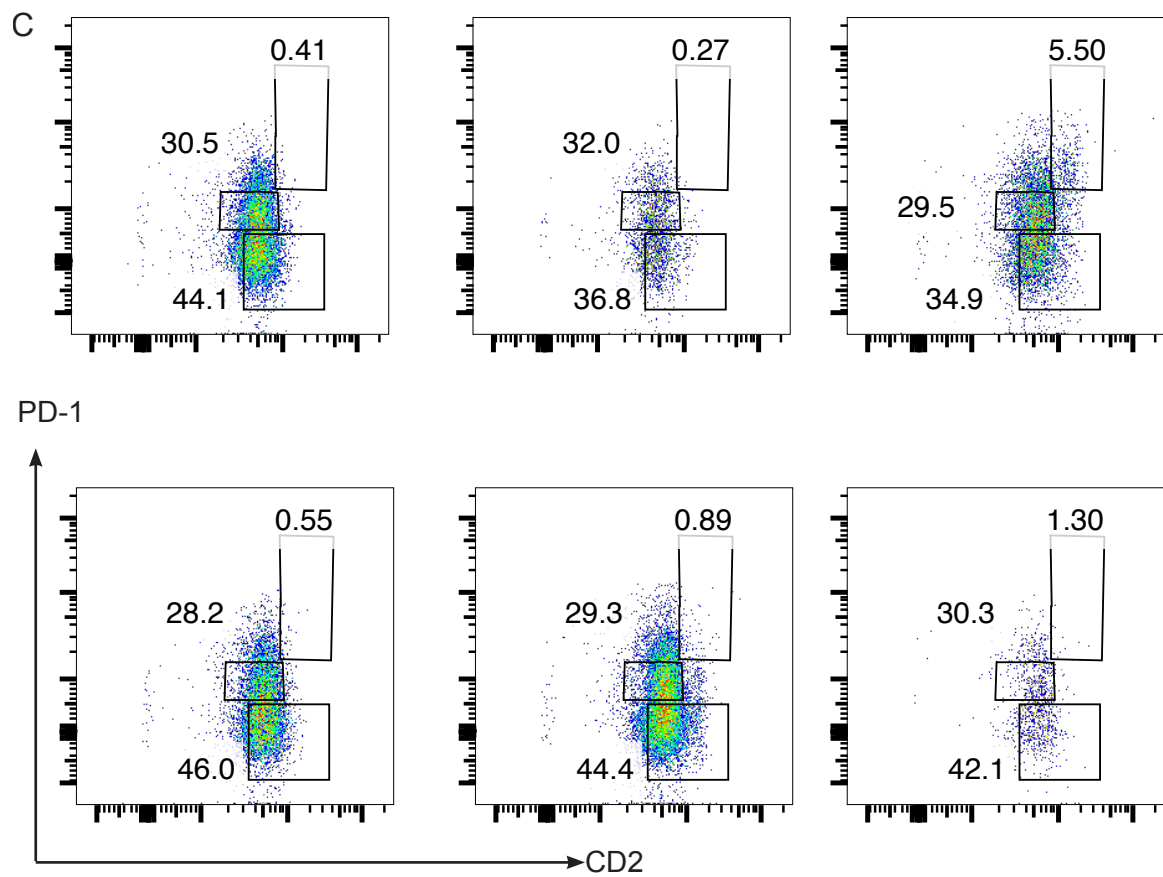


Figure 5.2 The effect of CD2 ligation in T cell activation in response to low levels of antigen and/or low affinity antigens.

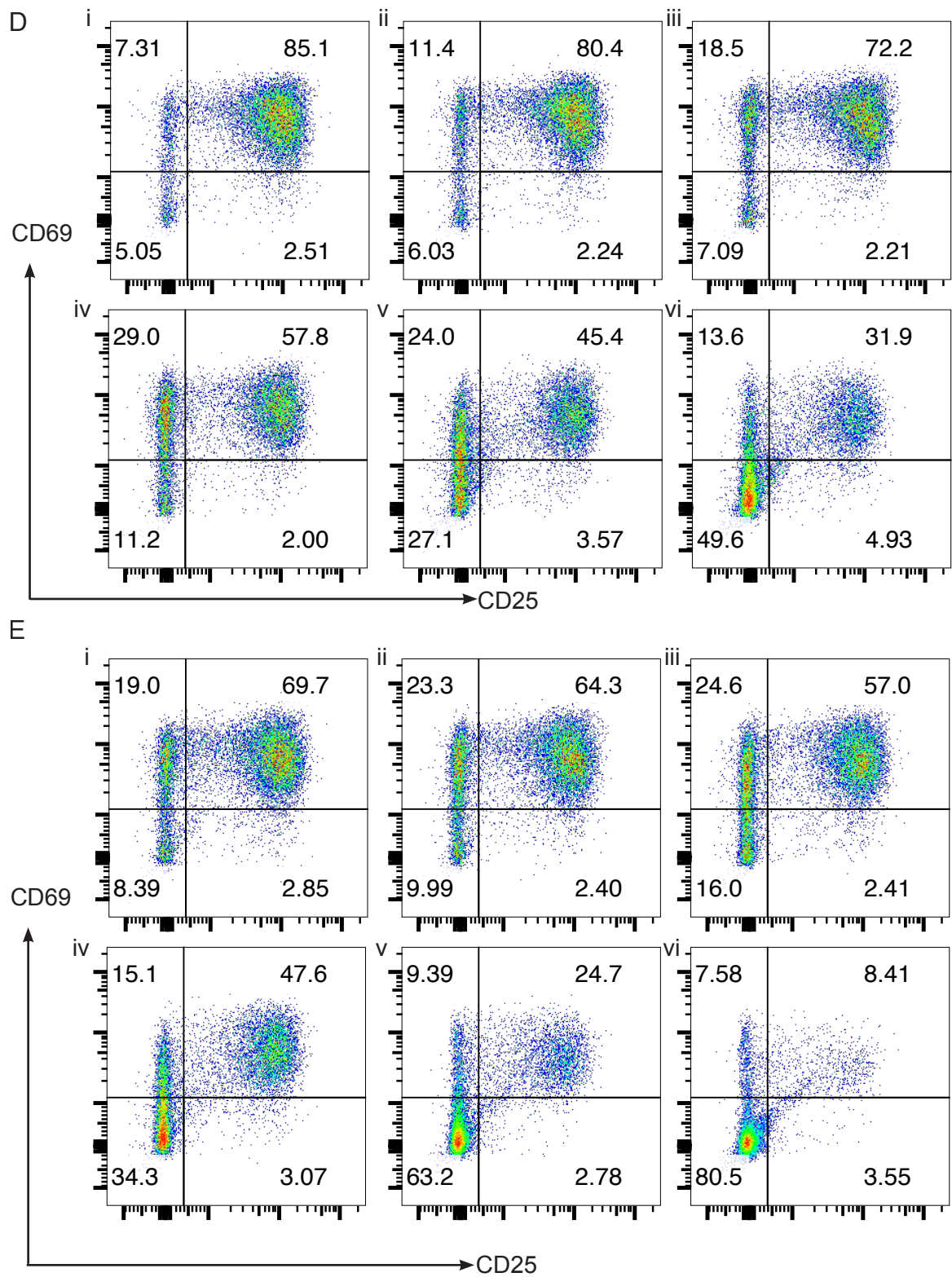
1G4 TCR transfected total CD8⁺ T cells were co-cultured NY-ESO-9V altered peptide ligands (APLs) loaded APCs(EBV-transformed B cell line) for 24hr before flow cytometric analysis of T cell activation in the presence of blocking anti-CD58 mAb, TS2/9 clone, or isotype control. The activation profile of T cells was quantified by determining their CD25-CD69 expression profile as well as their PD-1-CD2 expression profile; for the latter PD-1^{HI}CD2^{HI} were quantified. A representative (with one donor) of two experiments is shown. A) The gating strategy used for quantifying the activation of CD8⁺ T cells is shown. CTV; cell trace violet volumetric dye was used to label APCs prior to co-culture with T cells.



