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Role of CD2 and Its Ligands in T Cell Activation

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Abstract

CD2 is a transmembrane molecule and a “non-canonical” member of the signaling lymphocyte activation molecule (SLAM) family of receptors that is expressed on T cells and NK cells. Its ligands, mouse CD48 and human CD58, are widely expressed on hematopoietic cells including antigen-presenting cells (APCs) and T cells. Previous studies indicated that CD2 promotes T-cell receptor (TCR) signaling when it is engaged by its ligands displayed on APCs. However, the supporting experimental data were rather controversial, and there is no general agreement about the role of CD2 in T cell activation.

To study the function of CD2 and its ligands in T cells, we examined T cell functions in newly generated mouse strains lacking CD2 or CD48 in the C57BL/6 background. Compared to wild-type (WT) mice, T cells from CD2-deficient (“knock-out”; KO) mice had severe activation defects. Surprisingly, expression of CD48 on T cells, not on APCs, was also necessary for optimal T cell responses. We found evidence of CD2 interacted with CD48 in *cis* on T cells and observed their co-localization by confocal microscopy and fluorescence resonance energy transfer (FRET). The only exception was CD2-dependent cytotoxicity, which required CD48 both on T cells and on APCs. Mechanistic studies using mass spectrometry and structure-function analyses revealed that the *cis* interactions between CD2 and CD48 on T cells boosted TCR signaling, an effect that correlated with the capacity of CD2 to recruit the kinase Lck. Similarly, our further study revealed that the *cis* interactions between CD2 and CD58 on human T cells were also necessary for maximal TCR signaling and T cell activation.

Taken together, our studies provide clear evidence that *cis* interactions between CD2 and its ligands on T cells are important in TCR signaling and T cell activation. Modulation of these *cis* interactions can be a promising approach to suppress or enhance T cell activation in a therapeutic setting.

Keywords

T cell activation, graft-versus-host disease, CD2, CD48, CD58, Lck, *cis* interactions

Résumé

CD2 est une molécule transmembranaire et un membre “ non-canonique ” de la famille de la famille SLAM (« *signaling lymphocyte activation molecule* ») exprimée à la surface des lymphocytes T et des cellules NK (« *natural killer* »). Les ligands de CD2, CD48 chez la souris et CD58 chez l’humain, sont exprimés de manière ubiquitaire sur les cellules hématopoïétiques, y compris sur les cellules présentatrices d’antigène (CPA) et lymphocytes T. Des études antérieures ont indiqué que CD2 est impliqué dans la signalisation des récepteurs TCR (« *T-cell receptor* ») en réponse à son engagement par CD48 sur le CPA; cependant, les données expérimentales qui supportent ce modèle sont plutôt contradictoires et aucun accord n’a été trouvé sur les rôle de CD2 dans l’activation de lymphocytes T.

Pour étudier la fonction de CD2 et ses ligands, nous avons examiné les fonctions des lymphocytes T chez des souches de souris dépourvues de CD2 ou CD48 nouvellement générées à partir du “fond génétique” C57BL/6. Par rapport aux souris de type sauvage (WT; « *wild-type* »), les lymphocytes T de souris CD2-déficientes (« knock-out »; KO) présentent des sévères défauts d’activation. Il est intéressant de noter que l’expression de CD48 sur les lymphocytes T, mais non sur les CPA, était aussi nécessaire pour les réponses des lymphocytes T. Nous avons également démontré que CD2 interagit en *cis* avec CD48 sur les cellules T et avons observé leur co-localisation par microscopie confocale et FRET (« fluorescence resonance energy transfer) ». La seule exception était la cytotoxicité CD2-dépendante, qui nécessitait l’expression de CD48 à la fois sur les lymphocytes T et sur les

CPA. L'étude des mécanismes par la spectrométrie de masse et les analyses structure-fonction ont démontré que les interactions en *cis* entre CD2 et CD48 permettent de stimuler la signalisation du TCR, ce qui corrèle avec la capacité de CD2 à recruter la kinase Lck. De manière similaire, notre étude plus approfondie a démontré que les interactions en *cis* entre CD2 et CD58 sur les lymphocytes T humains sont nécessaires pour la signalisation maximale du TCR et l'activation cellulaire T.

L'ensemble de nos études ont mis en évidence que les interactions en *cis* entre CD2 et ses ligands sur les lymphocytes T jouent un rôle important dans la signalisation du TCR et l'activation de ces cellules. La modulation de ces interaction en *cis* pourrait être une approche potentielle pour augmenter ou interférer avec l'activation des lymphocytes T dans un contexte thérapeutique.

Les mots-clés

Activation des cellules T, maladie du greffon contre l'hôte, CD2, CD48, CD58, Lck, interaction en *cis*

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List of Acronyms and Abbreviations

Abbreviation	Full Name
ADAP	Adhesion and degranulation promoting adaptor protein
APCs	Antigen-presenting cells
Bcl-2	B-cell lymphoma-2
Bcl-6	B-cell lymphoma-6
CAR	Chimeric antigen receptor
CD4-CTLs	Cytotoxic $CD4^+$ T cells
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX-1	Cyclooxygenase-1
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CTLs	Cytotoxic T lymphocytes
CXCL1	C-X-C motif chemokine ligand 1
DAG	Diacylglycerol
DCs	Dendritic cells
DN	Double negative
DP	Double positive
ETP	Early thymic progenitor
FoxP3	Forkhead box P 3
GADS	GRB2-related adaptor downstream of shc
GATA-3	GATA sequence-binding factor-3
GCs	Germinal centers
G-CSF	Granulocyte colony stimulating factor
GPI	Glycosylphosphatidylinositol
GRB-2	Growth factor receptor-bound protein-2
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel diseases
ICOS	Inducible co-stimulatory molecule
IFN-γ	Interferon gamma
IgC2	Immunoglobulin constant 2
IgE	Immunoglobin E
IgV	Immunoglobulin variable
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-23R	Interleukin-23 receptor
ILCs	Innate lymphoid cells

iNKT	Invariant natural killer T
IP_3	Inositol 1,4,5-triphosphate
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
ITK	IL-2-inducible T cell kinase
KO	Knock-out
LAT	Linker for activation of T cells
LAG-3	Lymphocyte activation gene-3
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
MPP	Multipotent progenitor
NK	Natural killer
NKT	Natural killer T
PD-1	Programmed cell death protein-1
PDK-1	Phosphoinositide dependent protein kinase-1
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PKB	Protein kinase B
PLC-γ1	Phospholipase Cγ1
scFv	Single-chain variable fragment
SH2	Src-homology 2
SHIP-1	Inositol polyphosphate 5-phosphatase-1
Siglec-15	Sialic acid-binding immunoglobulin-type lectins-15
SLAM	Signaling lymphocyte activation molecule
SLE	Systemic lupus erythematosus
SLP76	SH2-domain-containing leukocyte protein of 76 kDa
SMACs	Supramolecular activation clusters
SP	Single positive
STAT4	Signal transducer and activator of transcription 4
STAT1	Signal transducer and activator of transcription 1
RAG-1	Recombination activating gene-1
RAG-2	Recombination activating gene-2
Runx 3	Runx family transcription factor 3
T-bet	T-box transcription factor TBX 21
TCF-1	T-cell factor-1

TCR	T cell receptor
Tfh	T follicular helper
TG-MΦ	Thioglycolate-induced peritoneal macrophages
TGF-β	Transforming growth factor beta
Th	T-helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T-cell immunoglobulin mucin-3
Tox2	TOX high mobility group box family member-2
Treg	T regulatory
XLP	X-linked lymphoproliferative
ZAP70	Zeta-chain associated protein kinase 70 kDa

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Chapter 1: General Introduction

Immunity and conventional T cells revisited

Biological immunity represents the body's capacity to recognize and reject what is non-self, such as microbial pathogens and tumour cells. The mammalian immune system consists of two cooperative subsystems: the innate immune system as well as the adaptive immune system. Innate immunity is considered the first part of defence against invading pathogens, and provides rapid and effective defences. To evoke an effective innate immune response, many cells are involved, such as natural killer (NK) cells, neutrophils, mast cells, innate lymphoid cells (ILCs), macrophages and dendritic cells (DCs) [1-6]. These innate immune cells are activated when their unique activating receptors recognize and bind soluble or cell-bound activating ligands, which can result in several processes.

For instance, macrophages use phagocytosis to capture and sterilize their "prey" through lysosomal degradation [7]. DCs can capture small fragments of foreign elements, process antigens and present antigens to adaptive immune cells [8]. NK cells can directly lyse infected cells or tumor cells through cytotoxicity, and they can rapidly produce a variety of cytokines to regulate other immune cell functions [9]. The highly motile neutrophils are the first innate immune responders against invading pathogens. They can destroy pathogens by phagocytosis and release of cytotoxic substances such as lysozyme, α-defensins and histones[10-12]. Mast cell activation requires their surface high-affinity IgE receptor, FcεRI, to interact with target cells coated with IgE. This binding triggers mast cell activation and degranulation, which releases a variety of compounds that can also regulate the immune response (e.g., cytokines, chemokines, histamine and proteases) [13]. ILCs are a newly explored family of innate immune cells. ILCs are divided into three groups—ILC1s, ILC2s and ILC3s—according to their transcription factor and cytokine production patterns [14]. The crucial roles of ILCs in immune defence against invading pathogens and the regulation of immune responses have been described in multiple studies [5, 14].

If microbial pathogens escape and persist after the innate immune response, the

adaptive immune system is recruited. The adaptive immune system consists of T lymphocytes (or T cells) and B lymphocytes (or B cells), and can generate a stronger and more rapid immune response when a pathogen is encountered again. This phenomenon is termed immune memory. T cells and B cells possess highly polymorphic antigen receptors that recognize specific antigens. These receptors are named TCR (T cell antigen receptor) and BCR (B cell antigen receptor), respectively. T cells are subdivided into T-helper (Th) cells (marked as $CD4^+$ T cells) and cytotoxic T cells (marked as $CD8^+$ T cells). $CD8^+$ cytotoxic T cells directly eliminate pathogen-infected cells or tumour cells through the delivery of pro-apoptotic granules such as perforin and granzymes [15]. $CD4^+$ Th cells are critical for orchestrating and maximizing immune responses through the release of cytokines that regulate the activity of many immune cell types, including themselves [16, 17].

After antigen-pulsed DCs activate B cells via the BCR, $CD4^+$ Th cells can assist B cells in further differentiating into plasma cells, which secrete antigen-specific antibodies [18, 19]. T cells are particularly critical for adaptive immunity since they not only mediate a very efficient immune response but also control B cell functional responses through cell-cell interactions involving receptors and ligands [20-22]. Furthermore, cytokines released from T cells can bind to the specific cytokine receptors on the cells of the innate and adaptive immune system to influence their functions [23-25].

T cell activation requires the TCR to recognize matching foreign antigens presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Multiple types of cell surface molecules can positively or negatively modulate the strength and duration of the activating signals. These include co-stimulatory receptors such as CD28 and the inducible co-stimulatory molecule (ICOS, CD278)[26-29], co-inhibitory receptors such as the programmed cell death protein 1 (PD-1, CD279), the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), and T-cell immunoglobulin mucin-3 (TIM-3) [30-36]. Other receptors such as the SLAM family of

immune cell receptors and sialic acid-binding immunoglobulin-type lectins-15 (Siglec-15) are also implicated [37-41].

Since T cell activation is an essential event in immunity [42, 43], it is necessary to understand how T cells develop and how T cell activation is modulated. We will first review T cell development and then discuss the processes controlling T cell activation.

1.1 Understanding T-cell development

Mature T cells, together with other immune cells, appear in the peripheral blood, spleen, lymph nodes as well as mucosal organs, such as lungs and intestine [44]. Based on cell surface markers CD4 and CD8, T cells are divided into two major sub-lineages—$CD4^+$ T cells and $CD8^+$ T cells—that execute distinct functions. All T cells originate from hematopoietic stem cells (HSCs) found in the bone marrow [45, 46]. Their fate-decided precursors then migrate into the thymus to initiate T cell maturation, selection as well as subsequent export to the periphery [47].

1.1.1 The origin of T cells in the bone marrow

Like other lymphocytes, T cells arise from HSCs [45]. According to the universally accepted model, HSCs first differentiate into multipotent progenitor (MPP) cells. These MPP cells give rise to two intermediate progenitors: common myeloid progenitor (CMP) cells as well as common lymphoid progenitor (CLP) cells [48, 49]. CLP cells have the potential to differentiate into a variety of lymphocyte lineages, including T cells, B cells, and NK cells [45, 50, 51]. CLP cells are identified based on the expression of IL-7R and Flk-2, as well as low C-kit and Sca-1 expression levels; however, they have no expression of specific markers for mature immune cells, such as TCR, CD3, CD4, CD8 and BCR [51-56]. Subsequently, CLP cells differentiate into early thymic progenitor (ETP) cells. ETP cells are identified as $CD34^+CD1A^-$IL-$7R\alpha^+CD44^+CD45RA^+$GM-$CSFR^-$Thy-1^{high} cells [55, 57-59]. Then,

the committed progenitor cells enter the thymus and initiate the T cell development program.

1.1.2 Development of T cells in the thymus

The thymus is the specialized organ responsible for the generation and maturation of T cells, which are needed for the development of the functional mature T cells [47, 59]. Figure 1.1 depicts the important steps within the development of T cells in the thymus. ETPs undergo a series of maturation steps, including rearrangement and expression of the TCR gene, cell expansion, thymic education by positive and negative selection, and establishment of T cell competence [60-62]. The maturation of T cells can be divided into several successive stages according to the expression of their cell surface markers, which including $CD4^-$ and $CD8^-$ double negative (DN), $CD4^+$ and $CD8^+$ double positive (DP) and $CD4^+$ or $CD8^+$ single positive (SP), respectively [63, 64]. While the majority of progenitor cells in the thymus differentiate into TCR αβ T cells, approximately 5% differentiate into γδ TCR [65]. Besides T-cell potential, ETPs also possess NK and B lymphoid potentials [66, 67].

Lineage specification events are the first essential steps in T cell development and require the irreversible loss of alternative non-T lineage potential. There are multiple signaling pathways involved in the T-lineage specification; for example, blocking Notch signals leads to arrest of T cell development at a very early stage [68]. It has been shown that Notch signaling can inhibit alternative lineage fate potentials (including B lymphoid and myeloid cells), and also promotes the expression of T lineage-specific genes and the expansion of committed T lineage precursors [68-71]. In addition, GATA sequence-binding factor 3 (GATA-3) [72], T-cell factor 1 (TCF-1) [73-75] and E-box proteins [76, 77] are thought to serve roles in the induction of T lineage commitment.

One essential event in T-lineage fate determination is the expression of the TCR genes, including TCR αβ or γδ. As T-lineage precursor cells enter the subcapsular zone in

the thymus, they begin expressing recombination-activating gene 1 (RAG-1) and RAG-2 recombinase. RAG protein induces the rearrangement of TCR genes leading to the generation of highly diverse TCRs with their capacity to recognize various antigens [78-80]. The diversity of TCRs is generated by rearranging germline variable (V), junctional (J) and diversity (D) gene fragments. For TCRαβ T cells, the proper rearrangement of the *Tcrb* gene will lead to the exclusion of the *Tcrg* and *Tcrd* genes [81-83]. Meanwhile, the T lineage precursor cells also start expressing the CD3 chains and the protein tyrosine kinase Lck for TCR signaling [84, 85]. The properly rearranged TCRβ chains are coupled to the pre-Tα chain and the CD3 molecules, to form the pre-TCR complex in TCRαβ T cells. The functional pre-TCR signals can induce several rounds of proliferation to enter the DP stage. Before entering the DP stage, αβ precursor cells will encounter the first checkpoint for verifying the proper TCR gene rearrangement at DN3, which is known as β-selection [86-88]. This requires the thymocytes to have a properly rearranged TCRβ gene. Alternatively, the successful recombination of *Tcrd* and *Tcrg* leads to the assembly of a γδ TCR. γδ precursor cells must also go through a checkpoint to verify their TCR rearrangement. Moreover, the checkpoint requires proper signals from mature TCRγδ-CD3 complexes [89-91].