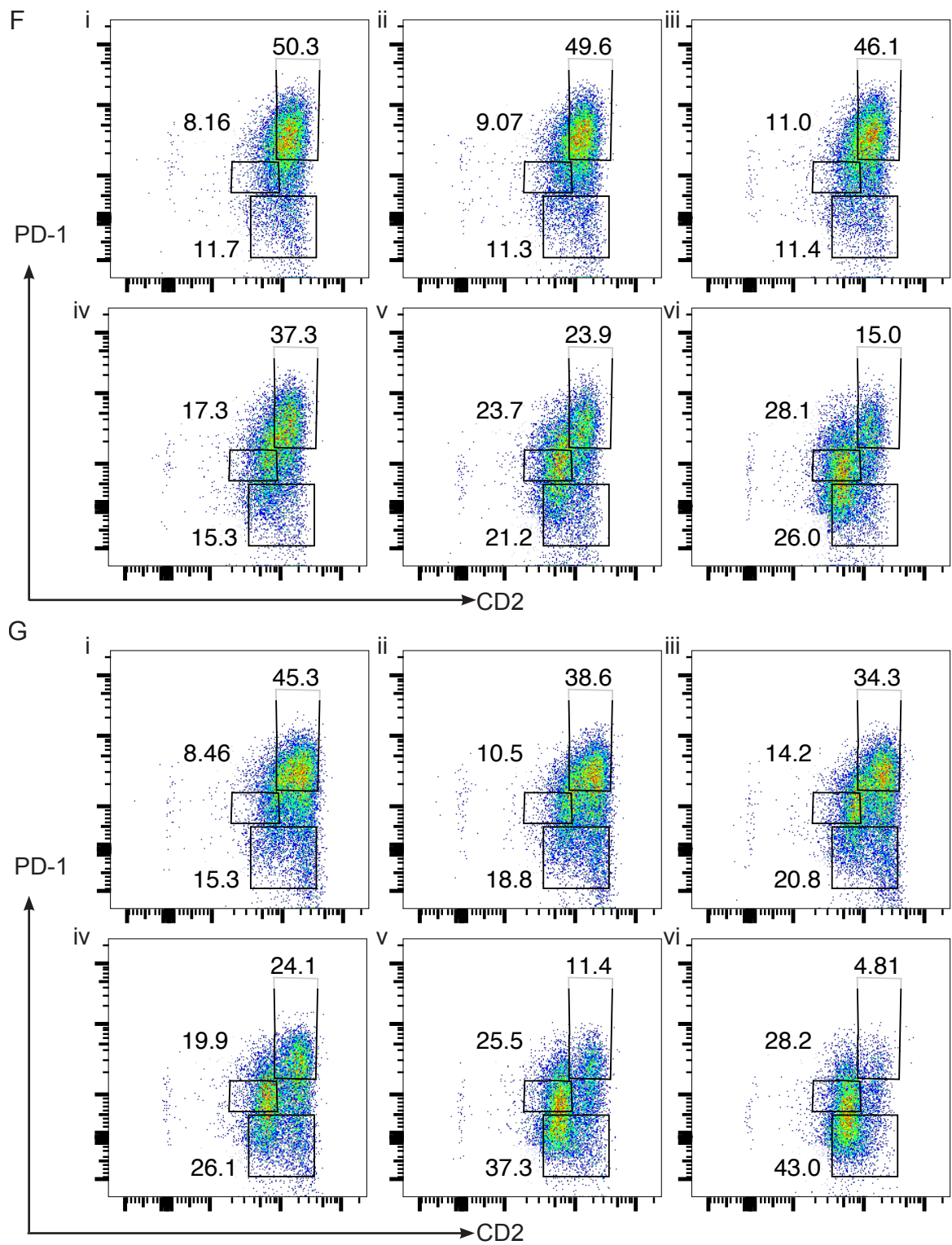
B) The CD69-CD25 expression profiles of CD8⁺ T cells in stimulation control conditions are shown here; isotype control (top), blocking anti-CD58 mAb (bottom). T cells cultured alone (left), 1G4 TCR negative T cells co-cultured with no peptide loaded APCs (middle), 1G4 TCR⁺ T cells co-cultured with no peptide loaded APCs (right).



C) The PD-1-CD2 expression profiles of CD8⁺ T cells in stimulation control conditions are shown here; isotype control (top), blocking anti-CD58 mAb (bottom). T cells cultured alone (left), 1G4 TCR negative T cells co-cultured with no peptide loaded APCs (middle), 1G4 TCR⁺ T cells co-cultured with no peptide loaded APCs (right).



The CD69-CD25 expression profiles of CD8⁺ T cells co-cultured with APCs loaded with decreasing levels of NY-ESO-9V (left-right: i) 1000, ii) 200, iii) 40, iv) 8, v) 1.6, vi) 0.32 nM) in the presence of isotype control mAb (D) or blocking anti-CD58 mAb (E) are shown.



The PD-1-CD2 expression profiles of CD8⁺ T cells co-cultured with APCs loaded with decreasing levels of NY-ESO-9V (left-right: i) 1000, ii) 200, iii) 40, iv) 8, v) 1.6, vi) 0.32 nM) in the presence of isotype control mAb (F) or blocking anti-CD58 mAb (G) are shown.

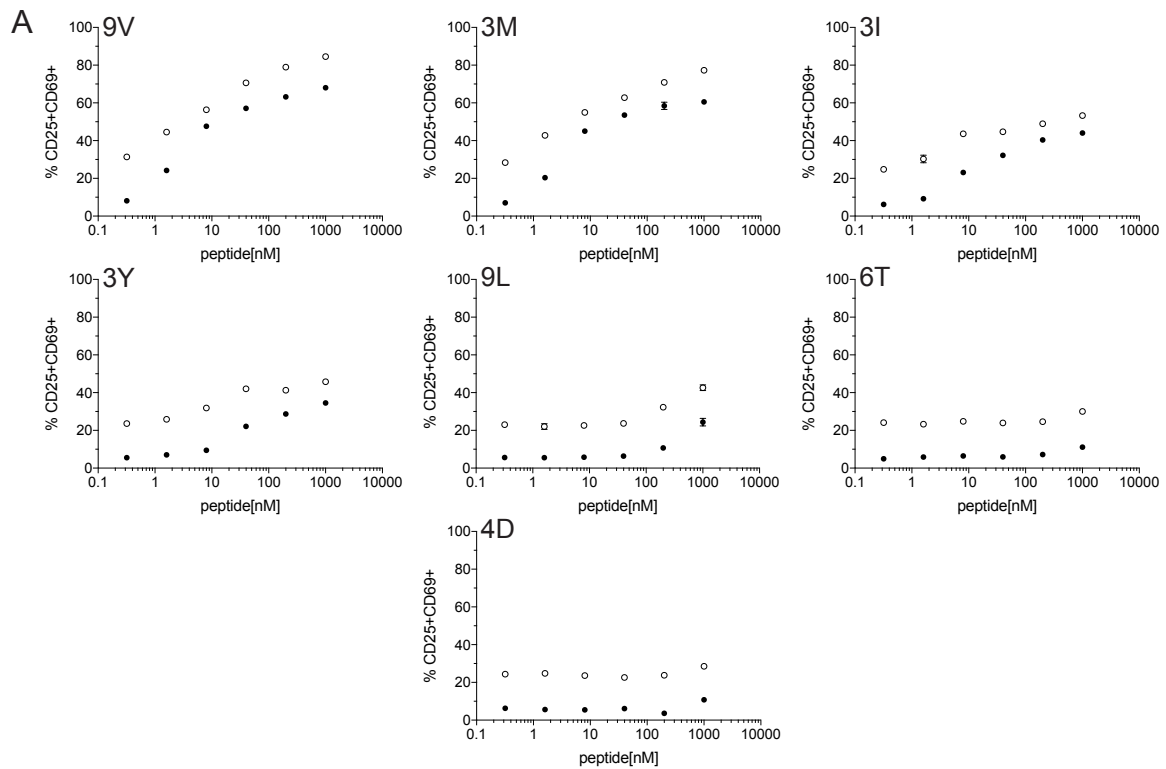
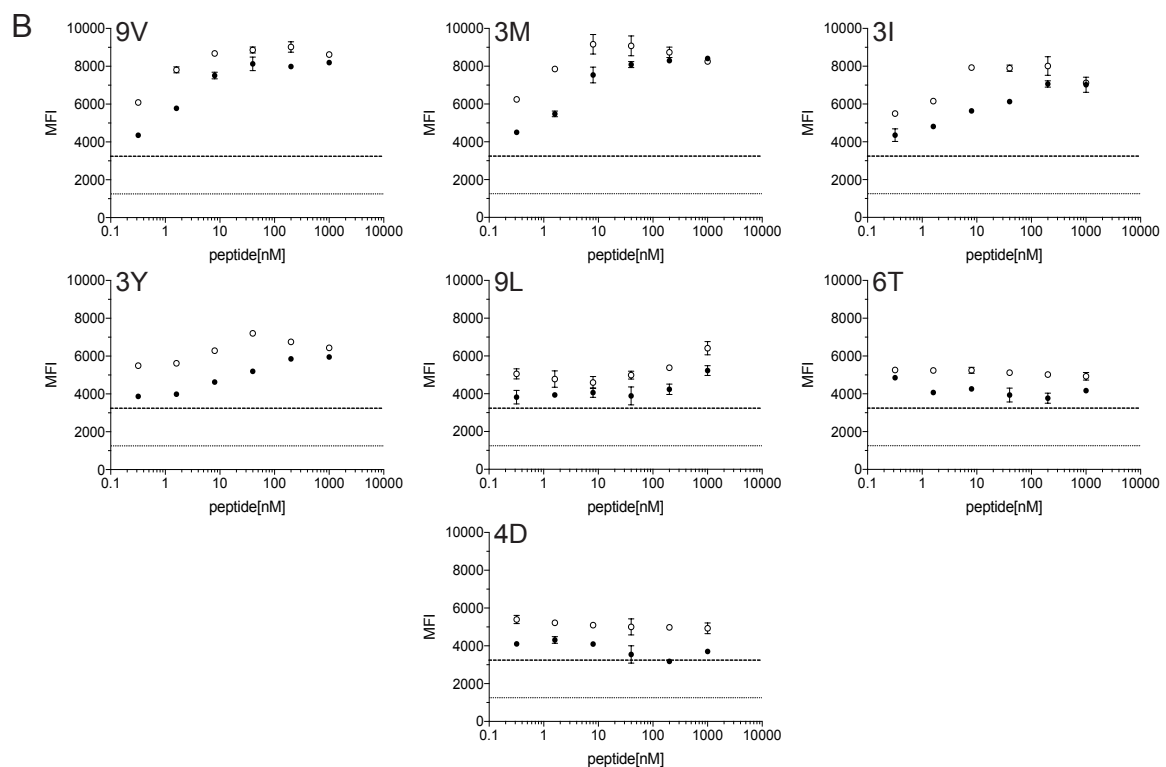
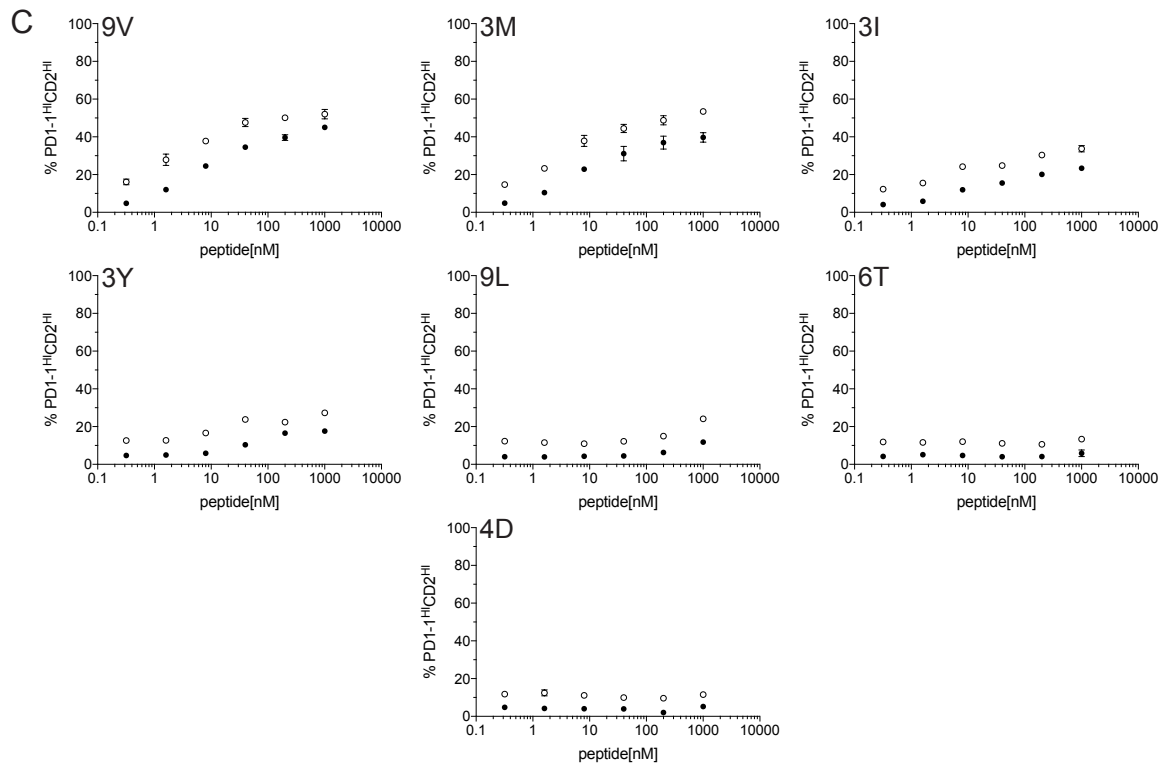


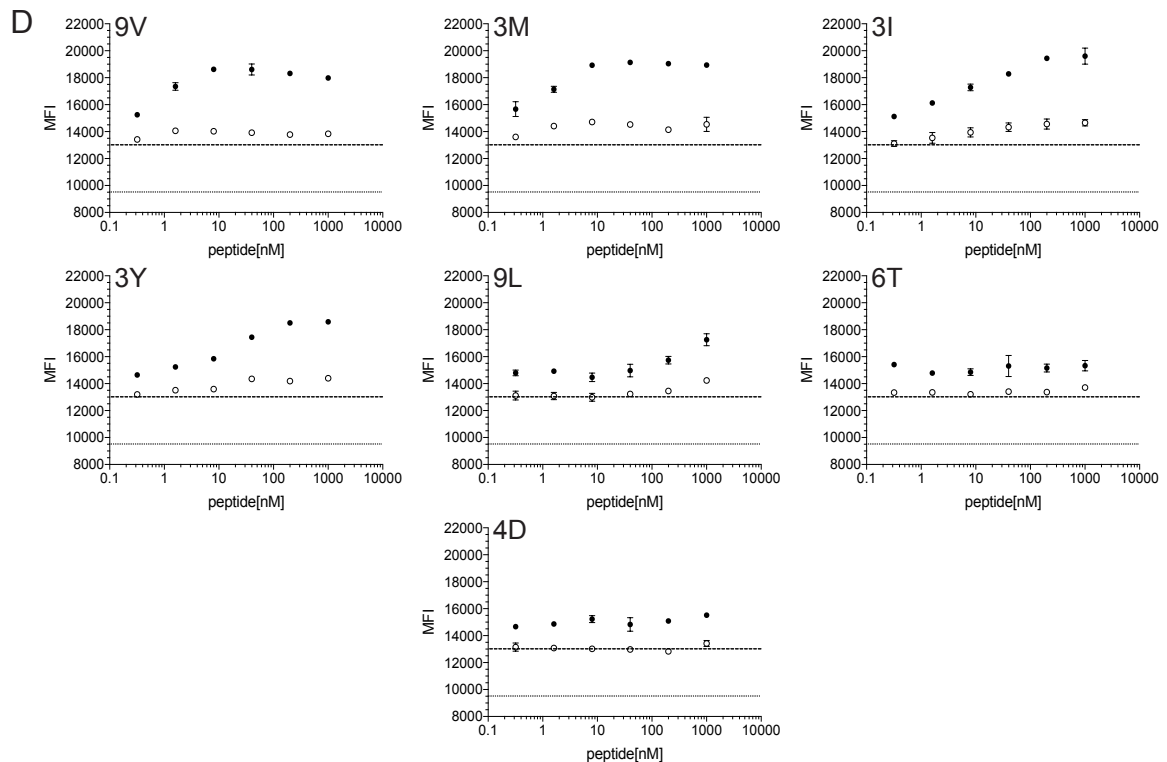
Figure 5.3 The effect of blocking CD2-CD58 interactions on T cell activation by TCR-pMHC interactions of different affinities, at decreasing concentrations of antigen. Collective results of from experiment presented in Figure 5.2

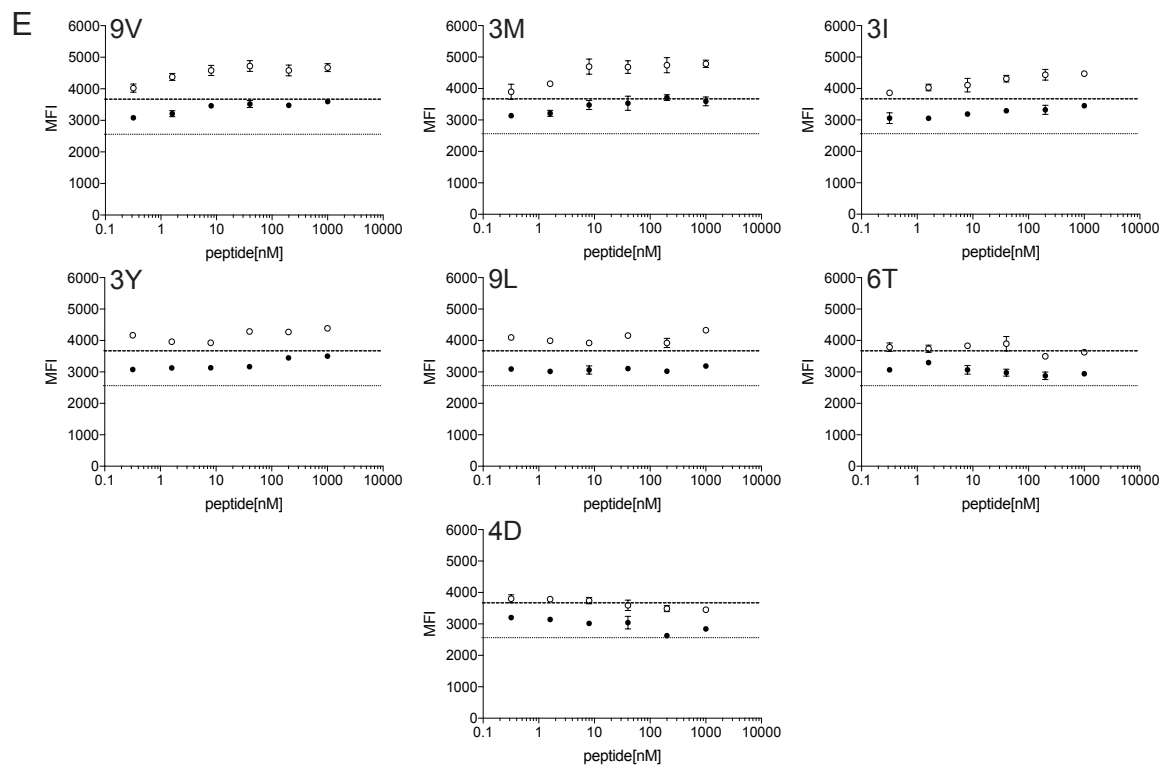
A) The percentage of CD69+CD25+ CD8+ T cells is shown at decreasing concentration of APL of NY-ESO-9V (9V, 3I, 3M, 3Y, 9L, 4D, 6T; high to low affinity). B) The graphs show the CD25 mean fluorescence intensity, MFI, of CD69+CD25+ CD8+ T cells shown in (A); dashed line, CD25 MFI of 1G4 T cells+no peptide loaded APCs(isotype), dotted line, CD25 MFI of 1G4 T cells+no peptide loaded APCs(blocking anti-CD58 mAb)