After passing the first checkpoint, thymocytes proceed to differentiate into the last stage of DN cells ($CD44^-CD25^-CD117^-$) [92, 93]. Thymocytes then express CD4 and CD8 molecules, thereby entering the DP stage [94]. At this stage, TCRα rearrangement is initiated by the re-expression of RAG genes [81, 95-97] and results in the generation of mature TCR complexes on the cell surface. Eventually, immature cells DP TCRαβ constitute 90% of the cell population in the thymus. However, only 1–3% of these cells will develop into either CD4 or CD8 SP cells [64, 98].

ab DP thymocytes differentiate into mature T cells after positive and negative selection. The binding specificity and strength of TCR engagement with self-peptide-MHC ligands determines cell survival and further development [99, 100]. In addition to

presenting antigens and binding TCRs, class I MHC molecules recognize CD8 and favor the differentiation of $CD8^+$ SP cytotoxic T cells. Conversely, class II MHC molecules bind CD4 and dictate the differentiation of $CD4^+$ helper T cells. Most DP cells express TCR complexes that either weakly recognize or do not interact with self-peptide-MHC molecules. The signals that normally contribute to the survival of these cells are not generated and consequently the cells die by "neglect" [101-103]. The surviving cells subsequently go through the negative selection process. DP cells with TCRs that recognize self-peptide-MHC molecules with high affinity display strong intracellular signals that lead to apoptosis [98, 104, 105]. The surviving DP thymocytes develop into either the $CD4^+$ SP or $CD8^+$ SP sublineages [106, 107]. Eventually, mature SP thymocytes leave the thymus through the blood vessels and circulate to the peripheral lymphoid organs.

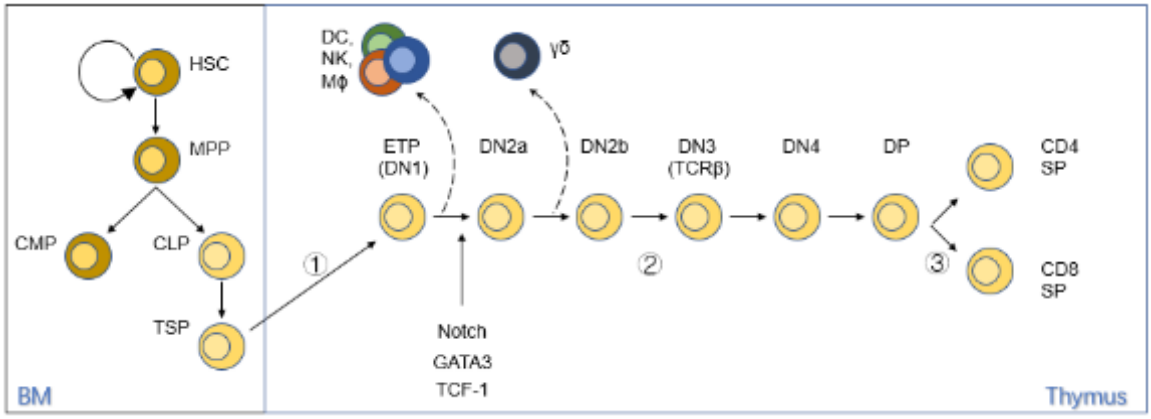

Figure 1.1 Overview of the T cell development in thymus. T cell lineage arise from the hematopoietic stem cells (HSCs) in bone marrow (BW). HSCs differentiate into multipotent progenitors (MPPs). MPPs can further differentiate into common myeloid progenitor (CMP) cells as well as common lymphoid progenitor (CLP) cells. Thymic seeding progenitor (TSP) cells are derived from the CLP cells. TSP cells migrate into the thymus and initiate the maturation of functions T cells. In the cortex of thymus, TSP progress through the early T-lineage progenitor (ETP) period and enter the double negative (DN) stage. Once the

progenitor cells pass the positive and negative selection, the mature T cells migrate out of the thymus and enter peripheral.

1.2 Different types of T cells and their functions

T cells are essential components of the adaptive immune response against pathogens and tumor cells [108]. T cells can also participate in the pathogenesis of auto-immune diseases such as type I diabetes and systemic lupus erythematosus. In this section, we will overview the different subsets of T lymphocytes in sections 1.2.1 and 1.2.2 and is also depicted in Figure 1.2.

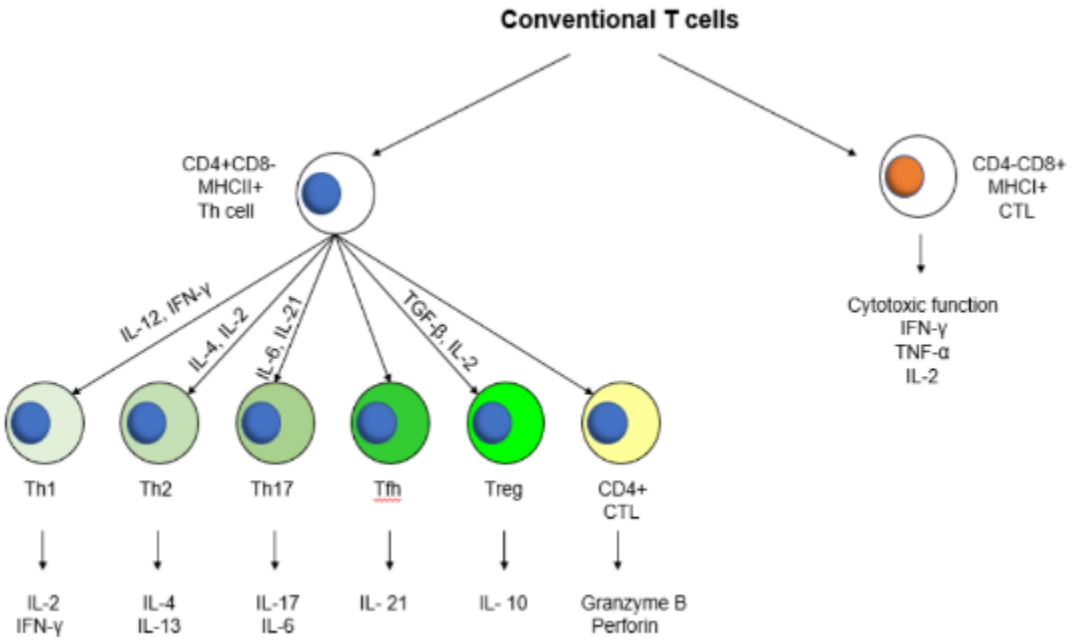

Figure 1.2 Overview of the different types of conventional T cells. Conventional T cells include the $CD4^+$ and $CD8^+$ T cells. CD4+ helper T(Th) cells are divided into five different subsets based on their different transcription factors and cytokines secretion. Th1 cells secrete IFN-γand IL-2, on stimulation with IL-2 from activated DCs and macrophage or in response to IFN-γstimulation from NK cells. Th2 cells produce IL-4 and IL-13, on stimulation with endogenous IL-4. Th17 cells produce IL-17A, IL-25 and IL-6. The retinoic

acid-related orphan receptor gamma t (RORγt) and forkhead box p3 (Foxp3) are required for the differentiation of Th17 cells. Follicular helper T (Tfh) cells collaborate with B cells to form the humoral immunity. Regulatory T (Treg) cells play an essential role in maintaining immune homeostasis and prevent autoimmunity. One approach by which Tregs to harness the immune responses is through the production of inhibitory cytokines such as IL-10. CD4+ cytotoxic T lymphocytes (CTLs) is a small percentage of unexpected CD4 subgroup with the capacity of secreting of granzyme B and perforin to lysis the pathogen infected cells in an MHC class II-restricted approach. In addition to lyse the target cells directly, CD8+ CTLs can secrete several kinds of cytokines such as IFN-γ, TNF-α, IL-2 to mediate their effector functions.

1.2.1 Th cells

Th cells, usually marked as the expression of CD4 molecules and differ from the cytotoxic T cells (CTLs), are required for nearly all adaptive immune responses [16, 109-111]. Th cells not only help antigen-loaded B cells further mature into antibody-producing plasma cells [112, 113], but they also release a variety of cytokines to modulate the activation of other immune cells [109, 114-116]. After exposure to proper antigen-loaded APCs, naïve $CD4^+$ T lymphocytes undergo cell expansion and subsequently differentiate into effector $CD4^+$ T cell populations. Th cells have been categorized into five subsets according to their secreted cytokines and the expression of lineage-specific master transcription factors: Th1, Th2, Th17, T follicular helper (Tfh) cells and T regulatory (Treg) cells [117-120]. A small subset of $CD4^+$ T cells with cytotoxic activity was also identified *in vitro* and *in vivo* [121-123]. These cytotoxic $CD4^+$ T cells (CD4-CTLs) can produce cytotoxic granules containing granzyme B and perforin, which enable to kill their target cells expressing class II MHC (MHC II) molecules [123].

Th1 cells

1.2.1.1

After recognizing peptide–MHC II complexes with their TCR, naïve $CD4^+$ T cells can differentiate into Th1 cells. It has been reported that IL-12 and IFN-γ are essential for the generation of Th1 lineage [124, 125]. IL-12 is produced by activated DCs and macrophages [126, 127], and induce the activation of signal transducer and activator of transcription 4 (STAT4) [128]. IFN-γ is secreted by NK cells and T cells, and result in the activation of STAT1 [129]. Both IL-12/STAT4 and IFN-γ/STAT1 signals are capable of inducing the expression of T-bet [128, 130-134]. It was established that T-bet acts as the master regulator of Th1 differentiation. Meanwhile, T-bet coupled to GATA-3 can directly repress the differentiation potential of Th2 cells [135-138].

Th1 cells are essential in the defense against infections by producing IFN-γ and IL-2 [139, 140]. IFN-γ activates macrophages to eliminate intracellular microbes and parasites [141]. IFN-γ also stimulates B cells to secrete IgG [142, 143], which opsonizes invading microbes and promotes macrophage-mediated phagocytosis. Th1 cells have also been proven to mediate several kinds of autoimmune diseases, such as inflammatory bowel diseases (IBDs) [144-146]. Notably, Crohn's disease is one type of IBD that results in affected patients having a higher amount of IL-12 in their intestinal mucosa [147, 148]. Moreover, neutralizing IL-12 with antibodies is an effective treatment for intestinal inflammation [146].

To prevent aberrant activation, Th1 activation is tightly regulated, usually via IL-10 [149, 150]. IL-10 is secreted by Th2 cells [151]. However, further research revealed that the production of IL-10 was also relevant to Treg cells [152], DCs [153], macrophages [154], B cells [155] and mast cells [156, 157]. To play its immunosuppressive functions, IL-10 represses the cytokine production of Th1 cells, and the expression of B7 molecules [158-161] when it acts on DCs or macrophages. Moreover, Th1 cells have been shown to secrete IL-10 [162], suggesting that Th1 cells possesse an auto-regulation mechanism for limiting tissue damage during immune responses [163].

1.2.1.2 Th2 cells

After activation, naive $CD4^+$ T cells can also differentiate into another effector lineage, Th2 cells. Th2 cells can secrete IL-4, IL-5, IL-13 and IL-10 [116]. IL-4 and IL-2 are critical for the differentiation of naïve $CD4^+$ T cells into Th2 cells [164]. The IL-4 receptor (IL-4R) signaling pathways initiate the differentiation of Th2 cells [164, 165]. The phosphorylated tails of IL-4R recruits and, via Jak protein tyrosine kinases, trigger phosphorylation of STAT6. Notably, STAT6 induces the expression of the transcription factor GATA3 [166-168]. It was established that GATA3 is the master factor of Th2 lineage. GATA3 collaborates with STAT5 in initial IL-4 production [120, 169]. Although Th2 T cells are the primary cell for IL-4 production, Th2 cell differentiation is largely determined by IL-4 [165]. Naive CD4 T cells endogenously express GATA3, which initiates the production of low levels of IL-4, and then IL-4 initiates Th2 differentiation. The expression levels of GATA3 are upregulated following the differentiation of Th2 cells, thus creating a positive cycle of regulation[170]. Furthermore, GATA3 is essential for the production of IL-5 and IL-13 [171, 172]. IL-2 is also critical for Th2 differentiation. Notably, IL-2 also activates STAT5 signalling, which promotes Th2 responsiveness by inducing IL-4Rα expression [173, 174].

Th2 cells play an important role in B cell-mediated antibody response and defence against extracellular pathogens. Th2 cells secrete IL-5, and IL-5 recruits eosinophils to the site of infection [175, 176]. Th2 cells can also secrete IL-10 and IL-13. IL-10 plays an important role in limiting Th1 functions, while IL-13 can regulate the activation of macrophages [177].

1.2.1.3 Th17 cells

Th17 cells are another subpopulation of Th cells that are defined by their hallmark cytokines IL-17A and IL-25 [164, 178]. The cytokines of the IL-17 family include IL-17A-E and IL-25 [179-181]. IL-17A and IL-25 are commonly produced by Th17 cells. Meanwhile, IL-17B-E are generated by a broad spectrum of immune cells.

As for other Th cells, a variety of cytokines are capable of inducing the differentiation of Th17 from activated $CD4^+$ T cells [182-185]. It was initially believed that Th17 differentiation was induced by IL-23; however, it was later suggested that Th17 differentiation is independent of IL-23 since there is absence of the IL-23 receptor (IL-23R) in naïve T cells. Further studies showed that IL-23 is essential for Th17 maintenance and proliferation, and that activated Th17 cells are capable of expressing IL-23R [186-188]. TGF-β together with IL-6 or IL-21 are essential for Th17 differentiation *in vitro* [189-191]. To explore the function of TGF-β signaling in Th17 differentiation, mice bearing a targeted deletion of TGF-β receptor in $CD4^+$ T cells were generated. $CD4^+$ T cells from these knockout mice could not differentiate into Th17 cells, even when given the appropriate stimulation [192]. Moreover, IL-6 cooperating with IL-21 initiate a signal mediated by the transcription factor STAT3, which elevate the level of the transcription factor RORγt. RORγt stimulates the expression of IL-17A, IL-17F and IL-23R to promote the differentiation of activated $CD4^+$ T cells to the Th17 lineage [193-196].

Th17 effector cells are essential for defending against invading pathogens by generation of various cytokines, including IL-17A, IL-25 and IL-21. It has been well established that IL-17A promotes proinflammatory gene expression in nonimmune and immune cells, such as TNFα, IL-1β, IL-6, IL-8, C-X-C motif chemokine ligand 1 (CXCL1), CXCL8 and granulocyte colony-stimulating factor (G-CSF) [197-201]. Notably, IL-21 signalling is critical for the differentiation of functional B cells [202]. IL-21 also promotes the cytotoxicity function of $CD8^+$ T cells and NK cells [203, 204]. Th17 cells are highly relevant to inflammatory and autoimmune diseases. Patients with mutant RORγt are susceptible to infections (e.g., *Mycobacterium*) and fungi (e.g., *Candida albicans*) [205, 206]. Furthermore, Th17 cells are involved in inflammatory autoimmune diseases [207, 208]. Augmented levels of Th17 cytokines have been observed in rheumatoid arthritis. Currently, the manipulation of IL-17 signalling was adopted to treat different types of autoimmune diseases [209].

1.2.1.4 Tfh cells

Tfh cells are required for humoral responses and mainly distributed in the germinal centres (GCs) of secondary lymphoid tissues. Tfh cells are responsible for directing the differentiation of GC B cells into antibody-secreting plasma cells[210].

Tfh cell differentiation and maturation require TCR stimulation and specific cytokines. TCR stimulation with MHC II-specific peptides together with IL-6 initiates the differentiation of Tfh cells by inducing B-cell lymphoma 6 (Bcl6) expression [211, 212]. IL-21 is also essential for successful Tfh differentiation. IL-6 and IL-21 activate STAT3 to induce Tfh cell differentiation [213, 214]. After activation by APCs, Tfh cells move into the GC where they mediate their functions. It was reported that CXCR5, ICOSL, IL-37, cyclooxygenase-1 (COX-1), TOX high mobility group box family member 2 (Tox2) and SAP family adaptors also serve critical roles in Tfh cell differentiation and functions [215-219].

Since Tfh cells are essential for promoting B cell function, aberrant Tfh responses can lead to various diseases. For example, Tfh cell frequency and autoantibodies are significantly augmented in patients with SLE [220-222].

1.2.2 $CD8^+$ T cells and cytotoxic T lymphocytes (CTLs)

$CD8^+$ T cells, often termed CTLs, are critical for human adaptive immune responses to clear intracellular pathogens and tumors. Like Th cells, $CD8^+$ T cells are differentiated in the thymus and express αβ TCRs. However, unlike the $CD4^+$ Th cells, $CD8^+$ T cells express the CD8 molecule, which is consisted of two chains, CD8α and CD8β. $CD8^+$ T cells respond to antigen peptides presented by MHC class I (MHC I) molecules on APCs.