C) The percentage of PD-1^{HI}-CD2^{HI} CD8⁺ T cells is shown at decreasing concentration of APL of NY-ESO-9V (9V, 3I, 3M, 3Y, 9L, 4D, 6T; high to low affinity). D) The graphs show the CD2 mean fluorescence intensity, MFI, of PD-1^{HI}-CD2^{HI} T cells shown in (C); dashed line, CD2 MFI of 1G4 T cells+no peptide loaded APCs(isotype), dotted line, CD2 MFI of 1G4 T cells+no peptide loaded APCs (blocking anti-CD58 mAb).





E) The graphs show the PD-1 mean fluorescence intensity, MFI, of PD-1^{HI}-CD2^{HI} T cells shown in (C); dashed line, PD-1 MFI of 1G4 T cells+no peptide loaded APCs(isotype), dotted line, PD-1 MFI of 1G4 T cells+no peptide loaded APCs (blocking anti-CD58 mAb).

5.1.3 The expression levels of CD2 in colorectal cancer (CRC)

We established a collaboration with the lab of Fiona Powrie and with the support of the NIHR Biomedical Research Center, Oxford, we gained access to colorectal cancer patient samples. We quantified the mean number of CD2 molecules in CD4⁺ and CD8⁺ T cells found in the tumour (CD4⁺ and CD8⁺ TILs) and compared them to the CD2 levels expressed in CD4⁺ and CD8⁺ T cells from normal adjacent tissue to the tumour tissue of the same patient. At the same time, the exhaustion profile of those subsets was determined by looking at their CD127, PD-1, CD28 expression profiles (Figure 5.4A).

Initially, the CD2 levels of all CD4⁺ and CD8⁺ T cells, without any focus on specific subpopulations were quantified and compared to normal tissue CD2 T cell expression (Figure 5.4B-E). Three out of eight patients showed reduced levels of CD2 in both CD4⁺ and CD8⁺ TILs, a fourth patient only in CD8⁺ TILs; this patient showed no changes in CD2 levels of CD4⁺ TILs. The CD2 reduction was more pronounced in CD8⁺ T cells; there was an average decrease of $\sim 1.56 \times 10^4$ molecules/cell and $\sim 1.00 \times 10^4$ molecules/cell in CD8⁺ and CD4⁺ TILs, respectively (Figure 5.4C-D). In contrast, three out of eight patients showed increased CD2 levels in CD4⁺ T cells and interestingly, no changes in CD2 levels of CD8⁺ TILs. An eighth patient showed an increase in CD2 levels in both T cell compartments. On average, the increase in CD2 in CD4⁺ T cells was $\sim 1.85 \times 10^4$ molecules/cell (ranging from 4.9×10^3 - 27.8×10^4 molecules/cell). These changes are summarised in Figure 5.4E.

We looked further into the aforementioned changes in CD2 levels by determining the CD2 levels in T cell subsets defined by CD28 and PD-1 expression, examples of these profiles, from three CRC patients, are shown in Figure 5.5A for the CD4⁺ compartment and in Figure 5.5B for the CD8⁺

compartment. Within the CD4⁺ compartment, we detected mainly CD28+PD-1⁻ and CD28+PD-1⁺ CD4⁺ T cell subsets. In the three patients that showed a reduction in CD2 levels in total CD4⁺ T cell compartment, this reduction was more obvious in the CD28+PD-1⁻ CD4⁺ T cell subset (Figure 5.5C, top panel and 5.5D, left). In contrast, in the four patients that showed an increase of CD2 molecules/cell, this increase was reflected in the CD28+PD-1⁺ CD4⁺ T cell subset (Figure 5.5C, bottom panel and 5.5D, right). A similar analysis was performed for the CD8⁺ compartment of the patients. In the CD8⁺ T cell compartment, T cell subsets expressing all four CD28[±] and PD-1[±] combination profiles were detectable in the majority of the patients. (Figure 5.5B). The CD2 levels of all four subsets for the patients that showed a significant reduction in CD2 levels of the CD8⁺ T cell compartment are shown in Figure 5.6 A, top panel and 5.6B, left). The reduction in CD2 levels was seen in all four subsets but was more pronounced in CD28+PD-1⁻, CD28-PD1⁺, and CD28-PD-1⁻ T cell subsets. The latter three CD8⁺ T cell subsets found in the tumour, expressed on average 4.25×10^4 CD2 molecules/cell while the same subsets in the normal adjacent tissue expressed on average 6.34×10^4 CD2 molecules/cell. The raw values of CD2 molecules/cell for each T cell subsets along with which subsets were found in each compartment of each patient are shown in Figure 5.7. Moreover, this significant reduction in the CD2 levels of the CD8⁺ T cell compartment of the tumour microenvironment, seemed to correlate with higher PD-1 levels in PD1⁺ CD8⁺ T cell compartment, on average 1.47×10^4 molecules/cell (Figure 5.6C, left); this was not statistically significant thought due to significant donor variability and the small number of patients. The CRC patients that showed no changes in CD2 levels in their CD8⁺ T cell compartment had lower PD-1 levels, on average 5.6×10^3 molecules/cell (Figure 5.8). A similar analysis was performed for the CD2 level changes detected

in the CD4+ T cell compartment of the tumour microenvironment; there were no clear correlations in this occasion.

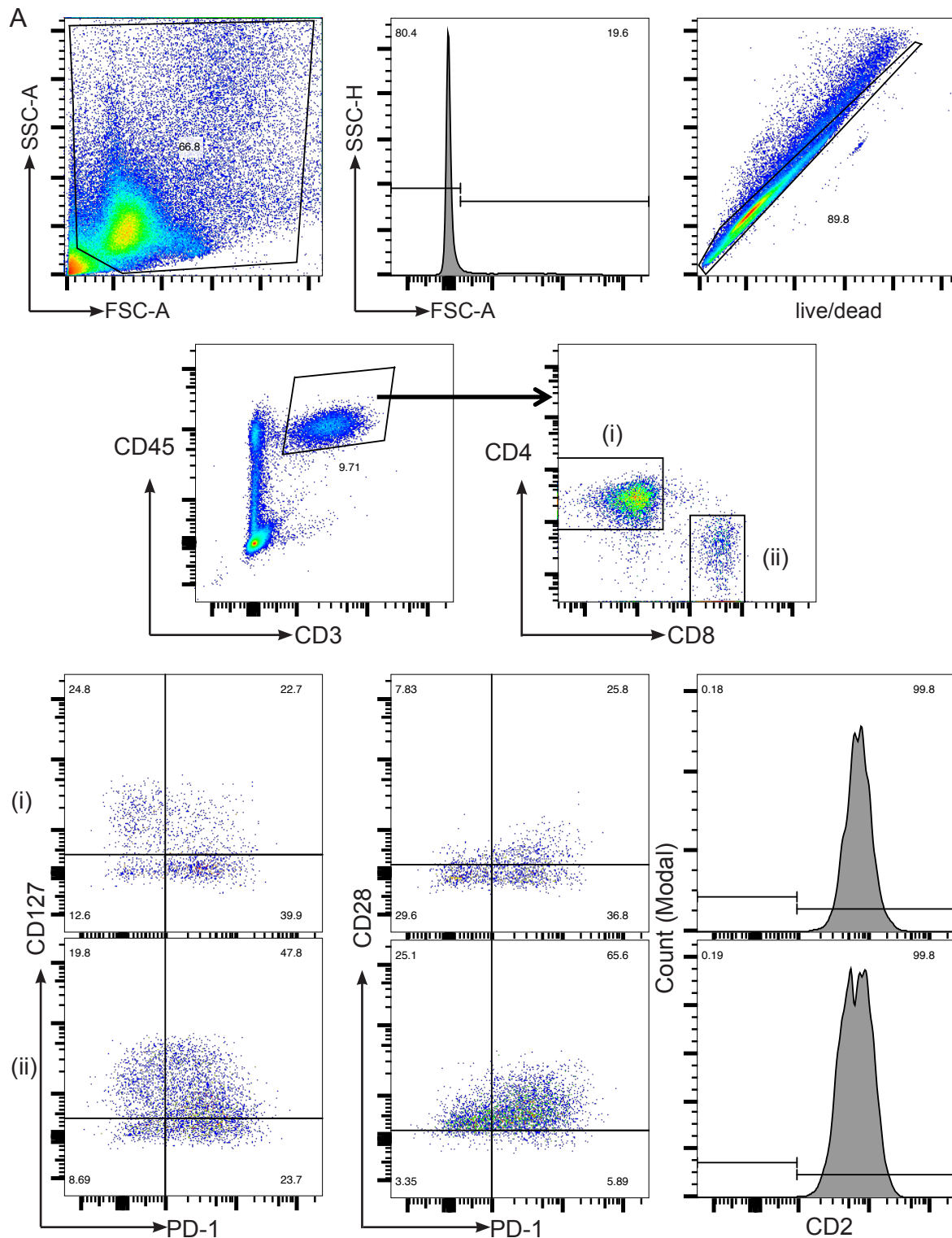
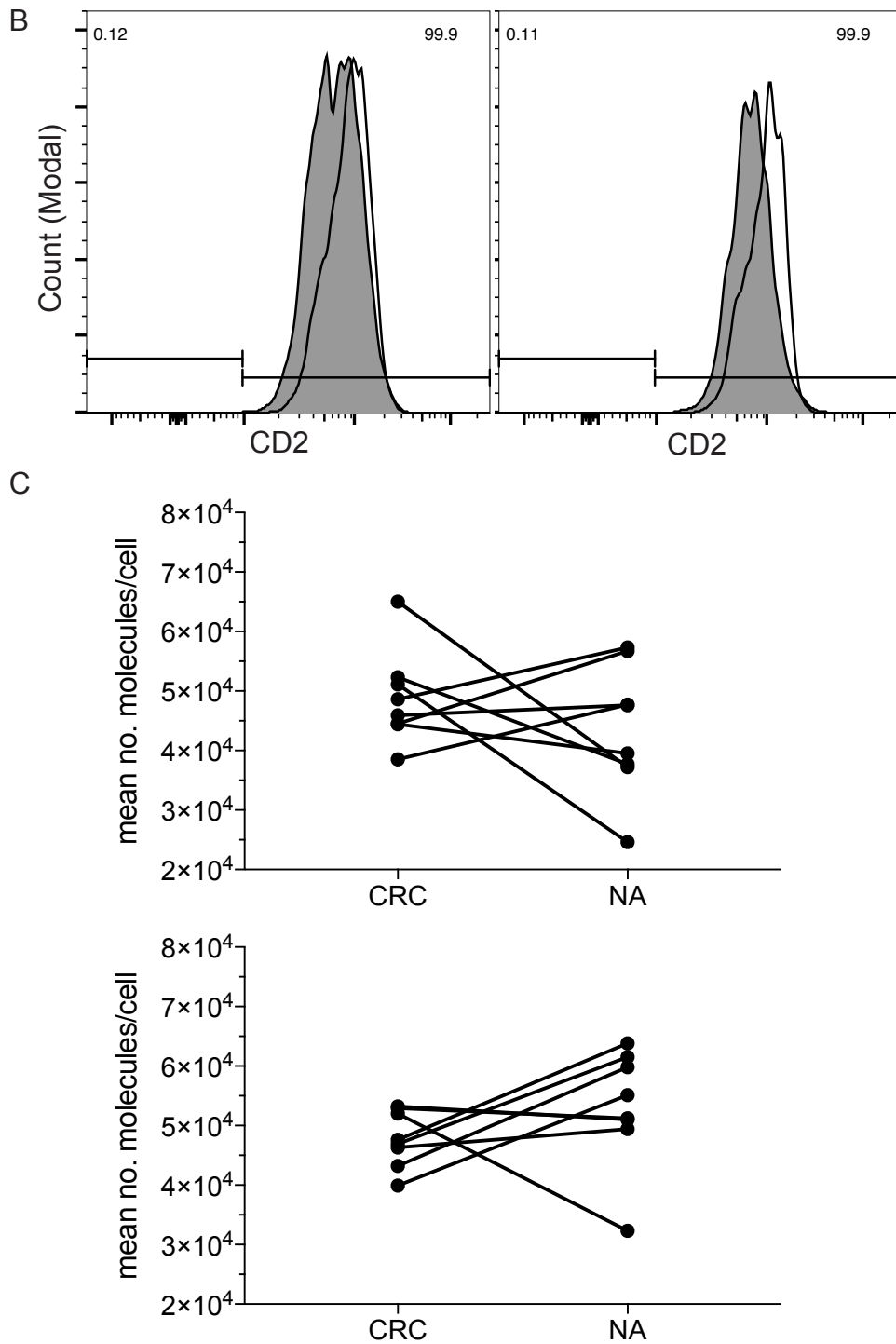
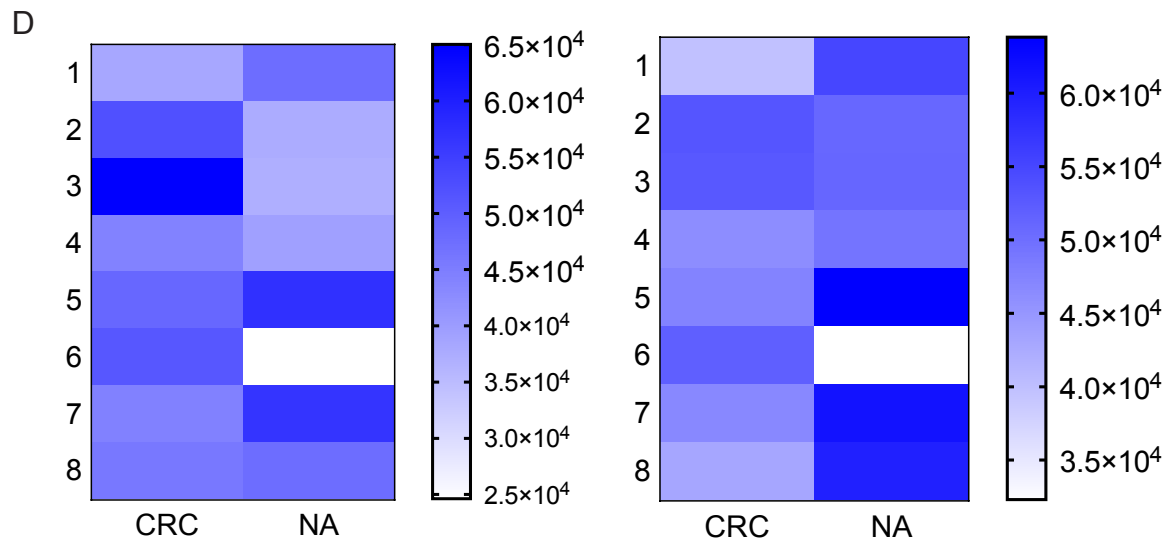


Figure 5.4 CD2 expression in TILs compared to T cells in normal adjacent tissue.

A) The gating strategy to determine the exhaustion profile, (CD127-PD1 and CD28-PD-1) and CD2 levels of CD8+ (i) and CD4+ T cells (ii) in CRC compared to corresponding normal adjacent tissue. PD-1 and CD2 gates were based on isotype stained controls.



B) An example of the CD2 expression in CD4+ T cells (left) and CD8+ T cells (right) from one CRC patient; CRC tissue (dark grey), normal adjacent tissue (empty). C) CD2 MFI expression levels were translated to mean number of molecules/cell using antibody-capture calibration beads. Data from 8 donors is shown, CD4+ T cells (top), CD8+ T cells (bottom). For each patient, CD2 levels in TILs (CRC) are linked to CD2 levels in T cells from normal adjacent tissue (NA) for the better comparison.



E

<i>Patient ID</i>	CD4+ T cells	CD8+ T cells
1	reduction	reduction
2	increase	similar
3	increase	similar
4	increase	similar
5	reduction	reduction
6	increase	increase
7	reduction	reduction
8	similar	reduction

D) The same data, shown in (C), are shown in a two-colour heat map.

E) Summarized in a tablet are the changes in CD2 expression levels of 8 patients by looking at the CD2 levels in TILs compared to CD2 levels in T cells from normal adjacent tissue. Reduction represents lower CD2 levels in TILs compared to T cells in NA, increase means higher CD2 levels in TILs compared to T cells in NA, similar means there was no significant change and CD2 levels were almost the same in TILs and NA T cells.