$CD8^+$ T cells become activated after recognizing a foreign antigen, thereby resulting effector functions mediated by a dual mechanism. The first is the secretion of inflammatory cytokines, primarily IFN-γ and TNF-α, which work against microbial pathogens and tumors. The second is the destruction of pathogen-infected cells and

tumour cells by the release of cytotoxic granules [223]. This activity requires previous activation ("priming") of $CD8^+$ T cells by antigenic stimulation. When $CD8^+$ T cells recognize their target cells, they form the "immunological synapse" (IS). As a result of productive activation, the cytotoxic granules from primed $CD8^+$ T cells then move towards the target cells and are released in the IS.

The cytotoxic granules contain perforin and granzymes. While perforin is capable of forming a pore in the target cell membrane [223, 224], granzyme is a family of serine proteases that cleave proteins within cells, resulting in the apoptosis of target cells [225]. Eight granzymes were reported in mice, with only five of these being identified in humans [226]. Granzyme B is the most potent member of this family. Granzyme B efficiently cleaves and activates caspase-3 and caspase-7 [227-229]. Moreover, granzyme B cleaves the pro-apoptotic B-cell lymphoma 2 (Bcl-2) family protein Bid. The carboxyl terminus of Bid moves into the mitochondria, where it induces the release of cytochrome c [230]. $CD8^+$ T cells also eliminate target cells via their Fas ligand that binds to Fas on the cell surface of target cells [231]. The interaction between FasL and Fas leads to activation of the caspase cascade, which enables lysis of the target cell.

Many studies have established that $CD8^+$ T cells serve critical roles in the control of infections. $CD8^+$ T cells are critical for the defence against intracellular pathogens, including viruses and bacteria such as *Listeria monocytogenes*, *Salmonella typhimurium* and *Mycobacterium tuberculosis* [232-235]. They also play a role in pathological conditions such as graft rejection and graft-versus-host disease (GVHD). Moreover, $CD8^+$ T cells are the most effective cytotoxic cells in antitumor immune responses. As a result of the latter, they are being targeted with great clinical success for cancer immunotherapies.

1.2.3 T cell exhaustion and alloreactivity

After priming by an antigen, $CD8^+$ T cells differentiate into effector T cells that

exhibit cytotoxic activity and can differentiate into memory $CD8^+$ T cells. When $CD8^+$ T cells are sub-optimally primed or chronically stimulated, they can develop into a state known as “exhaustion” [236]. Many studies have proven that exhausted $CD8^+$ T cells overexpress several inhibitory receptors, such as PD-1, CTLA-4 as well as lymphocyte activation gene 3 (LAG-3). These inhibitory receptors have been reported to be among the main factors accounting for T cell exhaustion, seemingly by acting at the level of exhausted T cell progenitors [237-239]. Thus, blocking these receptors, also known as “inhibitory” immune checkpoints, has been used with great success to improve $CD8^+$ T cell responses against cancer cells.

Immune checkpoint-blocking agents, either antibodies or soluble receptors, are created to block the functions of inhibitory receptors and to restore T cell responses against cancer cells. Ipilimumab acts by blocking the checkpoint protein CTLA-4 [240], pembrolizumab and nivolumab block the checkpoint protein PD-1 [240], and atezolizumab and durvalumab block programmed death-ligand 1 (PD-L1) [241, 242]. T cell checkpoint-blocking antibodies are now used as single agents or in combination with other therapies as treatments for approximately 50 different types of cancer [243]. PD-1/PD-L1 blocking antibodies are among the most widely studied immune therapies for cancer. In 2020, more than 4400 clinical trials tested PD-1/PD-L1 checkpoint inhibitors [244]. However, further investigations revealed that even for patients with tumours highly expressing PD-L1, more than half of them did not respond to PD-1/PD-L1 inhibitors [245-247]. Furthermore, immune checkpoint inhibitors can have adverse inflammatory effects that can impact people in a variety of ways, such as inflammation of the lungs, colon, pancreas, liver, kidneys and pituitary gland. Ultimately, although immune checkpoint inhibitors have not completely resolved the issue of cancer treatment, they represent a significant stride forward in cancer therapy (Figure 1.3)[248, 249].

Patients with terminal organ failure can benefit from organ transplantation as a replacement therapy. The primary barrier to long-term transplantation is recipient

alloimmunity. In allograft responses, recipient T cells are stimulated through the interaction between host TCRs and allogeneic MHC molecules on donor cells. $CD8^+$ T cells are the primary effector cells in allograft responses, due to their cytotoxic effector function and their ability to produce proinflammatory cytokines such as IFN-γ [250].

Taken together, this section provides a general review covering the development, characteristics and biological functions of conventional T cells. We will not discuss Tregs cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells or γδ T cells in greater detail due to the scope of our research. In the following subsection, a critical event related to our topic, T cell activation, will be introduced.

1.3 T cell activation

T cell activation is a key event of antigen-specific immunity that is required for protection against microbial pathogens and tumour cells [251-253]. It is also involved in the pathophysiology of autoimmune diseases such as diabetes and rheumatoid arthritis [254-256].

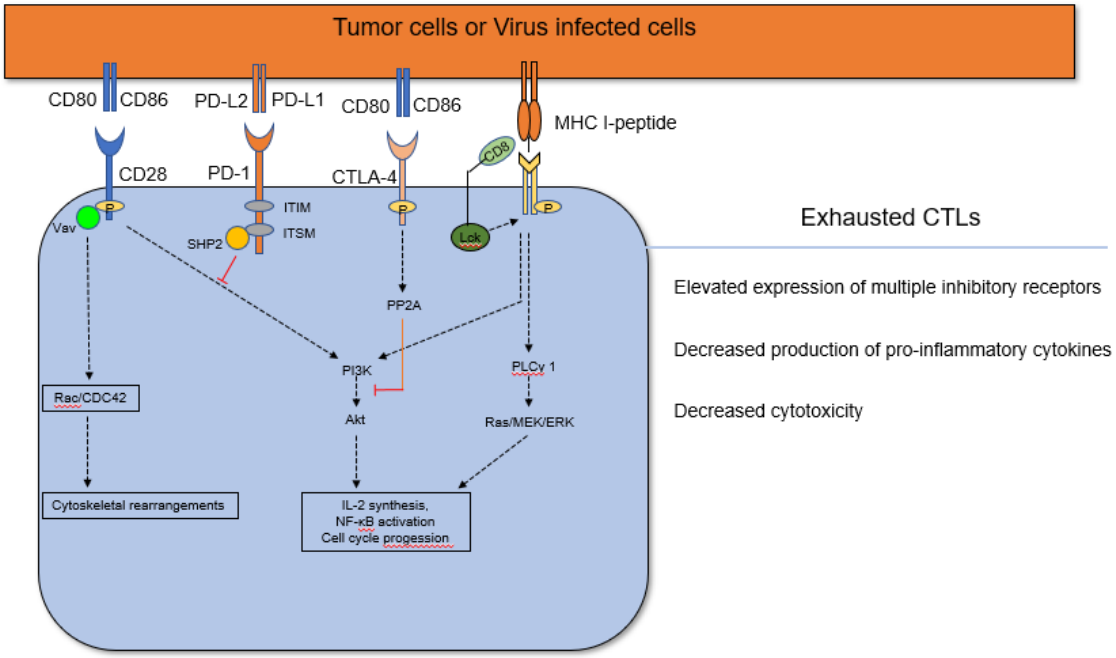

Figure 1.3 Overview of the mechanism for regulating T cell activation. A variety of signals are required for regulating the activation of $CD8^+$ T cells. The chief activating signal is generated from the T cell antigen receptor (TCR), which recognizes antigens associated MHC molecules on APCs. The second essential signal for $CD8^+$ T cells activation is from the interaction between CD28 on $CD8^+$ T cells with their ligands B7 molecules expressed on APCs. T cells also express the co-inhibitory receptors such as PD-1 and CTLA-4. PD-1 and CTLA-4 are the key co-inhibitory receptors lead to T cells T cell "exhaustion".

1.3.1 T cell priming

Once developed, T cells migrate out of the thymus and circulate through the peripheral lymphoid organs until they recognize their proper antigenic peptides, which are presented by the MHC molecules on the surface of APCs. For T cells to respond adequately to antigens, multiple types of receptors—either activating or inhibitory—recognize ligands that may or may not be expressed on APCs. Chief amongst them is the TCR, which is triggered by antigens presented by the MHC molecules on APCs.

On the surface of most conventional T cells, the TCR consists of two highly variable

protein chains: α and β. The somatic recombination of TCR gene segments is used to generate antigen-binding diversity. The TCRα-chain is generated from the recombination of variable (Vα) and joining (Jα) gene segments, while the TCRβ-chain is generated from the recombination of a D gene (Dβ) segment as well as Vβ and Jβ segments [257]. These recombination events generate more than 10^{15} combinations for TCRαβ [258].

The priming of T cells is controlled by TCR triggering; however, the TCR itself does not activate downstream signalling pathways to activate the T cells. Instead, the TCR is coupled to a complex of signalling molecules, named the CD3-z complex, which induces the signals leading to T cell activation. The TCR-CD3 complex comprises the TCRαβ, CD3εγ, CD3εδ and CD3ζζ dimers [259]. Once the TCRαβ heterodimers are properly engaged by the peptide-MHC complex, the ensuing conformational changes in the TCR-CD3 complex induce the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3-z cytoplasmic tails. This event is mediated by the Src family of protein tyrosine kinases, including Lck and Fyn. The phosphorylated tails then use SH2 domains to recruit and activate the Syk family kinase zeta-chain-associated protein kinase 70 kDa (ZAP-70) [260-263]. Activated ZAP-70 then phosphorylates the adaptor LAT [264, 265]. Phosphorylated LAT provides a docking platform for recruiting other adaptor molecules and enzymes, which form the LAT signalosome, including growth factor receptor-bound protein 2 (GRB-2), GRB2-related adaptor downstream of Shc (GADS), SH2-domain-containing leukocyte protein of 76 kDa (SLP76), adhesion and degranulation promoting adaptor protein (ADAP), phospholipase Cγ1 (PLC-γ1), IL-2-inducible T cell kinase (ITK) and the exchange factor VAV-1 [265]. The LAT signalosome enables a signalling cascade, involving SLP76 phosphorylation by ZAP-70, that results in the recruitment of ITK and subsequent phosphorylation of PLC-γ1. Phosphorylated PLC-γ1 cleaves the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the intracellular second messengers inositol 1,4,5-triphosphate

(IP_3) and diacylglycerol (DAG). Increased IP_3 leads to the release of Ca^{2+} from the endoplasmic reticulum (ER) and then activates the calcium-dependent signalling pathways. Notably, DAG can activate the NF-κB and Ras-MAPK pathways. Activated SLP76 can also recruit VAV-1, which is involved in the remodelling of the actin cytoskeleton (Figure 1.3).

Activated T cells are capable of multiple responses, such as proliferation, cytokine production and effector functions [264, 266-269]. However, TCR signalling alone is insufficient for optimal T cell activation and can lead to T cells switching to an anergic state and dysfunction [270].

T cells also express co-receptors (e.g., CD4 and CD8 [271, 272]), co-stimulatory receptors (e.g., CD28 and ICOS [28, 273, 274]) and co-inhibitory receptors (e.g., PD-1 and CTLA-4) that recognize potential ligands on APCs and modulate T cell activation [275-278].

1.3.2 Co-signaling receptors CD4 and CD8

According to their cell surface proteins CD4 or CD8, conventional T cells are subcategorized into two groups—each playing distinct effector roles in adaptive immune responses. Both CD4 and CD8 molecules belong to the immunoglobulin superfamily, which consists of extracellular IgSF domains connected to a transmembrane region. The extracellular domain of CD4 interacts with MHC class II molecules and is composed of four Ig-like domains with a short cytoplasmic domain that bears a conserved Lck-binding site [271, 279]. The CD8 molecule is a disulfide-linked heterodimer that consists of two chains: one α chain and one β chain. The extracellular domain of CD8 binds to the MHC class I molecule and its intracellular domain binds to Lck [271, 280].

The most widely accepted proposal for co-receptors to exert their function is the recruitment and delivery of TCR-phosphorylating Src family kinase Lck to stimulated TCRs [280, 281]. Lck initiates the phosphorylation of the ITAMs of TCRs when TCRs bind to antigen peptide-MHC molecules [281]. Then, ZAP-70 is recruited by the phosphorylated

ITAMs and induces the downstream activation signals. This model was supported by early experimental observations, which indicated that the TCR-CD3 complex itself lacks intrinsic protein tyrosine kinase activity, T cell activation is aided by a synergistic interaction between CD4, and the TCR and co-receptors recruit protein tyrosine kinase Lck [271, 282-285].

Later, Davis et al. proposed a new mechanism of TCR triggering known as the "pseudodimer" model, which explains the role of self-peptides in T cell activation [286, 287]. In this model, CD4 or CD8 molecules are thought to bridge the gap between an antigenic peptide-MHC complex and an endogenous peptide-MHC complex to form receptor pseudodimers. This model is supported by research arguing that endogenous peptides can activate T cells [287]. However, there is very little data to support this proposal. Moreover, the notion of CD4 physically connecting with the TCR in an orientation to suit pseudodimerization has received minimal support in structural studies.

Recently, new models were created according to experimental observations [288, 289]. However, we will not discuss these new models here. Overall, all of the models support the notion that the co-receptors recruiting Lck are critical for T cell activation.

1.3.3 Co-stimulatory receptor CD28

Upon TCR stimulation, an IS at the interface between the T cell and the APC is formed. This IS is composed of distinct supramolecular activation clusters (SMACs) arranged like an eye with the TCR complexes. CD28 is colocalized in the central SMAC region of immunological synapses [290]. Notably, CD28 is a stimulatory receptor of the immunoglobulin superfamily and is primarily expressed on the surface of T lineage cells. It interacts with B7-1 (CD80) and B7-2 (CD86) on APCs. While CD28 is constitutively expressed on mouse T cells, it is expressed on more than 80% of human Th cells and 30% of human CTLs [291-293]. CD28 interacts with CD80/86 to cause signal transduction events that are dependent on its cytoplasmic tail, which can recruit several adaptors.

There are two regions on its cytoplasmic tail that have received a lot of attention—one is the membrane-proximal YMNM motif and the other is the distal PYAP motif [294]. It was reported that the YMNM motif recruits phosphoinositide 3-kinase (PI3K), GRB2 and inositol 5' polyphosphatase 1 (SHIP-1). For example, PI3K can catalyze the production of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which recruits protein kinase B (PKB/Akt) and phosphoinositide-dependent protein kinase 1 (PDK-1). PDK-1 phosphorylates PKB, while phosphorylated PKB activates its downstream targets—including mTOR and IκB. Active mTOR and IκB lead to increased NF-kB transcriptional activity, which induces the transcription of both the pro-survival factor Bcl-XL and IL-2. GRB2 contains two SH3 domains and one SH2 domain, which are capable of binding the PYAP motif and the YMNM motif of CD28, respectively. Furthermore, GRB2 recruits VAV-1 and Sos, which lead to cdc42/Rac1 activation to induce the cytoskeletal rearrangement and downstream MAPK activation (JNK) signals. VAV-1 also interacts with the SLP-76-LAT complex, which induces the PLCγ-1 signalling cascade [295-303]. CD28-deficient mice exhibit reduced GC formation and reduced antibody responses to T-dependent antigens [302, 304, 305]. CD28 signals also are essential for the survival of activated T cells.

Currently, targeting the co-inhibitory receptor PD-1, which will be discussed in more details below, has demonstrated significant clinical benefits for a variety of tumours. However, the molecular mechanism of PD-1 function has remained poorly understood for many years. Hui et al. and Kamphorst et al. found that CD28 is the primary target of PD-1. Phosphorylated PD-1 recruits the phosphatase SHP-2, which ultimately results in the dephosphorylation of CD28 and the termination of its signalling cascade [306, 307].

Chimeric antigen receptor *(*CAR*)* T-cell therapy has achieved impressive outcomes in the treatment of certain subsets of B-cell leukemia and lymphoma. CAR T-cell therapy involves modifying a person's T cells to recognize specific antigens in an MHC-independent approach. The chimeric antigen receptor typically contains an extracellular domain, which is a single-chain variable fragment (scFv), a transmembrane domain for the stability of the

receptor, and an intracellular domain containing a co-stimulatory signalling domain (most often CD28 and/or 4-1BB) and a TCR signalling domain (typically CD3ζ) for T cell activation. First-generation CARs lacked co-stimulatory domains, thereby providing very low antitumor responses. In the following generation of CARs, the addition of the CD28 co-stimulatory domain led to markedly increased T cell proliferation and cytokine secretion. Multiple studies have reported that CARs with a co-stimulatory domain are more efficient at controlling tumour growth [308-314].

1.3.4 Co-inhibitory receptor PD-1

Co-inhibitory receptors, including PD-1, CTLA-4, TIM-3 and LAG-3, are critical regulators of T cell function and guarantee immune homeostasis by limiting T cell proliferation and effector functions. Currently, PD-1 and CTLA-4 are at the forefront of cancer therapy.

The PD-1: PD-L pathway is pivotal for the regulation of T cell tolerance and protects normal tissues from inflammatory damage. Despite being absent in naïve T cells, PD-1 is rapidly upregulated upon T cell activation. Subsequently, the interactions between PD-1 and its ligands PD-L1 or PD-L2 generate inhibitory signals for T cell activation. The signals can attenuate the priming of T cells or inhibit effector T cell functions. Particularly in the context of chronic infections, T cells are consistently exposed to antigens and continue to express high levels of PD-1, which eventually diminish the T cells' effector functions. PD-1 binds to either of its two ligands, PD-L1 (CD274 or B7-H1) or PD-L2 (CD273 or B7-DC). PD-L1 is expressed on immune and non-immune cells in inflamed tissues. Meanwhile, PD-L2 is primarily expressed on APCs and Th2 cells [315]. The induction and upregulation of PD-1 ligands in an inflammatory environment may provide a negative feedback mechanism to prevent overactive immunity [316]. Tumour cells mainly express PD-L1, which is the main inhibitory ligand for T cells in humans. Thus, PD-L1 has been of greater interest than PD-L2 for cancer therapy.

It was initially proposed that PD-1 attenuates TCR signaling [317, 318]. However, Hui et al. determined that CD28 is the primary target of PD-1 [306]. Moreover, Kamphorst et al. reported that CD28 is critical for PD-1 therapies to kill efficiently cancer in mice. The intracellular domain of PD-1 consists of an ITIM as well as an immunoreceptor tyrosine-based switch motif (ITSM) [307]. After stimulation, the phosphorylated ITIM and ITSM recruit and activate SHP-2. SHP-2 dephosphorylates CD28 to terminate its signalling cascade and inhibit T cell effector functions.

Notably, Sugiura et al. also discovered that PD-L1 and CD80 interact in cis on activated DCs, thereby blocking the trans interaction between PD-1 and PD-L1 and promoting T cell responses[319]. Recently, Sugiura et al. generated anti-CD80 antibodies for detaching the CD80 from the cis-CD80/PD-L1 duplex and enabling the trans interaction between PD-1 and its ligand. Notably, the elimination of PD-1 restriction has an important role in reducing autoimmune disease symptoms[320].

Blocking monoclonal antibodies targeting PD-1/PD-L1 have made breakthroughs for treating multiple tumours, making inhibition of PD-1/PD-L1 signaling of clinical importance. Currently, monoclonal antibodies against PD-1 or PD-L1 are routinely used in clinical settings, including nivolumab [321] and pembrolizumab [322]. Furthermore, it has been proven that combining PD-1/PD-L1 inhibitors with CTLA-4 inhibitors can remarkably improve the overall efficacy of treating various kinds of cancer. After combination, their effectiveness could reach 60%—compared to only 25% efficiency when administering each drug independently [323].

1.4 Extended SLAM-family receptors (SFRs) CD2 and CD48

The canonical SFRs include six molecules: SLAMF1, 2B4, SLAMF6, SLAMF7, SLAMF5 and Ly-9 [324, 325]. All six SFR-encoding genes are located in the same locus (*SLAM* locus) on chromosome 1 [326]. SFRs are only expressed in hematopoietic cells. Using their cytoplasmic domain, SFRs interact with the SH2 domain-only SAP adaptors, which control

the signals emanating from SFRs. Most SFRs are homotypic receptors. The only exception is receptor 2B4, which is activated by ligand CD48. Until recently, SFRs were believed to interact with their ligands in trans (between two different types of cells). However, recent data suggest that they may also interact in cis (within one cell). Depending on the expression status of SAP adaptors and the extent of engagement of SFRs by ligands, SFRs can mediate inhibitory or activating signals and functions. In humans and mice lacking SAP adaptors, SFRs display a non-physiological "super-inhibitory" function that suppresses other activating receptors, leading to reduced NK cell activation, reduced $CD8^+$ T cell cytotoxicity, abolished T cell-dependent antibody production and blocked invariant natural killer T (iNKT) cell development. X-linked lymphoproliferative (XLP) disease is caused by these defects due to SAP deficiency. However, with SAP adaptors, SFRs are less inhibitory and sometimes become activating [326-328].

CD2 and CD48 are part of the "extended" family of SFRs. Unlike canonical SFRs, these non-canonical SFRs do not interact with SAP adaptors and their signalling mechanisms and functions are more elusive. Like the genes coding for canonical SFRs, the gene coding for CD48 is located within the *SLAM* locus. However, the gene coding for CD2 is located on another chromosome. Moreover, CD48 is a glycosylphosphatidylinositol (GPI)-linked molecule found on all hematopoietic cells [329]. As previously stated, it binds 2B4 as well as CD2. CD2 binds CD48 in mice and another GPI-linked molecule, CD58, in humans. CD2 is a transmembrane molecule and a member of the immunoglobulin superfamily found on the surface of T cells, NK cells as well as mouse B cells. The extracellular fragment of CD2 contains a proximal IgC2 domain followed by a distal IgV domain. CD2 interacts with its ligands CD48 (in mice) and CD58 (also known as LFA-3; in humans). It was reported that the interactions between CD2 and its ligands was found at the periphery of mature immune synapses[330]. In its cytoplasmic domain, mouse CD2 possesses 5 proline-rich sequences. These motifs were proposed to mediate binding to Src homology 3 (SH3) domain-containing effectors, such as Lck, Itk, CD2-AP and CD2-BP,

although much confusion exists regarding the functional relevance of these interactions.

1.4.1 The binding affinity between CD2 and its ligands

The affinity between CD2 and its ligand is relatively low (interactions between mouse CD2 and CD48 have a K_d value of 90 μM and interactions between human CD2 and CD58 have K_d values clustering in the region of 10–30 μM), and the dissociation rate is rapid[331, 332]. Without additional interactions, it may be hard to understand how such low affinity and high dissociation rates would lead to stable IS formation. Thus, it remains unclear how CD2 and its ligands interact with each other in cells.

1.4.2 Roles of CD2 and its ligand in immune cells

CD2 is expressed on all T cells, both immature and mature. It was initially thought to be critical for T cell development in thymus [333]. However, in an early study, the authors investigated the function of CD2 during thymocyte development in mice by using a monoclonal antibody that targets mouse CD2. Anti-CD2 antibody treatment diminished CD2 expression in thymocytes; however, no effect on the development of mature $CD3^+$ thymocytes was detected and large numbers of $CD3^+CD2^-$ cells were present in thymocytes [334]. In subsequent studies, two CD2-deficient mouse strains were analyzed and it was found that CD2 deficiency might promote positive cell selection [335]. Further studies showed that the lack of CD2 enhanced positive selection [335]. This finding suggested a CD2-independent regulation of thymocyte selection by TCR signalling. However, it also contradicted other findings that implied an effect of CD2 on TCR signalling.

Although the CD2 molecule has been described as a co-stimulatory receptor on T cells over the past decade, an initial analysis of genetically-engineered CD2 KO mice showed that these animals had normal T cell responses, including the function of cytotoxic T cells and the production of antibodies in response to immunization[336]. Notably, these

mice were created in the 129 background and then backcrossed with C57BL/6 mice [B6.129] [337]. A subsequent study of the same mouse strain showed reduced responses to low doses—but not high doses—of antigenic peptides [338]. Later, another group showed that, although CD2 KO mice displayed normal or minimally compromised T cell responses to anti-CD3 antibodies or antigenic peptides, CD2 deficiency accentuated the T cell defects observed in mice lacking CD28 [339]. Hence, although it was proposed that CD2 has a role in promoting T cell responses to sub-optimal antigen receptor stimulation, this role overlaps with that of CD28 in this function.

Kato reported that CD48 is a ligand for mouse CD2, which is involved in the regulation of T cell function. They generated hamster mAb HM48-1, which was able to block the binding of the extracellular domain of mouse CD2 to T cells. HM48-1 inhibited T cell activation induced by lectins (Con A and PHA). On the other hand, HM48-1 greatly augmented the proliferative response when crosslinked with an anti-CD3 mAb [340]. Additionally, Gonzalez-Cabrero et al. generated CD48 KO [B6.129] mice. Although the activation of $CD4^+$ T cells in CD48 KO mice was severely impaired, they did not examine further the mechanisms involved [341].

CD48 is anchored to the lipid raft of the cell membrane using GPI linkages. Previous studies showed CD48 could serve a role in T cell signalling. Earlier results suggested that CD48 maybe associated with Lck and that cross-linking CD48 led to the phosphorylation of Lck. It was shown that CD48 co-stimulation increased the phosphorylation of Lck and TCR-associated z chains. Further studies showed that the cross-linking of CD48 resulted in increased intracellular calcium flux and enhanced IL-2 production and TCR signalling. In summary, CD48 on T cells may be involved in regulating TCR signalling by binding to signalling molecules in lipid rafts [342]. On the other hand, it has been reported that CD48 on APCs plays its role in trans for regulating T cell activation. For example, in allogeneic mixed lymphocyte responses, CD48 absent from APCs displayed a less stimulatory to T cells [341]. Investigators reported that in the absence of intracellular structural domains,

CD48 stimulation induces the rearrangement of signalling factors as well as Lck kinase activity in lipid rafts. Moran et al. activated T cells with artificial CD2-expressing APCs and investigated the consequences of CD48 and its associated lipid rafts upon being recruited to the responder: stimulator contact site. They observed the qualitatively and quantitatively enhanced lipid raft-dependent binding of ζ to the actin cytoskeleton and the tyrosine phosphorylation of ζ following CD2:CD48 interactions. This suggests that CD48 coexists with intracellular protein tyrosine kinases in "lipid rafts" and is involved in signal transduction and cytoskeletal reorganization. However, the study lacked strong evidence to show that CD48 can directly interact with Lck in lipid rafts [343].

1.5 Working hypothesis of the project

Currently, therapeutic anti-CD2 antibodies are being tested as antibodies in phase 1 studies of CD2-positive tumours and psoriasis (www.clinicaltrials.gov). Earlier studies showed that stimulation with anti-CD2 Abs can regulate human T cell activation [344]. Imaging analysis using CD2 ligands immobilized on artificial lipid bilayers showed that CD2 is a key part of the so-called "immune synapse" [330]. However, the analysis of CD2 KO mice does not support the importance of CD2 [337]. As noted in Section 1.4.2, the importance of CD2 in conventional T cell activation remains unclear.

We aimed to investigate the role and mechanism of action of CD2-CD48 in conventional T cell activation. To date, all studies—and the models derived from these studies—have focused on the role of CD2 when engaged in trans by its ligands on APCs. Notably, however, these ligands—namely CD48 and CD58—are also expressed in T cells. Given the growing evidence that immune cell receptors such as PD-1 and 2B4 can be regulated by ligands in cis, we wanted to discern the relative impact of these two types of splicing on CD2 function.

1.5.1 Subject 1

The first aim was to study the function of CD2 and its ligands in T cell function. This aim was achieved through the following objectives:

1. Identify whether CD2 and its ligands support the development of T cells.
2. Investigate whether CD2 and its ligands are required for T cell activation.
3. Further investigate whether cis interaction between CD2 and its ligands on T cells is required for T cell activation.
4. Investigate whether CD2 and CD48 are required for T cell-mediated cytotoxicity.
5. Further investigate whether cis interaction between CD2 and CD48 on T cells is critical for T cell-mediated cytotoxicity.

1.5.2 Subject 2

The focus of Aim 2 was to explore the signalling molecules downstream of CD2 that support T cell activation.

1. Investigate whether the intracellular domain of CD2 is needed for TCR signalling.
2. Further investigate which motif of the intracellular domain of CD2 is involved in the signalling transduction.
3. Investigate the downstream effectors of CD2 and its ligand in T cells.
4. To investigate the function of CD2-CD58 interaction in humans.

Chapter 2 The role of CD2 and its ligands in T cell activation

***cis* interactions between CD2 and its ligands on T cells are required for T cell activation**

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2.1 Summary

CD2 reportedly promotes T cell activation when engaged by its ligands, CD48 (mice) and CD58 (humans) found on APCs. Herein, we determined that, while CD2 on T cells was necessary for T cell activation, its ligands were not required on APCs. Rather, the ligands were needed on T cells. Thus, T cell-intrinsic interactions of CD2 with its ligands are required for TCR signaling and T cell activation.

2.2 Introduction

T cell activation is a critical component of antigen-specific immunity needed for protection against microbial pathogens and tumor cells[345-347]. It is implicated in the pathophysiology of auto-immune diseases such as diabetes and rheumatoid arthritis. For T cells to respond adequately to antigens, multiple receptors, activating or inhibitory, recognize ligands that may or may not be expressed on APCs. Chief amongst them is the T cell antigen receptor (TCR), which recognizes antigens associated with MHC molecules on APCs. T cells also express co-receptors, namely CD4 and CD8; co-stimulatory receptors, including CD28 and ICOS; as well as co-inhibitory receptors, like PD-1 and CTLA-4, that can also be triggered by ligands on APCs to modulate T cell activation.

CD2 is a transmembrane immunoglobulin superfamily member broadly expressed on T cells and NK cells[329, 348, 349]. It is distantly related to the SLAM family of receptors[38]. CD2 recognizes as ligands CD48 (in mice) and CD58 (also known as LFA-3; in humans), two glycosylphosphatidylinositol (GPI)-linked receptors abundantly expressed on APCs. CD48 is also highly expressed on all T cells in mice, whereas CD58 is highly expressed in effector-memory and innate-like T cells in humans (www.immgen.org; https://singlecell.broadinstitute.org/). Imaging analyses using CD58 immobilized on artificial lipid bilayers suggested that, upon engagement by ligands on APCs (hereafter termed "*trans*" interactions), CD2 becomes part of the T cell "immunological synapse"[350-352]. As stimulation of T cells with agonistic anti-CD2 antibodies (Abs)

resulted in markedly enhanced T cell activation, it has been implied that CD2 is a potent T cell activating receptor[329, 348, 349]. Conversely, it was shown that blocking anti-CD2 Abs diminished T cell activation, leading to the idea that CD2 blockade may be useful in the treatment of T cell-mediated diseases such as graft rejection and autoimmunity in humans[353].

Genetic studies have provided more conflicting evidence on the importance of CD2 in T cell activation. Initial analyses of genetically-engineered CD2-deficient (knock-out; KO) mice in a mixed genetic background [129-C57BL/6 (B6)] suggested that these animals had normal T cell responses[337]. However, a subsequent study showed reduced responses to low-affinity, but not high-affinity, antigenic peptides[354, 355]. Another study found that the CD2 KO mice displayed normal or only minimally compromised T cell responses to anti-CD3 Abs or antigenic peptides, although CD2 deficiency accentuated the defects caused by lack of CD28[339].

Although CD2 has been presumed to be engaged by ligands displayed on APCs[350, 351], , these ligands are also expressed on T cells, suggesting that interactions within individual T cells or between adjacent T cells (hereafter termed "*cis*^{T}" *and* "*cis*$^{T\text{-}T}$" interactions, respectively) may also exist. To address this possibility, we analyzed T cell functions in newly generated mouse strains lacking CD2 or CD48, in a pure B6 background. We found that T cells lacking CD2 had broad activation defects *in vitro* and *in vivo*. Surprisingly, identical defects were seen in T cells lacking CD48. Imaging analyses provided evidence that CD2 and CD48 interacted in *cis* at the surface of individual T cells.