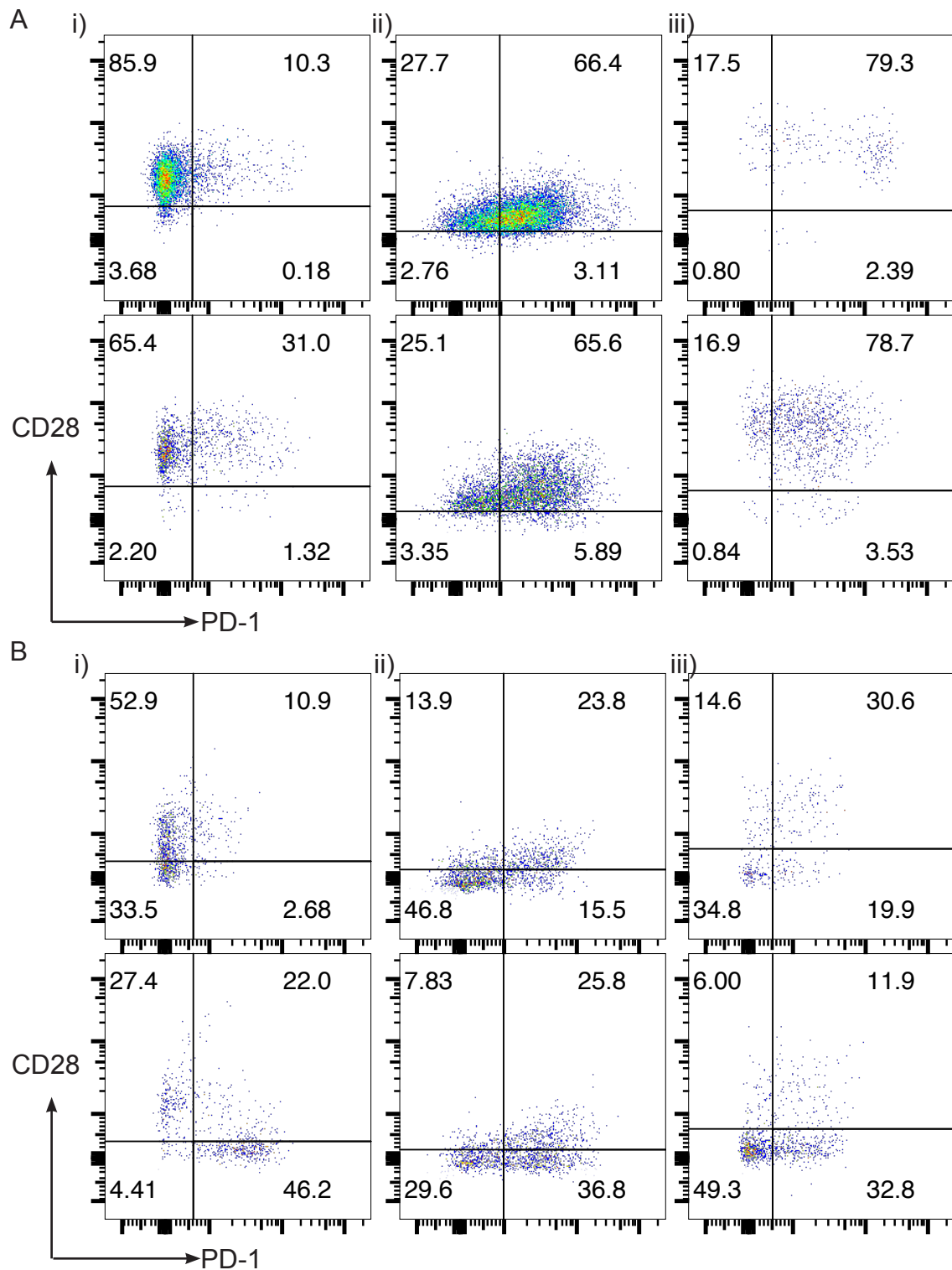
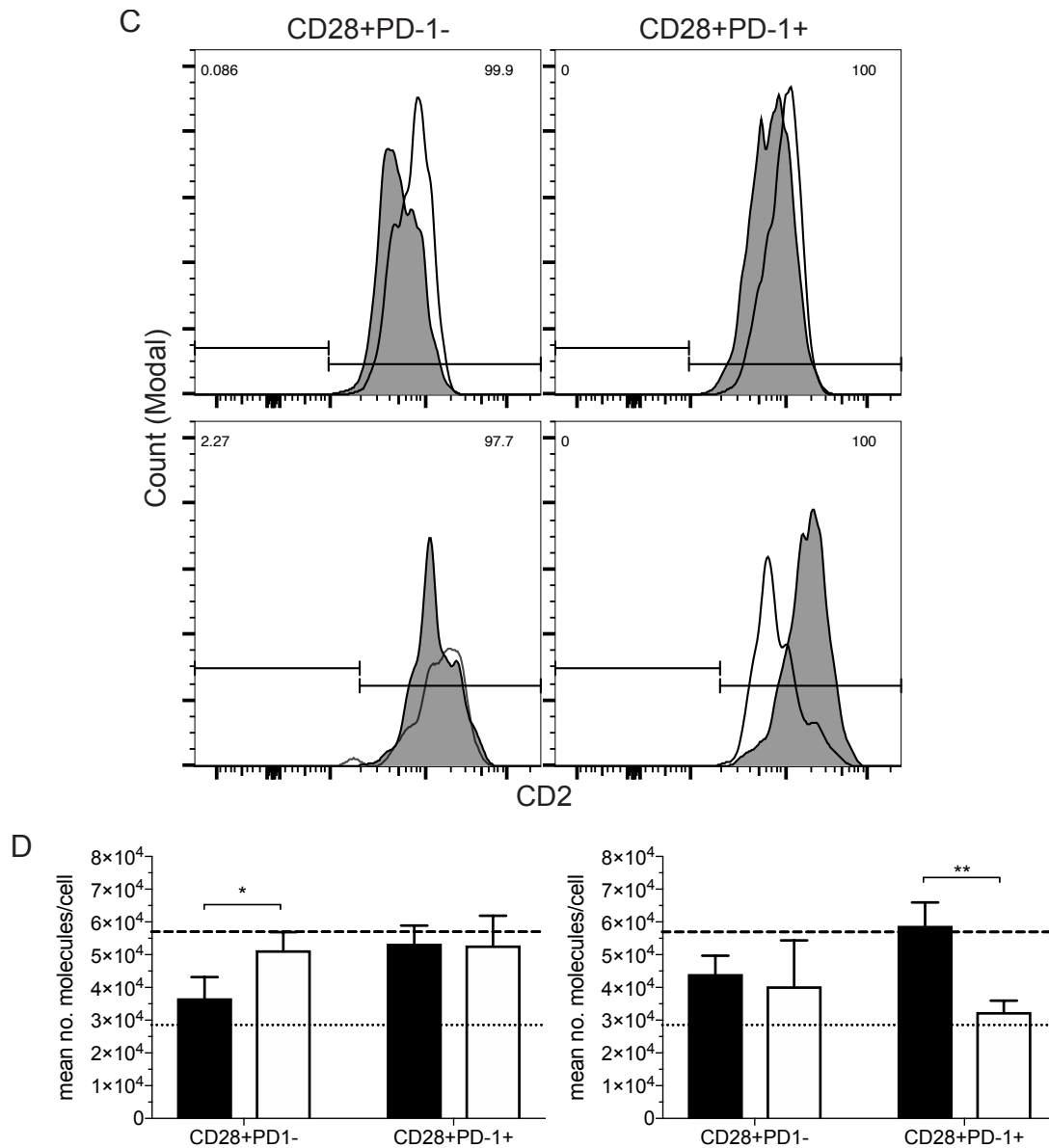


Figure 5.5 CD2 expression levels of T cell subsets defined by their CD28-PD-1 expression profile.

A) The plots show the CD28-PD-1 expression profiles of CD4⁺ T cells from 3 patients (i-iii, correspond to Patient ID 1, 7, and 3 (shown in Figure 5.4E), respectively. NA (top), CRC (bottom). B) The plots show the CD28-PD-1 expression profiles of CD8⁺ T cells from 3 patients (i-iii, correspond to Patient ID 1, 7, and 3 (shown in Figure 5.4E), respectively. NA (top), CRC (bottom).



C) The histograms show representative examples, from two CRC patients, of the CD2 expression levels of CD28+PD-1- CD4+ T cells and CD28+PD-1+ CD4+T cell subsets found in CRC tissue (dark grey) and normal adjacent tissue, NA (empty). Patient ID, 7 (top) and Patient ID, 3 (bottom) D) The graphs show the means of CD2 levels, as mean number of molecules/cell, in CD4+ T cells subsets shown in (C). The means of patients that showed a reduced CD2 expression in total CD4+ TILs compared to total NA CD4+ T cells (left) and the means of patients that showed an increased CD2 expression in total CD4+ TILs compared to total NA CD4+ T cells (right). *, $p < 0.05$, **, $p < 0.005$, with parametric Welch's *U*-test. Dotted line represents the mean CD2 expression levels in naïve CD4+ T cells in human peripheral blood. Dashed line represents the mean CD2 expression levels in memory CD4+ T cells in human peripheral blood of healthy individuals. Error bars represented SD.