2.3 Materials and Methods

2.3.1 Mice

CD2 KO mice and CD48 KO mice were generated by CRISPR-Cas9, using the guide RNA sequences 5'-TGATATTGATGAGGTGCGAT-3' (for CD2) and 5'-

GGTTACATTGCTGCCGGTGG-3' (for CD48). DNA was injected in fertilized oocytes of C57BL/6J mice. For CD2 KO mice, mice bearing a 28-nucleotide (clone 914) or 25-nucleotide (clone 919) deletion in exon 2 of *Cd2* were identified. For CD48 KO mice, mice bearing a 10-nucleotide (clone T6), a 1-nucleotide (clone T87) or a 147-nucleotide (clone del-147) deletion in exon 2 of *Slamf2* were selected. Once identified, heterozygous KO mice were backcrossed to the C57BL/6J background for 10-12 (CD2 KO) or 6-12 (CD48 KO) generations, and subsequently bred to homozygosity for experimentation. All deletions resulted in a frameshift in the second coding exon of the *Cd2* or *Slamf2* gene, and caused loss of CD2 or CD48 expression by flow cytometry.

OT-I TCR transgenic *mice* and OT-II TCR transgenic *mice* were obtained from the Jackson Laboratory, and bred with CD2 KO or CD48 KO mice. B6. SJL mice were purchased from the Jackson Laboratory, and bred with CD2 KO mice. Balb/c mice were purchased from the Jackson Laboratory.

2.3.2 Plasmids

For production of the Fc fusion proteins encompassing the extracellular domain of mouse CD2 or mouse CD48, the relevant cDNA fragments were amplified by PCR and cloned into pSK-Fc, which also encodes the Fc portion of human IgG1. Subsequently, fusion constructs were cloned into pXM139 for transient expression in Cos-1 cells. For production of the Fc fusion proteins encompassing the extracellular domain of human CD2, human CD48 or human CD58, the appropriate cDNA fragments were amplified by PCR and cloned into phIgG1-Fc, which also encodes the Fc portion of human IgG1, for transient expression in HEK293T cells. Fc-fusion proteins were purified from supernatants using rProtein A Sepharose® Fast Flow (MilliporeSigma, GE 17-1279-03).

2.3.3 Functional assays using freshly purified T cells

Purified $CD4^+$ or $CD8^+$ splenic T cells (10^6 cells per ml) from the indicated mice were

stimulated anti-CD3 MAb, with or without soluble anti-CD28 (1 μg ml^{-1}), concanavalin A (Con A; 4 μg ml^{-1}; Sigma-Aldrich), or PMA (100 ng ml^{-1}; Sigma-Aldrich) plus ionomycin (1 μg ml^{-1}; Sigma-Aldrich). For anti-CD2 Ab blockade experiments, assays were as usual except that rat anti-CD2 RM2-5 or isotype control rat IgG2b G013B8 (1 μg ml^{-1}, coated on plastic) was also added. For the alloreactivity assays, purified $CD8^+$ T cells (10^6cells per ml) were incubated for 2 days with MHC-mismatched irradiated splenocytes (2,500 rad). For stimulation with the superantigen SEB, $CD4^+$ T cells (10^6cells per ml) were stimulated for 2 days with the indicated concentrations of SEB, in the presence of irradiated splenocytes as APCs [356]. For OT-I mice, $CD8^+$ T cells (10^6cells per ml) were stimulated for 24 hours with the OVA-derived SIINFEKL (N4) agonist peptide or SIIGFEKL (G4) weak agonist peptide (0.01-1000 nM), with APCs.

2.3.4 Graft-versus-host disease

A 3:1 mixture of $CD8^+$ T cells (1.8×10^6) and $CD4^+$ T cells (0.6×10^6) from the indicated mice in the C57BL/6 background was injected intravenously into sub-lethally irradiated (5 Gy, gamma irradiator with a $Cesium^{137}$ source) Balb/c mice. PBS was injected as control. Mice were then monitored every day for weight loss and survival. Mice exhibiting a body weight loss greater than 30% were immediately euthanized, and their death was recorded as having occurred the next day. Otherwise, mice were sacrificed at the indicated times. The number and cell surface markers of donor cells in the spleen were determined using flow cytometry.

2.3.5 Binding to soluble Fc fusion protein

Previously activated OT-II mouse T cells or Jurkat human T cells were incubated for 30 min on ice with the indicated Fc fusion proteins, which all carry the Fc portion of human IgG1. Subsequently, cells were washed and stained with the secondary antibody for 30 min on ice.

2.3.6 Immunofluorescence microscopy

Images were acquired using a laser-scanning confocal microscope LSM-700 (Zeiss). For quantification of fluorescence, raw images were reconstructed by the Zeiss Zen software processing tool (Black Edition). Fluorescence intensity (FI) profiles for Brilliant Violet 421 (CD2) and Alexa Fluor 647 (CD48) were determined along a solid line drawn on the images, using Zeiss Zen 2012 (Blue *Edition*). FI profiles along the solid line are represented by the curves.

2.3.7 Fluorescence resonance energy transfer (FRET) assay

The acceptor photobleaching FRET assay was performed as described [357]. In essence, a SNAP tag or a CLIP tag was added to the amino-terminus of mouse CD48 or mouse CD2, respectively, using PCR. After cloning into the vector pFB-Neo, constructs were co-transfected into HEK293T cells, using polyethylenimine (Polysciences, 23966-1). After 48 hours, cells were harvested and seeded into poly-L-lysine-treated 96-well plates (MatTek Corporation, P35G-0-20-C). After 24 hours, cells were labeled with the dye for 45 minutes at 37°C, followed by 3 washes with PBS. In some cases, Abs (3 mg ml^{-1}) were included. Cells were then fixed for 10 minutes at room temperature and used for the this assay[358].

2.3.8 Cytotoxicity and assays with previously activated T cells

OT-I $CD8^+$ T cells were primed for 36 hours with SIINFEKL (20 µM) in the presence of irradiated splenocytes. They were subsequently expanded for 48 with IL-2. Then, primed $CD8^+$ T cells were incubated with APCs (EL-4 cells, RMA cells or previously activated $CD4^+$ T cells, 3000 cells per well in 96-well plate), which were previously labeled with ^{51}Cr and pulsed or not for 1 hour with SIINFEKL peptide (1 µM). Cytotoxicity assays were then performed in duplicates at the indicated E:T ratios. Maximal ^{51}Cr release and

spontaneous release were determined by adding 1.0% (v/v) *Triton* X-*100* or medium, respectively. Release of ^{51}Cr into the supernatant was measured with a γ-counter (Perkin Elmer, Wallac Wizard 1470). For induction of CD107a surface exposure, assays were conducted in the presence of anti-CD107a MAb 1D4B (BioLegend). After 4 hours, cells were stained and analyzed using a flow cytometer. OT-I T cells were identified by gating on $CD8^+$ cells. Experiments with primed $CD8^+$ T cells assessing proliferation or cytokine production were conducted using similar conditions.

2.4 Results

2.4.1 Normal T cell development in mice lacking CD2

To address further the role and triggering mechanism of CD2 in T cells, we generated new CD2 KO mouse strains in the pure C57BL/6 background. Compared to control wild-type mice, CD2 KO mice lacked CD2, but had normal expression of other T cell markers, including TCR and CD3, in thymus and spleen (Fig. 2.1A,B). They also had normal T cell subsets in spleen, with normal proportions and numbers of regulatory, naïve, effector-memory and central memory T cells (Fig. 2.1C,D).

2.4.2 Lack of CD2 broadly compromises T cell responses even in the absence of APCs

To test T cell activation *in vitro*, freshly isolated T cells from CD2 KO mice were stimulated with various stimuli, requiring or not requiring APCs. Compared to wild-type T cells, CD2 KO T cells, either $CD4^+$ or $CD8^+$, displayed reduced proliferation (as measured by thymidine incorporation) and production of cytokines (IL-2, IFN-γ and IL-4), in response to anti-CD3 Abs, in the absence or in the presence of anti-CD28 Abs (Fig. 2.2A). Similar defects were observed with the lectin concanavalin A (Con A; “C”), but not with the combination of phorbol myristate acetate (PMA) and ionomycin (P+I), which bypass the proximal TCR signaling apparatus (Fig. 2.2B). Analogous defects were noted when wild-

type T cells were treated with blocking anti-CD2 Ab RM2-5, compared to isotype control Ab (Fig. 2.2C).

2.4.3 Lack of CD2 broadly compromises T cell responses in the presence of APCs

In response to irradiated wild-type splenocytes as APCs plus the superantigen staphylococcal enterotoxin B (SEB), CD2 KO $CD4^+$ T cells also had a pronounced deficiency in T cell activation, when compared to wild-type $CD4^+$ T cells (Fig. 2.3A). SEB activates $CD4^+$ T cells expressing Vβ8.1 or Vβ8.2[359]. These T cell subsets were present in equal proportions in CD2 KO and wild-type mice (Fig. 2.3B). Likewise, in response to irradiated splenocytes from wild-type Balb/c mice, CD2 KO $CD8^+$ T cells showed a marked activation defect, compared to wild-type $CD8^+$ T cells (Fig. 2.3C). This "alloreactive response" occurs because TCRs from C57BL/6 mice recognize mismatched class I MHC from Balb/c mice as alloantigen.

To analyse antigen-specific responses, we also bred CD2 KO mice with OT-I or OT-II mice, which express a transgenic TCR reactive against ovalbumin (OVA) presented by class I or class II MHC, respectively. When activated by irradiated wild-type splenocytes and the high-affinity agonistic OVA peptide N4 (SII<u>N</u>FEKL), freshly isolated CD2 KO OT-I $CD8^+$ T cells displayed a severe decrease (up to 90%) in proliferation and cytokine secretion, in comparison to wild-type OT-I $CD8^+$ T cells (Fig. 2.3D). A more pronounced defect was noted with the low-affinity agonistic peptide G4 (SII<u>G</u>FEKL) (Fig. 2.3E). An analogous defect was noted when CD2 KO OT-II $CD4^+$ T cells were activated with irradiated wild-type splenocytes and the OVA peptide $OVA_{329-337}$, compared to wild-type OT-II $CD4^+$ T cells (Fig. 2.3F).

Hence, T cells from CD2 KO mice, both $CD4^+$ and $CD8^+$, displayed pronounced activation defects in response to multiple stimuli, in the absence or in the presence of APCs. Analogous deficits were caused by addition of a blocking anti-CD2 Ab.

2.4.4 CD2 on T cells is engaged by CD48 on T cells

The observation that CD2 was critical for T cell activation even in the absence of APCs implied that CD2 was engaged by ligands present on T cells. The only known ligand of CD2 in mice is CD48. To examine if other ligands may exist for CD2, we first performed binding assays using a soluble Fc fusion protein encompassing the extracellular domain of CD2, and purified T cells from wild-type, CD48 KO or CD2 KO mice. Using the CD2-Fc fusion protein (hereafter termed CD2-Fc) as probe, specific staining was noted on wild-type T cells (Fig. 2.4A). This staining was completely abolished on CD48 KO T cells. Of note, binding was greater (~3.5-fold) when CD2 KO T cells were used for staining. This difference was not due to increased CD48 expression on CD2 KO T cells, as shown by the calculated MFI ratio of CD2-Fc over CD48 (Fig. 2.4B).

In a similar way, when a CD48-Fc fusion protein was utilized as a probe, specific staining was seen on wild-type T cells, but not on CD2 KO T cells (Fig. 2.4A). Moreover, staining was greater (~3-fold) on CD48 KO T cells, compared to wild-type T cells, and this increase was not caused by greater CD2 expression (Fig. 2.4A,B). No expression of 2B4, another receptor capable of binding CD48 [360], was noted on these cells (Fig. 2.4A). This was seen whether T cells were activated or not (data not shown). In mice, 2B4 is expressed on exhausted T cells and γδ T cells, but not on most other T cells (www.immgen.org).

To address if CD2 and CD48 were interacting with each other at the surface of individual T cells, their colocalization was ascertained by confocal microscopy. Using fluorescence-coupled Abs, we observed that significant proportions of CD2 and CD48 were colocalized at the surface of individual T cells (Fig. 2.4C). No colocalization of the fluorochromes was seen with CD2 KO or CD48 KO T cells.

We also examined the ability of CD2 and CD48 to interact in *cis* using fluorescence resonance energy transfer (FRET), coupled to confocal microscopy. To this end, constructs encoding SNAP-tagged CD2 and CLIP-tagged CD48 were co-transfected into

HEK293T cells. After labeling the tags with an energy acceptor (Alexa Fluor 647 [AF 647]) or an energy donor (547), respectively, photobleaching of the acceptor was performed (Fig. 2.4D). Then, the impact of photobleaching on fluorescence of CD48-coupled 547 was measured. Photobleaching resulted in a pronounced increase (~30%) in the fluorescence of CD48-coupled 547 (Fig. 2.4D). This effect was markedly attenuated (reduced to ~10%) by addition of blocking anti-CD2 Ab RM2-5, compared to control Ab. The recovery of donor fluorescence after acceptor photobleaching implied that CD2 and CD48 were in close proximity at the cell surface.

Thus, CD2 and CD48 were an exclusive receptor-ligand pair and interacted in *cis* at the surface of individual T cells. In T cells lacking either molecule, the remaining component of the pair showed an increased capacity to recognize an exogenous fusion protein corresponding to the missing molecule, suggesting that the absence of *cis* interactions may facilitate *trans* interactions.

2.4.5 Normal T cell development in mice lacking CD48

If the *cis* interactions between CD2 and CD48 were critical for T cell activation, one would expect that CD48 KO T cells might display identical activation defects compared to CD2 KO T cells. No defect in T cell development or T cell subsets was observed in CD48 KO mice (Fig. 2.5A,B,C,D).

2.4.6 CD48 KO T cells phenocopy the functional defects of CD2 KO T cells

As was the case for CD2 KO T cells (Fig. 2.2), CD48 KO T cells, either $CD4^+$ or $CD8^+$, displayed greatly reduced responses to anti-CD3 Abs, with or without anti-CD28 Abs, or to Con A ("C"), when compared to wild-type T cells (Fig. 2.6A). Analogous defects were observed upon stimulation with wild-type APCs and SEB, or allogeneic wild-type Balb/c splenocytes (Fig. 2.6B,C). By opposition, when wild-type Balb/c $CD8^+$ T cells were activated by CD48 KO splenic cells, no defect in alloreactivity was seen (Fig. 2.6C). CD48

KO OT-I T cells and CD48 KO OT-II T cells also displayed markedly reduced responses to wild-type APCs and antigen, compared to their wild-type OT-I or OT-II T cell counterparts, respectively (Fig. 2.6D-F). These defects were seen whether APCs expressed or not CD48. Once again, wild-type OT-I or OT-II T cells activated by CD48 KO splenocytes as APCs plus antigen showed no defect.

Therefore, loss of CD48 on T cells mimicked the defects in T cell activation caused by loss of CD2, implying that the CD2-CD48 *cis* interactions were critical for T cell activation. Loss of CD48 on APCs had no appreciable impact on the responses studied.

2.4.7 Lack of CD2 compromises T cell-mediated cytotoxicity, but not its ligand

To examine if CD2-CD48 *cis* interactions were needed for T cell-mediated cytotoxicity, OT-I $CD8^+$ T cells from wild-type, CD2 KO or CD48 KO mice were primed by a first stimulation with wild-type APCs and a high concentration of OVA peptide (Fig. 2.7A). After expansion in IL-2-containing medium, cytotoxicity was measured using EL-4 T cell lymphoma cell line, RMA T cell lymphoma cell line or activated $CD4^+$ T cells as target cells, loaded or not with the OVA peptide. All these targets normally express CD48. Using a ^{51}Cr release assay, we found that wild-type T cells had marked cytotoxicity towards EL-4 cells in the presence of antigen, but not in the absence of antigen (Fig. 2.7B,C). This response was markedly compromised in CD2 KO T cells (Fig. 3B). However, no defect was observed in CD48 KO T cells (Fig. 2.7C). Similar results were obtained when cell surface exposure of CD107a, a marker of degranulation, was assessed (Fig. 2.7D,E).

2.4.8 CD48 on APCs is needed for CD2-dependent cytotoxicity

To assess if CD2 was engaged by CD48 on target cells to promote cytotoxicity, similar experiments were conducted with RMA cells, expressing or not CD48, as targets (Fig. 2.8A). Wild-type T cells displayed reduced cytotoxicity and diminished CD107a exposure in response to OVA peptide-loaded $CD48^{\square}$ RMA, compared to $CD48^+$ RMA (Fig.

2.8B,C; Fig. 2.8D,E). In contrast, CD2 KO T cells had similar defects towards $CD48^+$ and $CD48^-$ targets (Fig. 2.8B; Fig. 2.8D). While there was no difference in cytotoxicity mediated by wild-type or CD48 KO T cells towards $CD48^+$ RMA, as shown above, CD48 KO T cells had more severe defects in cytotoxicity towards $CD48^-$ RMA, compared to $CD48^+$ RMA (Fig. 2.8C; Fig. 2.8E). Similar results were obtained when $CD4^+$ T cells were used as APCs (Fig. 2.8F,G). In contrast to cytotoxicity, proliferation and cytokine production by antigen-primed cytotoxic $CD8^+$ T cells were not compromised by loss of CD48 on APCs (Fig. 2.8H).

Thus, unlike proliferation and cytokine production, CD2-dependent cytotoxicity required expression of CD48 on APCs. When APCs lacked CD48, expression of CD48 on T cells enabled a partial compensation of cytotoxicity.

2.4.9 CD2 and CD48 expressed on T cells are critical for graft-versus-host disease

To validate these findings *in vivo*, we studied graft-versus-host disease (GVHD), a T cell-dependent immunopathology in which donor T cells are activated by allogeneic MHC in the host, leading to T cell-dependent cytokine secretion and cytotoxicity towards host cells[361]. In brief, $CD8^+$ T cells and $CD4^+$ T cells (the latter to provide help) were purified from wild-type, CD2 KO or CD48 KO mice in the B6 background, and injected in previously irradiated wild-type Balb/c mice (Fig. 2.9A). Weight loss and survival were monitored.

While mice given phosphate-buffered saline (PBS) alone did not show weight loss or reduced survival, mice provided with wild-type T cells exhibited progressive weight loss (after ~day 5) and mortality (after ~day 9) (Fig. 2.9B). All mice given wild-type T cells died by day 16. In contrast, mice receiving CD2 KO T cells displayed less rapid weight loss and mortality. Approximately 40% of these mice survived. Furthermore, after an initial weight loss, several mice gained back weight. Compared to mice injected with wild-type T cells, mice inoculated with CD48 KO T cells also had less severe weight loss and mortality. Nevertheless, this effect was not as pronounced as that seen in mice receiving CD2 KO T

cells.

To examine if the reduced GVHD in CD2 KO or CD48 KO mice was due to compromised T cell activation, mice were sacrificed at day 7, prior to the onset of mortality (Fig. 2.9C). Donor T cells (identified by expression of H2-K^b) were enumerated in spleen and analyzed for expression of effector markers. Mice injected with PBS alone did not have donor T cells, as expected (Fig. 2.9D,E). However, all other mice did. Mice injected with CD2 KO T or CD48 KO T cells had lower proportions and numbers (reduced by ~50-70%) of donor T cells, compared to mice given wild-type T cells. This was true for $CD8^+$ and $CD4^+$ T cells. Nearly all donor T cells were $CD44^{hi}CD62L^{lo}$, indicating that they were activated (Fig. 2.9D).

To ensure that CD2 KO and CD48 KO T cells did not have a compromise in survival after transfer, we also sacrificed mice at day 3, prior to the onset of weight loss. Mice inoculated with wild-type, CD2 KO or CD48 KO T cells displayed no difference in the proportions or numbers of donor T cells (Fig. 2.9F).

Therefore, absence of CD2 or, to a lesser extent, CD48 on T cells resulted in reduced GVHD. This effect was not due to reduced T cell survival, but rather was due to compromised expansion of activated T cells. The intermediate defect observed with CD48 KO donor T cells, compared to CD2 KO T cells, was in keeping with a partial role of CD48 on APCs in cytotoxicity, which is involved in GVHD.

2.5 Discussion

TCR molecules recognize specific antigens to initiate T cell activation, with other signaling molecules assemble into microclusters, also known as supramolecular activation clusters (SMAC).

CD2 antigen, also known as LFA-2, is one of the earliest T cell marker molecules that have been identified[362]. In the early studies, CD2 was shown to be enriched in the central supramolecular activation cluster (c-SMAC)[363]. Recent studies have found that

CD2 is localized at the outer edge of the mature immunological synapse and forms a corolla-like structure[330]. Although the mechanism of these studies remains to be elucidated, the results suggest that CD2 may play a role in T-cell activation.

The function of CD2 in T-cell activation has been explored using a variety of approaches, including studies using anti-CD2 antibodies and mouse models of CD2 deficiency. One of these studies showed that mouse anti-CD2 antibodies induce a sustained decrease in mouse T-cell responses against anti-CD3 and SEB[344]. In another independent study, the results showed that anti-human CD2 mAb has a strong immunosuppressive effect in mixed lymphocyte reactions and was predicted for its potential efficacy in treating transplant rejection[364]. Conversely, some studies have stimulated T cells with agonistic anti-CD2 antibodies (Abs), resulting in significantly enhanced T cell activation[365]. Meanwhile, mouse genetics have been used to study the role of CD2 on T cells. Preliminary analysis of genetically engineered CD2-deficient mice of mixed genetic background indicates that these animals have normal T-cell responses[336]. However, a subsequent study showed, at low antigen densities but not at high antigen densities, CD2 has an essential role in T cell activation[366]. Another study found that T-cell responses to anti-CD3 antibodies or antigenic peptides were normal or only slightly affected in CD2 KO mice, although CD2 deficiency exacerbated the deficiency caused by CD28 deficiency[339]. These studies have provided conflicting evidence on the importance of CD2 in T cell activation.

CD2 recognizes its ligands CD48 (in mice) and CD58 (also known as LFA-3; in humans), two glycosylphosphatidylinositol (GPI)–linked receptors. CD48 is not only expressed on APCs but also abundantly expressed on the surface of T cells. It has been shown that CD48 KO mice have a severe impairment in the activation of $CD4^+$ T cells. Proliferative responses to mitogens, anti-CD3 mAb and alloantigens are reduced[367]. Another study suggested that GPI-linked CD48 contributes to early TCR signaling by recruiting intracellular adaptors through lipid rafts[368]. However, it is difficult to imagine

that CD48 has such a function because this GPI-molecule has no intracellular domain.

By creating new KO mouse strains in a pure B6 genetic background and using blocking anti-CD2 Abs, we found that CD2 was critical for T cell activation in response to a variety of stimuli, including antigen, superantigen, alloantigen, mitogenic lectins, and anti-TCR-CD3 Abs without or with anti-CD28 Abs. In contrast, CD2 was not needed for activation by PMA plus ionomycin. An identical role was ascribed to T cell-expressed CD48, the sole ligand of CD2 in mice. Since the activation defects observed in CD2 KO or CD48 KO T cells occurred in cells stimulated with anti-TCR Abs without APCs, these data implied that CD2 and CD48 were interacting with each other on T cells, and that these interactions were needed for T cell activation.

Our data indicated that the interactions between CD2 and CD48 on T cells occurred within individual T cells (*cis*^{T} interactions). In support of this idea, we found that CD2 and CD48 were co-localized at the surface of individual T cells, in immunofluorescence assays combined with confocal microscopy. Moreover, CD2 and CD48 were co-localized at the surface of transfected HEK293T cells in FRET assays. The latter proximity was compromised by addition of a blocking anti-CD2 Ab, which was also found to prevent activation when provided to wild-type T cells. Lastly, experiments in which CD2 KO T cells and CD48 T cells were mixed indicated that the presence on adjacent cells of the missing component of the receptor-ligand pair could not rescue TCR-triggered calcium fluxes in mutant T cells. We postulate that these *cis* interactions occur head-to-head and may be facilitated by membrane curvatures or membrane microvilli, as suggested for *cis* interactions involving other molecules[369].

Even though our results provided solid evidence that CD2-CD48 *cis* interactions took place in individual T cells, it should be pointed out that they did not exclude the possibility that CD2 and CD48 may also interact between adjacent T cells (*cis*$^{T\text{-}T}$ interactions). This may be especially prominent when T cells are juxtaposed, for instance as a consequence of T cell adhesion to APCs or as a result of T cell homotypic clustering

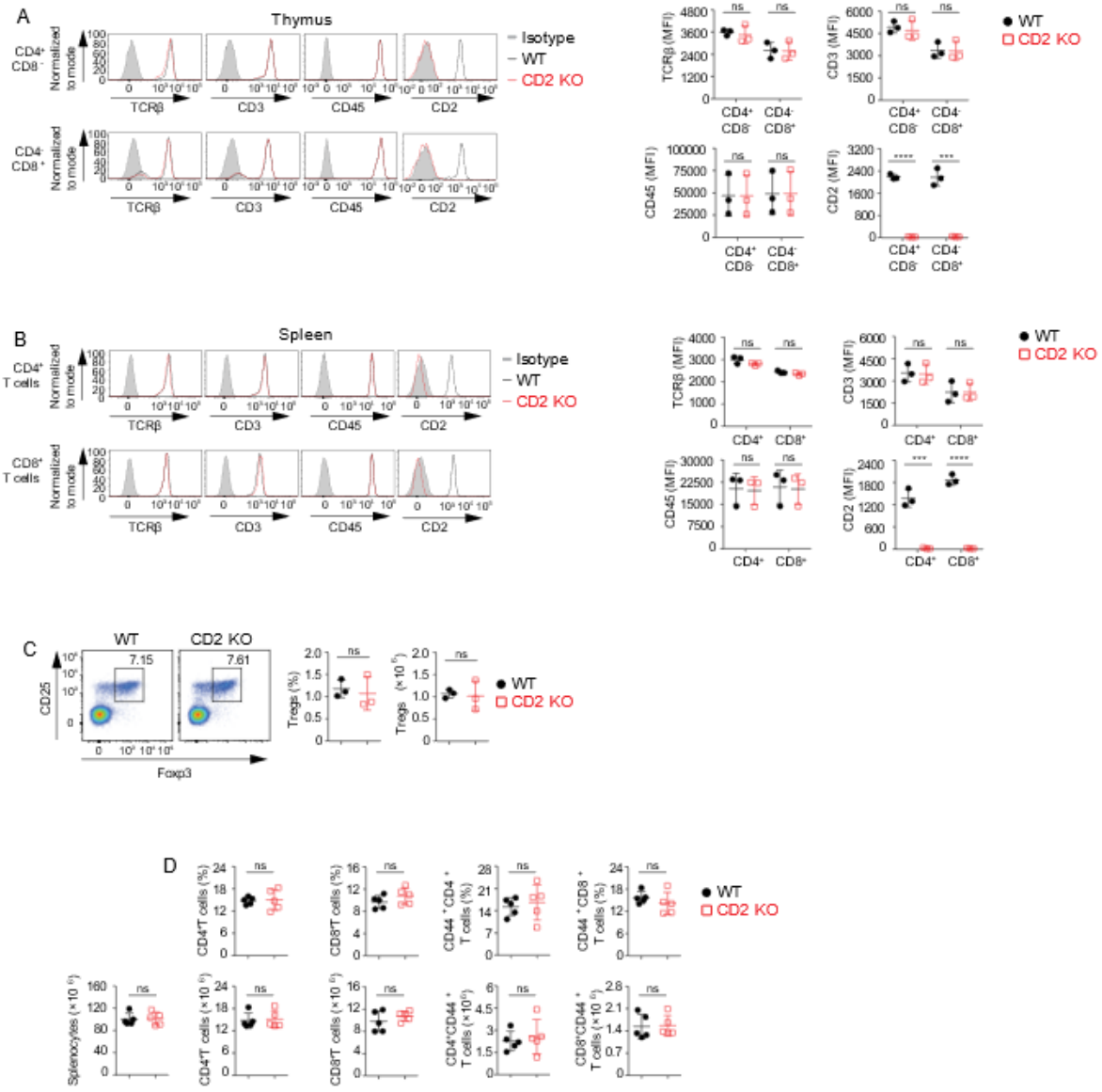
during proliferation. These possibilities deserve future consideration.

When T cells were activated by antigens and APCs, the presence of CD48 on APCs was not needed for proliferation and cytokine production. This was true in freshly isolated T cells and previously activated T cells. However, expression of CD48 on APCs, as well as on T cells, was needed for $CD8^+$ T cell-mediated cytotoxicity. This dual involvement of CD48 was in keeping with our observation that GVHD, a T cell-dependent model of immunopathology involving cytotoxicity, was more compromised in CD2 KO mice compared to CD48 KO mice. We postulate that CD2-CD48 interactions in *trans* are also required for cytotoxicity, because of the need of *trans* interactions to facilitate the release of cytotoxic granules towards target cells. Evidence of a key role of engagement of CD2 on human T cells by CD58 on APCs was provided by others for polarization of cytotoxic granules toward the microtubule-organizing center[352].

2.6 Author contributions

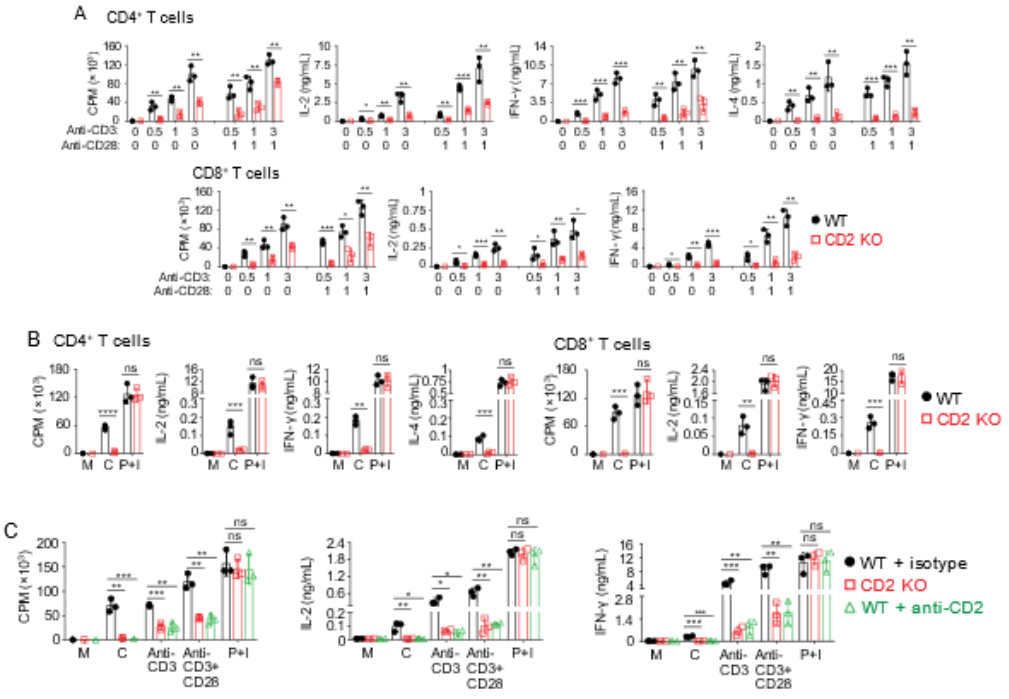
The following author contributions are noted: B.L., R.R. and A.V. conceptualized the study. Y.L., J.Q., R.L., D.D., Z.T. and K.Z. provided additional materials and resources. M.-C.Z., R.L., D.D. and Z.T. assisted with the methodology of the study. B.L, J.A., A.G.P., R.R. and A.V. conducted the investigations and assisted with interpretation of results. B.L., J.Q., R.R. and A.V. contributed to data visualization and figure generation. B.L., R.R. and A.V. wrote the original manuscript. B.L. and A.V. contributed to revisions and editing. B.M., R.R. and A.V. directed the study, provided supervision, and obtained funding.