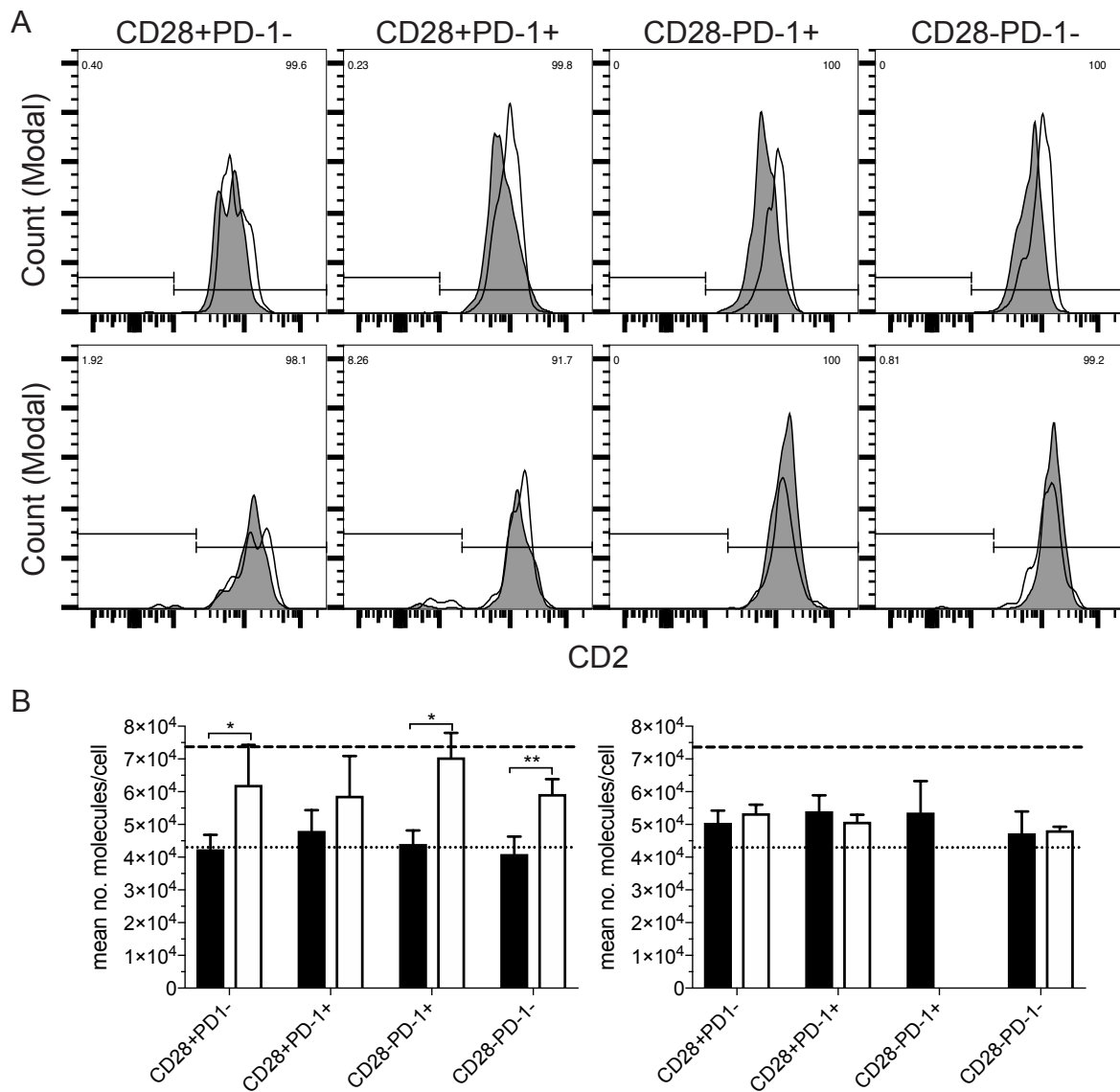


Figure 5.6 CD2 expression levels of CD8+ T cell subsets defined by their CD28-PD-1 expression profile.

A) The histograms show representative examples, from two CRC patients, of the CD2 expression levels of CD28+PD-1-, CD28+PD-1+, CD28-PD-1+, CD28-PD-1+ CD8+ T cell subsets found in CRC tissue (dark grey) and normal adjacent tissue, NA (empty). Patient ID, 7 (top) and Patient ID, 3 (bottom) B) The graphs show the means of CD2 levels, as mean number of molecules/cell, in CD8+ T cells subsets shown in (A). The means of patients that showed a reduced CD2 expression in total CD8+ TILs compared to total NA CD8+ T cells (left) and the means of patients that showed similar CD2 expression in total CD4+ TILs and total NA CD8+ T cells (right). *, $p < 0.05$, **, $p < 0.002$, with parametric Welch's *U*-test. Dotted line represents the mean CD2 expression levels in naïve CD8+ T cells in human peripheral blood. Dashed line represents the mean CD2 expression levels in memory CD8+ T cells in human peripheral blood of healthy individuals. Error bars represented SD.

A		CRC		NA		CRC		NA	
	<i>Patient ID</i>	CD28+PD-1	CD28+PD-1	CD28+PD-1	CD28+PD-1	CD28+PD-1+	CD28+PD-1+	CD28+PD-1+	CD28+PD-1+
	1	3.09E+04	4.88E+04	5.34E+04	4.33E+04				
	2	4.72E+04	3.96E+04	6.05E+04	3.54E+04				
	3	5.03E+04	5.72E+04	6.78E+04	3.24E+04				
	4	4.04E+04	4.16E+04	5.10E+04	3.46E+04				
	5	4.37E+04	5.77E+04	5.89E+04	5.40E+04				
	6	3.84E+04	2.30E+04	5.61E+04	2.77E+04				
	7	3.55E+04	4.75E+04	4.78E+04	6.13E+04				
	8	3.38E+04	4.05E+04	5.12E+04	5.54E+04				

B		CRC		NA		CRC		NA		CRC		NA	
	<i>Patient ID</i>	CD28+PD-1	CD28+PD-1	CD28+PD-1+	CD28+PD-1+	CD28-PD-1+	CD28-PD-1+	CD28-PD-1+	CD28-PD-1+	CD28-PD-1-	CD28-PD-1-	CD28-PD-1-	CD28-PD-1-
	1	3.69E+04	5.56E+04	4.30E+04	4.50E+04	4.05E+04	7.90E+04	3.52E+04	5.55E+04				
	2	5.48E+04	5.24E+04	5.93E+04	4.93E+04	4.53E+04	N/A	4.68E+04	4.93E+04				
	3	4.89E+04	5.64E+04	5.28E+04	5.32E+04	5.16E+04	5.00E+04	5.42E+04	4.82E+04				
	4	4.78E+04	5.16E+04	4.99E+04	4.97E+04	6.41E+04	N/A	4.09E+04	4.72E+04				
	5	4.73E+04	6.48E+04	N/A	N/A	4.96E+04	N/A	3.79E+04	6.36E+04				
	6	4.04E+04	4.13E+04	4.51E+04	3.03E+04	5.68E+04	3.10E+04	3.00E+04	3.09E+04				
	7	4.16E+04	5.01E+04	5.52E+04	6.34E+04	4.48E+04	6.49E+04	4.37E+04	6.28E+04				
	8	4.40E+04	7.80E+04	4.58E+04	6.79E+04	4.11E+04	6.76E+04	4.70E+04	5.54E+04				

Figure 5.7 CD2 expression levels as mean number of molecules per cells in CD4+ and CD8+ T cell subsets based on CD28-PD-1 expression profiles.

A) The Table shows the values for CD2 molecules/cell for CD4+ T cell subsets from CRC and NA tissue for all patients presented in Figures 5.4-5.6.

B) The Table shows the values for CD2 molecules/cell for CD8+ T cell subsets from CRC and NA tissue for all patients presented in Figures 5.4-5.6.

N/A, no T cells with that specific CD28-PD-1 expression profile.

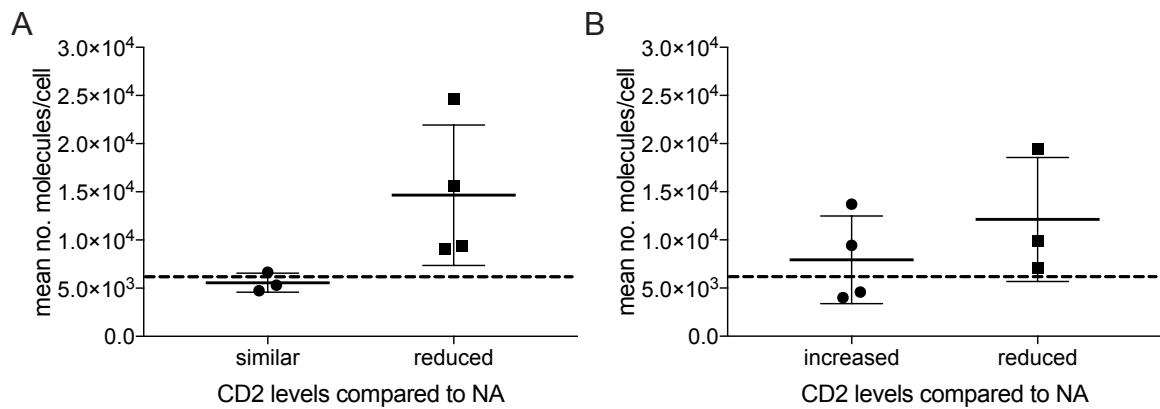


Figure 5.8 Reduced levels of CD2 in CD8+ TILs might correlate with higher levels of surface PD-1 expression in CD8+ TILs.