2.9 Figures and figure legends



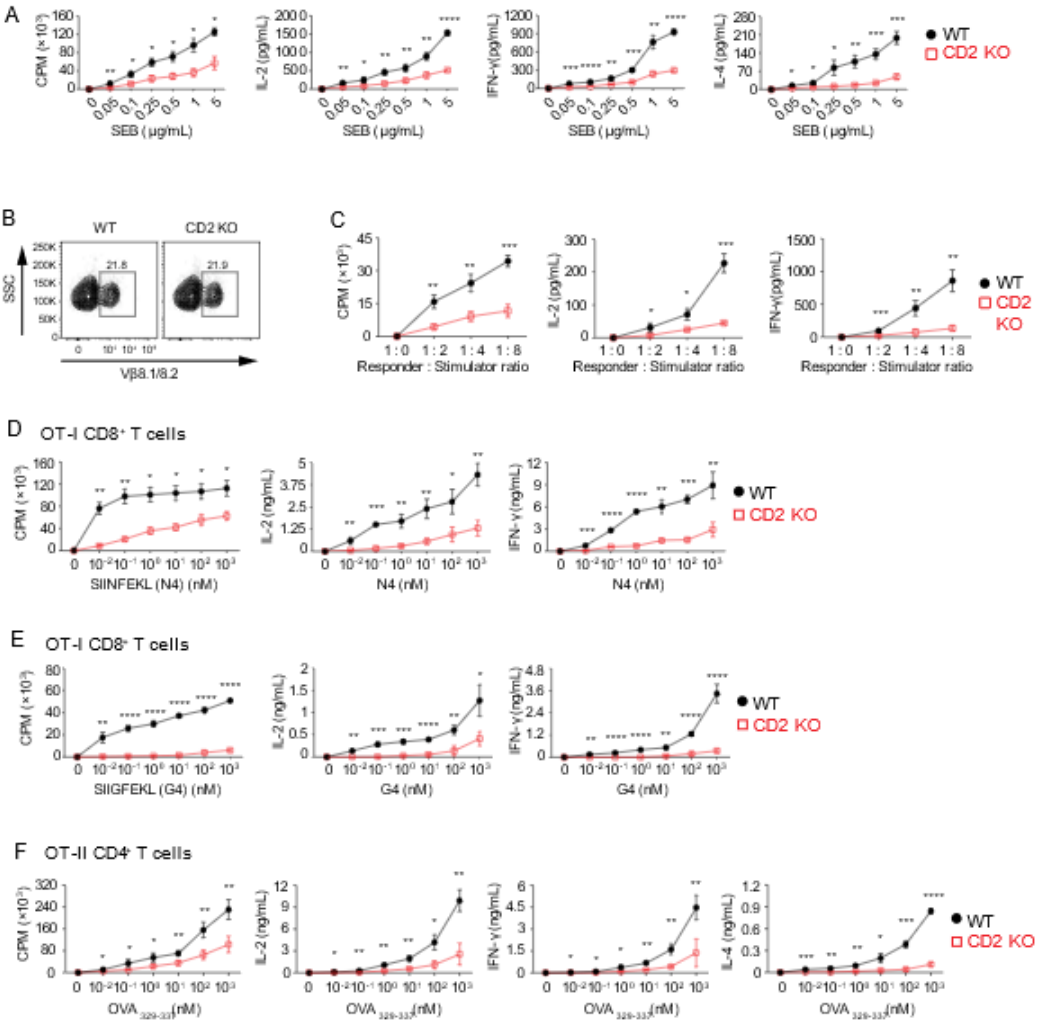
1

Fig 2.1 CD2 KO mice have normal expression of T cell markers and normal T cells subset. **(A** and **B)** The expression of cell markers (red lines) on $CD4^+CD8^-$ and $CD4^-CD8^+$ cells (A), or splenic $CD4^+$ and $CD8^+$ T cells (B), from WT and CD2 KO mice. Filled histograms, isotype control. **(C)** The numbers and frequencies of splenic regulatory T cells (Tregs) from WT and CD2 KO mice. Tregs were identified as $CD3^+CD4^+CD25^+Foxp3^+$ cells. The left pseudocolor blots depicts the percentage of Tregs. The percentage and number of Tregs in total splenocytes are summarized on the right. *n*=3. **(D)** Total splenocyte counts, and proportions of $CD4^+$ and $CD8^+$ splenic T cells, from WT and CD2 KO mice were determined. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).


A
CD4+ T cells
CD8+ T cells
CPM (×10³)
IL-2 (ng/mL)
IFN-γ (ng/mL)
IL-4 (ng/mL)
Anti-CD3:
Anti-CD28:
WT
CD2 KO
B
CD4+ T cells
CD8+ T cells
M
C
P+I
C
Anti-CD3
Anti-CD3+CD28
WT + isotype
CD2 KO
WT + anti-CD2

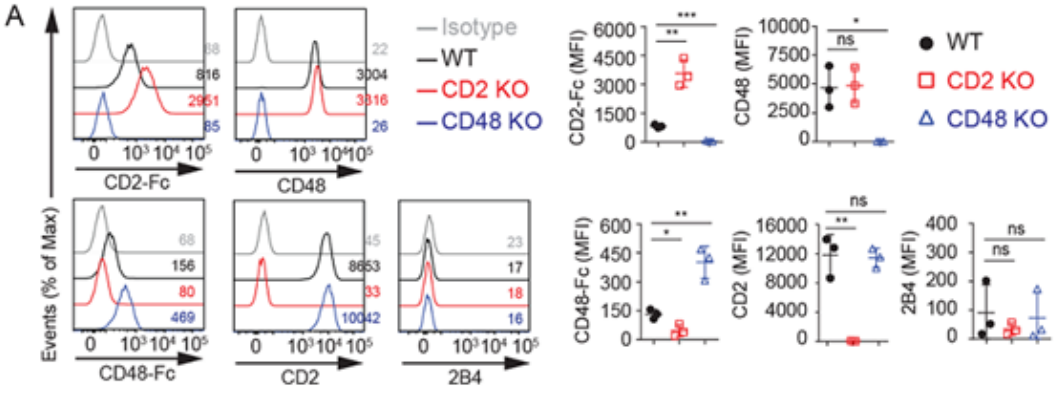
2

Fig 2.2 Loss of CD2 in T cells broadly compromises APC-independent T cell activation. **(A)** $CD4^+$ splenic T cells (depleted of iNKT cells) or $CD8^+$ splenic T cells (10^6 cells ml^{-1}) from WT or CD2 KO mice were stimulated for 48 hours, proliferation was assessed by measuring thymidine incorporation, while cytokine production was determined by ELISA. IL, interleukin; IFN, interferon. (**B)** Same as (A), except that cells were stimulated with concanavalin A (Con A, "C"; 4 μg ml^{-1}), or PMA (100 ng ml^{-1}) plus ionomycin (1μg ml^{-1}) (P+I). M, culture medium alone. (**C)** Anti-CD2 RM2-5 (anti-CD2) or its isotype-matched rat IgG2b, λ (isotype) coated on plastic, freshly isolated $CD8^+$ splenic T cells (10^6 cells ml^{-1}) from WT or CD2 KO mice were stimulated with the indicated conditions of Con A ("C", 4 μg ml^{-1}), anti-CD3 Abs (1 μg ml^{-1}) without or with anti-CD28 Abs (1 μg ml^{-1}), or PMA (100 ng ml^{-1}) plus ionomycin (1μg ml^{-1}) (P+I). M, culture medium alone. Each symbol in (A) and (B) represents an individual mouse; results are pooled from three independent experiments with a total of 3 mice in (A, B, C). Error bars represent means with s.d. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Students t-tests).


A
CPM (×10³)
IL-2 (pg/mL)
IFN-γ(pg/mL)
IL-4 (pg/mL)
SEB (μg/mL)
WT
CD2 KO
B
WT
CD2 KO
SSC
21.8
21.9
Vβ8.1/8.2
C
CPM (×10³)
IL-2 (pg/mL)
IFN-γ(pg/mL)
Responder : Stimulator ratio
WT
CD2 KO
D
OT-I CD8⁺ T cells
CPM (×10³)
IL-2 (ng/mL)
IFN-γ(ng/mL)
SIINFEKL (N4) (nM)
N4 (nM)
WT
CD2 KO
E
OT-I CD8⁺ T cells
CPM (×10³)
IL-2 (ng/mL)
IFN-γ(ng/mL)
SIIGFEKL (G4) (nM)
G4 (nM)
WT
CD2 KO
F
OT-II CD4⁺ T cells
CPM (×10³)
IL-2 (ng/mL)
IFN-γ(ng/mL)
IL-4 (ng/mL)
OVA 329-337 (nM)
WT
CD2 KO

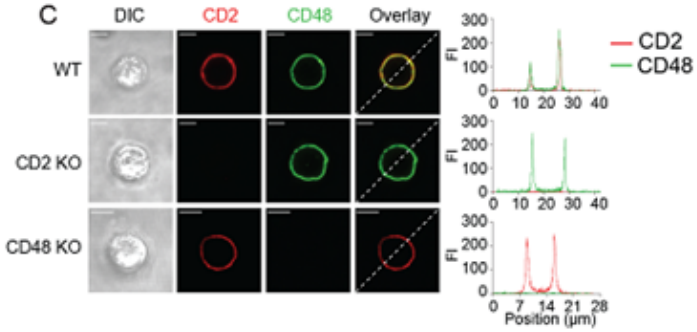
3

Fig 2.3 Loss of CD2 in T cells broadly compromises T cell activation. (A) Freshly isolated $CD4^+$ splenic T cells from WT and CD2 KO mice were stimulated with the superantigen staphylococcus enterotoxin B (SEB, 0.05, 0.1, 0.25, 0.5, 1 and 5 μg ml^{-1}) and irradiated splenocytes as APCs. Proliferation and cytokine production were monitored as in Fig. 2.2. **(B)** Flow cytometry analyses showing the proportions of the Vβ8.1/Vβ8.2^+ subsets in splenic $CD4^+$ T cells. One experiment representative of 3 experiments is shown. **(C)** Freshly isolated $CD8^+$ splenic T cells from WT or CD2 KO mice (B6 background) were stimulated with irradiated Balb/c splenocytes at the indicated responder : stimulator cell ratios. Proliferation and cytokine production were monitored as in Fig. 2.2. Responder, freshly isolated $CD8^+$ splenic T cells from WT or CD2 KO mice; stimulator, irradiated Balb/c splenocytes. Results are pooled from three independent experiments in (A, C). **(D** and **E)** Freshly isolated $CD8^+$ splenic T cells (10^6 cells ml^{-1}) from WT or CD2 KO mice bred with class I MHC-restricted TCR transgenic (Tg) mouse OT-I were activated with the indicated concentrations of high-affinity agonistic ovalbumin (OVA) amino-acids 257-264 peptide N4 (SII<u>N</u>FEKL) (A), or low-affinity agonistic OVA peptide G4 (SII<u>G</u>FEKL) (B) plus irradiated C57BL/6 splenocytes as APCs. After 24 hours, activation was monitored as detailed in (A). (**F)** Freshly isolated $CD4^+$ splenic T cells from WT or CD2 KO mice bred with OT-II mice were activated with $OVA_{329-337}$ plus irradiated C57BL/6 splenocytes as APCs. Activation was monitored as detailed in (A). Error bars represent means with s.d. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Students t-tests).



B

	WT	CD2 KO	CD48 KO
CD2-Fc / CD48	1.0	4.2	NA
CD48-Fc / CD2	1.0	NA	3.1



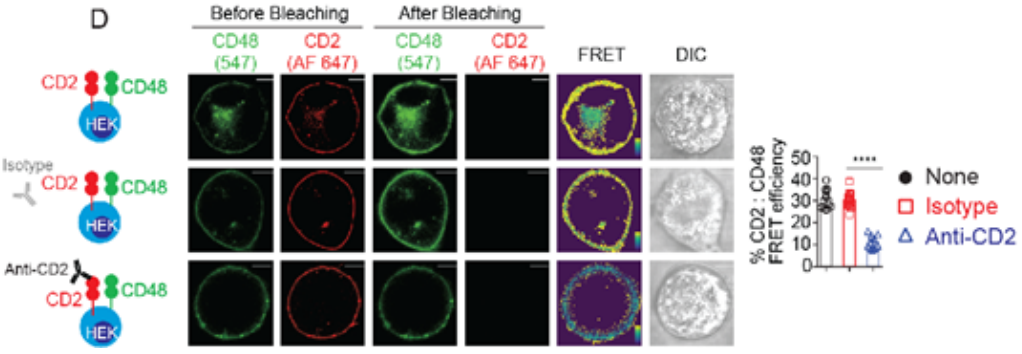
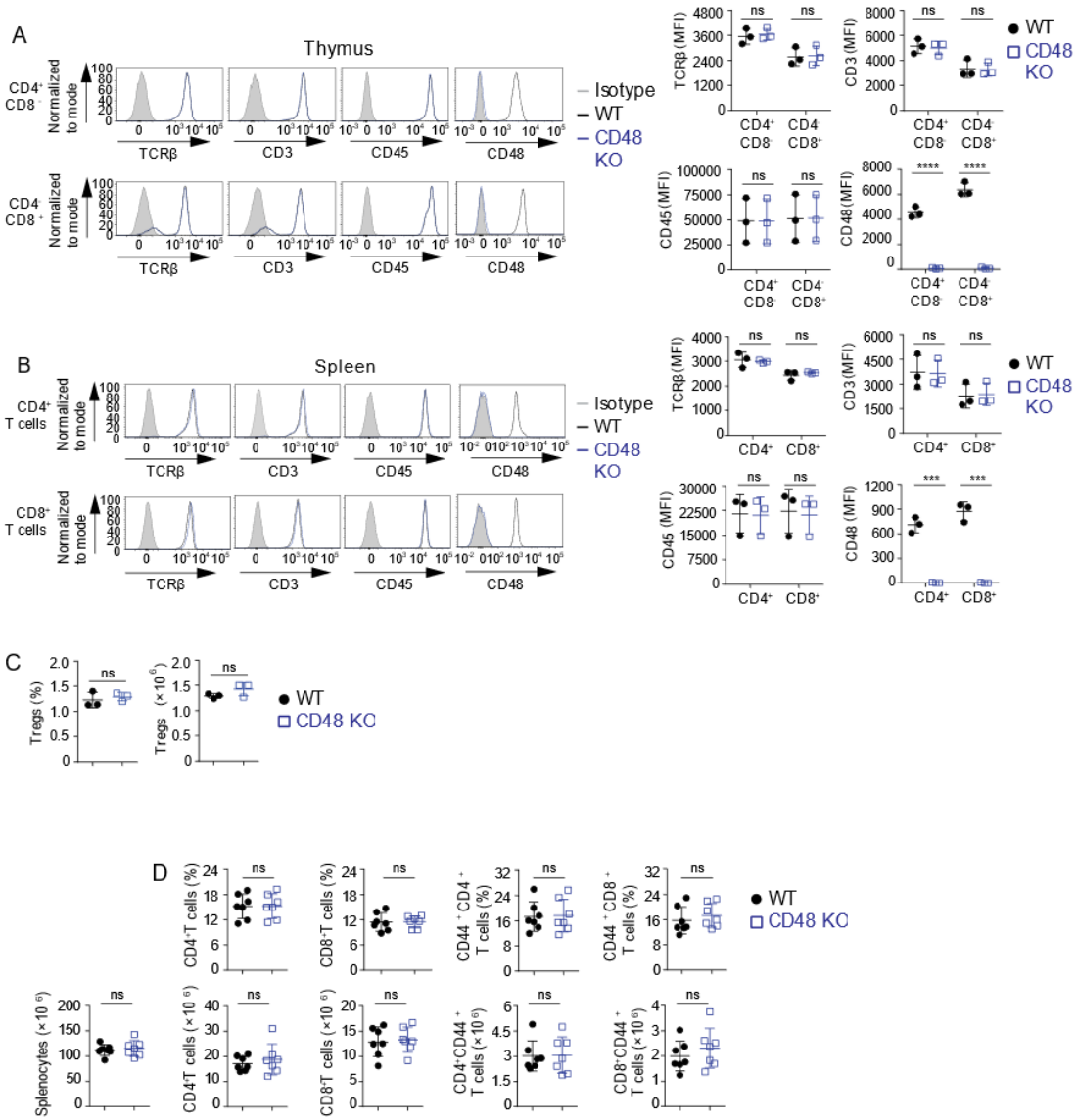
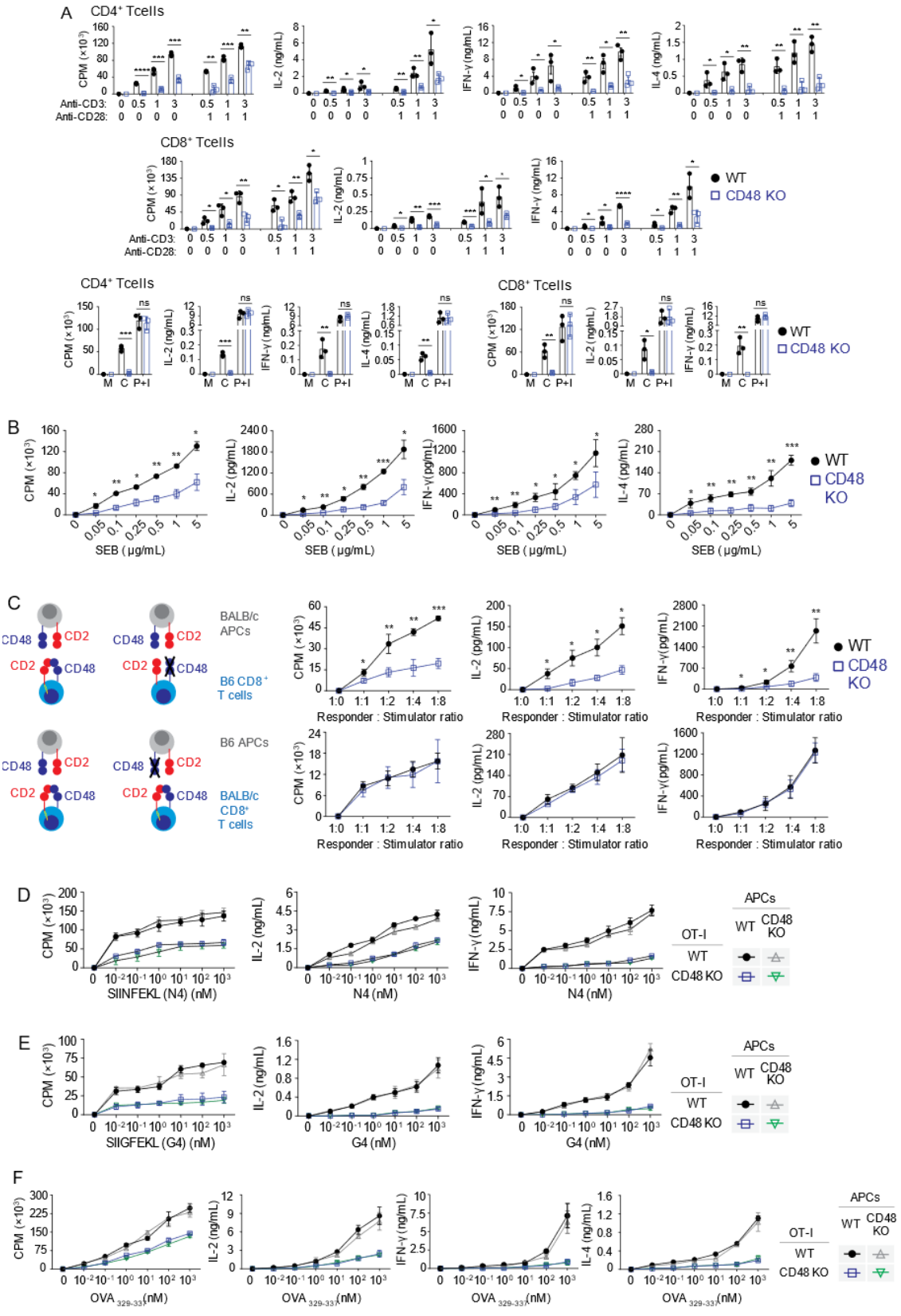