A) The graph shows the PD-1 expression levels, mean number of molecules/cell, of total CD8+ T cells in patients that had similar CD2 levels in CD8+ TILs and NA CD8+ T cells and of patients that had reduced CD2 levels in CD8+ TILs compared to NA CD8+ T cells.

B) The graph shows the PD-1 expression levels, mean number of molecules/cell, of total CD4+ in patients that had increased CD2 levels in CD4+ TILs compared to NA CD4+ T cells and of patients that had reduced CD2 levels in CD4+ TILs compared to NA CD4+ T cells. Dashed line represents the PD-1 levels found in peripheral blood PD+1 T cells of healthy individuals. Error bars represent SD.

5.2 Discussion

We have shown that CD2-CD58 interactions are contributing significantly to the CTL cytotoxicity against a melanoma cell line, *in vitro*, in agreement with previous reports^{166,167} and we now have a functional assay where we can test the effect of perturbing the CD2 corolla on CTL cytotoxicity.

We tried to address the contribution of CD2-CD58 interactions in the response of human T cells to low levels of antigen and to low affinity antigens. This becomes very important and relevant to us in the context of T cells responding to tumours, as these target cells tend to express low levels of cancer antigens and often of low affinity. Unfortunately, our results were masked by a significant 'background activation' in the absence of any exogenously loaded peptide thus we were unable to make firm conclusions on the above assay. An alternative APC line should be used to address this question. If one was to make a conclusion, we could say that CD2-CD58 interactions do not add to the T cells response to low antigen levels and/or low affinity antigens. However, this should be tested in another more suitable experimental setting.

Finally, we show for the first time, to our knowledge, that TILs, especially CD8⁺ TILs have reduced CD2 expression level compared to T cells found in normal adjacent to the tumour tissue. Interestingly, the exhausted TILs, which are in the majority of cases also CD28 negative, show the biggest reduction in CD2 levels compared to their NA CD8⁺ T cells. In fact, CD28⁻ CD8⁺ T cells found in human peripheral blood, is a population that increases with age and the CD2 signalling pathway is their main costimulatory pathway, enhancing cytokine production (IL-2, IFN γ), cytotoxicity and proliferation¹⁵³. Moreover, CD28⁻ CD8⁺ T cells were shown to express high levels of CD2 similar to CD28⁺CD2^{hi} CD8⁺ T cells⁵⁸. In our study, and in agreement with other reports⁵⁸, memory CD4⁺ and

CD8⁺ T cells are the subsets expressing high levels of CD2. PD-1⁺ TILs are expected to be of the memory subset hence they would express high levels of CD2; we report here, for the first time, that memory CD8⁺ T cells in peripheral blood express $\sim 7.3 \times 10^4$ molecules/cell. Instead, CD8⁺ TILs express low CD2 levels ($\sim 4.3 \times 10^4$ molecules/cell) that are same as the CD2 levels expressed by naïve CD8⁺ T cells found in peripheral blood. Higher CD2 levels could allow memory T cells to respond more quickly and efficiently in recall response or in recognizing tumour cells expressing low levels of antigen. We would, thus, like to propose that the lower CD2 levels in TILs hampers even more their cytotoxicity against tumours. This is further supported by our finding that the reduction in CD2 levels of CD8⁺ T cells correlates with higher levels of PD-1 in CD8⁺ T cells. Increased or no changes in the levels of CD2 in CD4⁺ and/or CD8⁺ T cells could mark an earlier phase of tumour progression where TILs are less or not “exhausted” and/or were recently activated; CD2 is up-regulated upon T cell activation. Conditions leading to CD2 down-regulation on activated T cells are not known and this requires further study.

6 Chapter 6. Final discussion and future directions

The discovery of the CD2 corolla and its role in the immune synapse fills gaps in our knowledge of how receptors organise themselves spatially in the IS and how this affects their function and the function of other receptors. We have investigated the role of the cytoplasmic tail in CD2 corolla formation. In this chapter I discuss potential future experiments that could be undertaken to further understand the role of CD2 and the corolla in T cell activation.

The formation of the corolla may depend upon the low affinity and fast off-rate of CD2-CD58 interactions. The low affinity interaction of CD2-CD48/CD58, with CD2-CD58 having a 5-10-fold higher affinity than CD2-CD48^{89,186} could also play a role in CD2 corolla formation. This could be tested by looking at the effect of crosslinking CD2 with agonistic anti-CD2 antibodies on SLBs or by mutagenesis studies that alter the affinity of CD2-CD48/CD58 interactions.

CD2 is expressed and shown to have significant functional roles also in NK cells ^{102–104} (as discussed in Chapter 1). It would be interesting to see whether NK cells form a CD2 corolla and whether this varies between CD56^{INT} and CD56^{HI} that express different levels of CD2, as we have shown here.

We have shown that CD2 corolla brings with it costimulatory receptors like CD28 and ICOS. At our TIRFM resolution of 300nm these receptors colocalised with CD2. However, it would be interesting to see if these receptors also colocalise with CD2 at 70 nm resolution offered by structured illumination microscopy or 20 nm resolution offered by Stochastic Optical Reconstruction Microscopy (STORM) imaging. This could provide further information as to the role of CD2-CD58 interaction that result in close contact similar to what is described by Chang V. *et al* ¹⁹⁰ in the formation of CD28 and ICOS clusters in the periphery of the IS. In fact, it would be very important to address whether the CD2 cluster in the periphery of

the IS, i.e. within the CD2 corolla is a site that favours enrichment of TCR microclusters when compared to any other peripheral area of the IS. We were not able to address this with our current settings and experimental conditions but it is possible to test. In addition, an important question would be to investigate how dependent the recruitment of CD28 and ICOS to the CD2 corolla is on the ligand densities on the SLBs and similarly for PD-1 localisation in the context of CD2 corolla. A potential limitation of our current RNA transfection of surface receptors could be that effector molecules like SHP-2, in the case of PD-1, might be limiting hence influence downstream PD-1-mediated effects despite the high levels of PD-1 expressed via transfection. Wei F. *et al* have used PD-1 RNA transfection alone and showed how PD-1 levels hence strength of PD-1 signalling affects T cell effectors functions¹⁴⁰; these experiments were done without transfection of additional Shp2 and yet they had still observed differential effects upon transfecting low, medium and high levels of PD-1.

We have shown that CD2 expression is reduced in CD8+ TILs from colorectal cancer patients and we have discussed how this reduction could be of significance for T cell responses against tumours. NK cells are also key players in cytotoxicity against tumours and it has been shown that their function can be compromised in the tumour microenvironment^{95,103,104,194}. It is possible that NK cells in the tumour microenvironment could exhibit a similar reduction in CD2 levels. Interestingly, CD2 signalling can be stimulated with subsequent up-regulation of CD2 expression in *in vivo* animal studies as shown by Sarkar S *et al*. Their study showed that exogenous application of purified soluble T11TS glycoprotein/soluble LFA-3/CD58 that binds CD2 can potentiate T cell responses against glioma¹⁹⁵. In light of our findings, similar studies looking at the expression of CD2 in different autoimmune diseases would allow further insight into the role of

CD2 in these diseases and new targets for treatments. Genome wide association studies (GWAS) have associated genetic variants at CD2/CD58 gene loci with autoimmunity^{196–198}. A study looking at expression of CD2 in T cells reported that there were no surface expression differences between patients and health donors but some functions of CD2, such as adhesion and enhancing T cell effector functions were defective in regulatory T cells of multiple sclerosis patients¹⁹⁹. A similar study has reported defective functions of CD2 but a parallel significant reduction of CD2 expression in total T cell populations of some systemic lupus erythematosus patients²⁰⁰. So it is also important to evaluate the function of CD2 along with the profiling expression levels.

In summary, we have presented a novel costimulatory structure, the CD2 corolla, in the IS of T cells. We have reported that the CD2 corolla can orchestrate co-stimulatory/inhibitory receptor localisation (CD28, ICOS and PD-1) and thus influence the signalling activity of these receptors and the activity of one relative to the other. We are proposing that the expression levels of CD2 and the rest of co-stimulatory receptors investigated here are key factors determining their localisation dynamics relative to the CD2 corolla. Importantly, we report that CD2 expression levels are reduced in CD8⁺ TILs from CRC patients and this could have implications when considering ways to improve immunotherapies for cancer patients.

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