Fig 2.4 CD2 interacts with its ligand CD48 in cis to promote T cell activation. **(A)** Freshly isolated OT-II $CD4^+$ splenic T from WT, CD2 KO, CD48 KO mice were activated with peptide + APCs, and stained with Fc portion of CD2 or CD48, followed by a secondary antibody directed against the Fc segment of the fusion proteins. Expression of CD2, CD48 and 2B4 was analyzed in parallel by staining cells with Abs directed against these molecules. Controls were WT cells stained with isotype control IgG (isotype). Numbers in histograms represent mean fluorescence intensity (MFI). **(B)** Relative ratios of mean fluorescence intensity (MFI) for staining with Fc fusion proteins over staining with Abs against the indicated molecules on CD2 KO or CD48 KO $CD4^+$ T cells, compared with WT $CD4^+$ T cells. Values were set as 1.0 for WT cells. NA, not applicable. **(C)** $CD4^+$ splenic T cells were stimulated with indicated antibody. Fixed cells were then stained with fluorescently-labeled anti-CD2 (red) and anti-CD48 (green) Abs. Cells were analyzed by laser-scanning confocal microscope. Representative photographs are shown on the left, whereas fluorescence intensity distribution along the solid line in the overlaid image is depicted on the right. Scale bars, 5 μm. **(D)** A FRET assay probing CD2 and its ligand CD48 on HEK-293T cells. Scale bars, 5 μm. Error bars represent means with s.d. $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).


A
Thymus
B
Spleen
Isotype
WT
CD48 KO
TCRβ
CD3
CD45
CD48
CD4+ T cells
CD8+ T cells
Normalized to mode
TCRβ (MFI)
CD3 (MFI)
CD45 (MFI)
CD48 (MFI)
ns
****
***
C
Tregs (%)
Tregs (×10^6)
D
CD4+T cells (%)
CD8+T cells (%)
CD44+CD4+ T cells (%)
CD44+CD8+ T cells (%)
Splenocytes (×10^6)
CD4T cells (×10^6)
CD8T cells (×10^6)
CD4+CD44+ T cells (×10^6)
CD8+CD44+ T cells (×10^6)

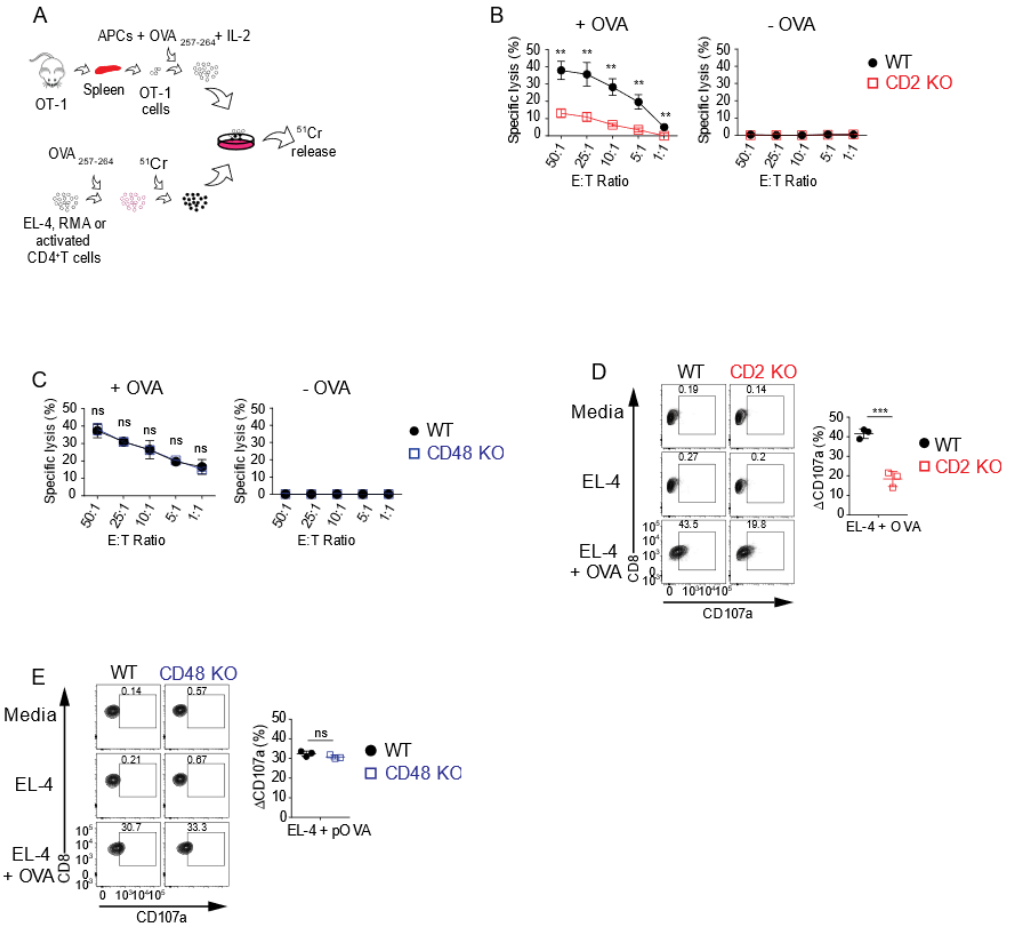
5

Fig 2.5 CD48 KO mice have normal T cells. (A and **B)** Same as Fig. 2.1(A and B), except that WT or CD48 KO thymic $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were analyzed in (A), WT or CD48 KO splenic $CD4^+$ and $CD8^+$ T cells were analyzed in (B). **(C)** Same as Fig. 2.1(C), except that WT or CD48 KO Tregs were analyzed. **(D)** Same as Fig. 2.1(D), except that WT or CD48 KO splenic $CD4^+$ and $CD8^+$ T cells were analyzed. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).


A
CD4⁺ Tcells
CD8⁺ Tcells
CPM (×10³)
IL-2 (ng/mL)
IFN-γ (ng/mL)
IL-4 (ng/mL)
Anti-CD3:
Anti-CD28:
WT
CD48 KO
M C P+I
ns
B
SEB (μg/mL)
IL-2 (pg/mL)
IFN-γ(pg/mL)
IL-4 (pg/mL)
C
CD48
CD2
BALB/c APCs
B6 CD8⁺ T cells
B6 APCs
BALB/c CD8⁺ T cells
Responder : Stimulator ratio
D
SIINFEKL (N4) (nM)
N4 (nM)
APCs
OT-I
E
SIIGFEKL (G4) (nM)
G4 (nM)
F
OVA 329-337 (nM)

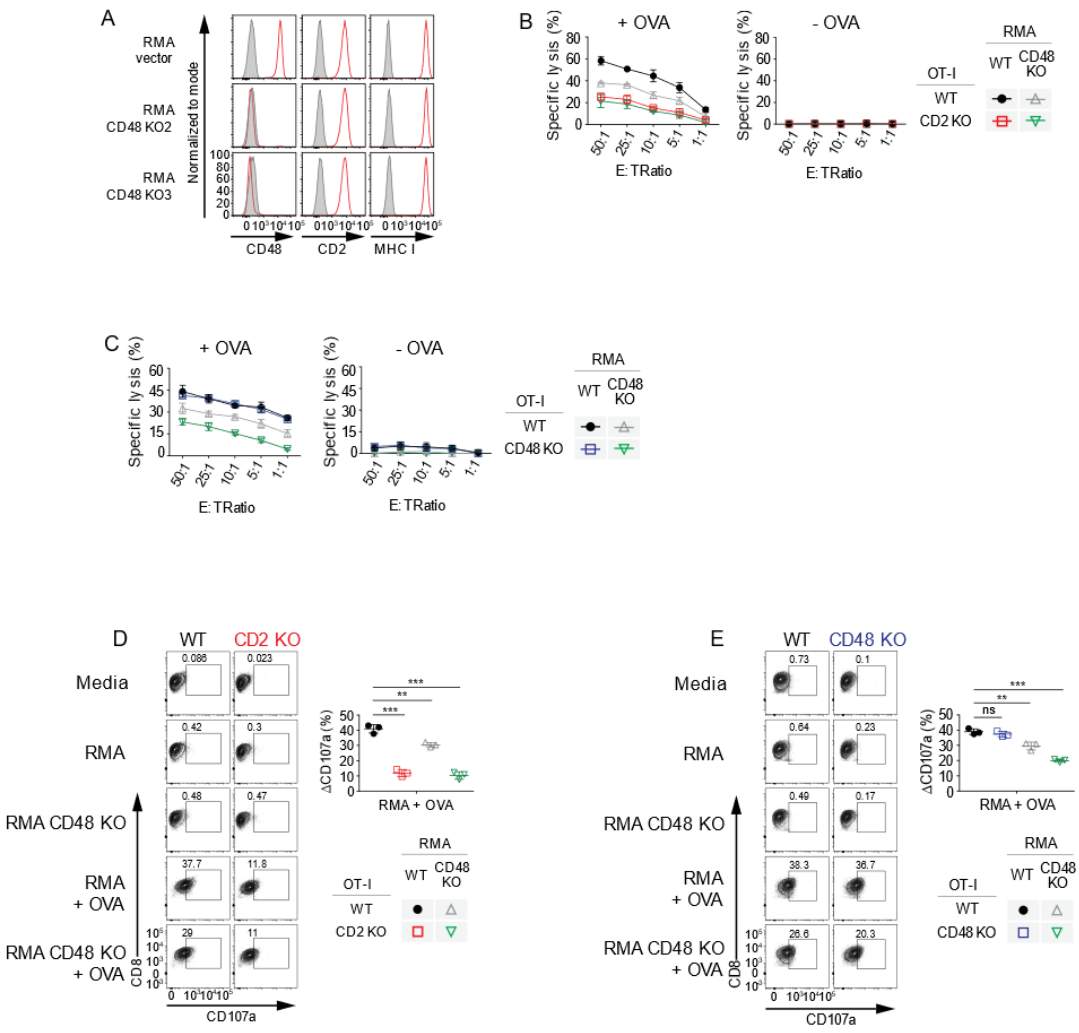
6

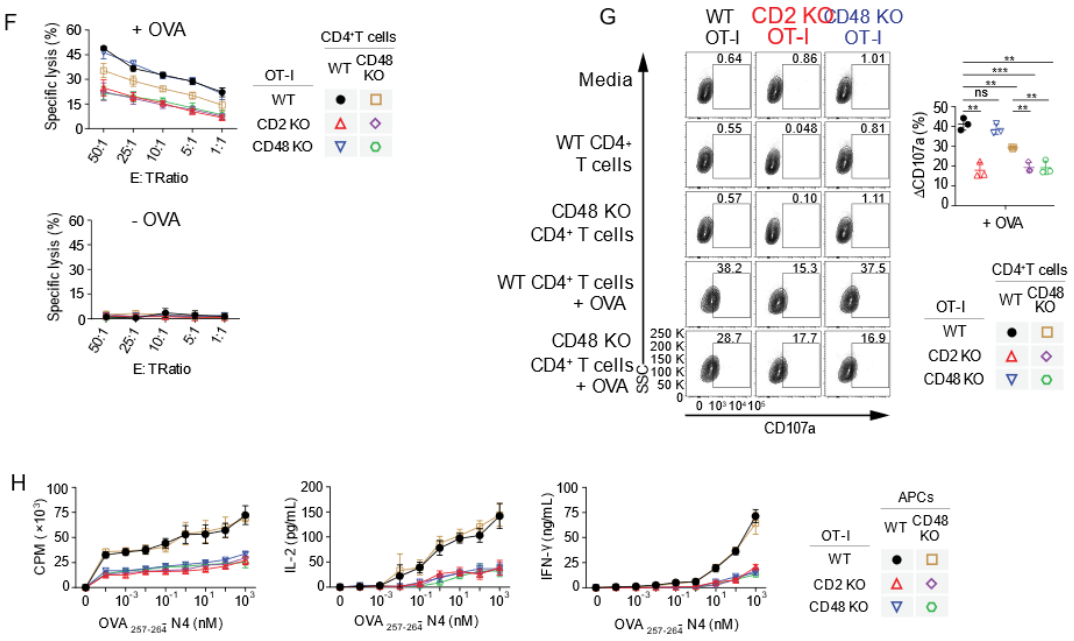
Fig 2.6 CD48 KO mice exhibit reduced T cell responses. (A) Same as Fig. 2.2(A and B), except that WT or CD48 KO splenic $CD4^+$ T cells and $CD8^+$ T cells were analyzed. **(B)** Same as Fig. 2.3 (A), except that WT or CD48 KO splenic $CD4^+$ T cells were analyzed. **(C)** The leftmost cartoon depicts the different set of stimulation. At the top, freshly isolated $CD8^+$ splenic T cells from WT or CD48 KO mice (B6 background) were stimulated with irradiated Balb/c splenocytes at the indicated responder : stimulator cell ratios. Proliferation and cytokine production were monitored as in Fig. 2.2(A). Responder, freshly isolated $CD8^+$ splenic T cells from WT or CD48 KO mice; stimulator, irradiated Balb/c splenocytes. At the bottom, freshly isolated $CD8^+$ splenic T cells from Balb/c mice were stimulated with irradiated WT or CD48 KO mice (B6 background) splenocytes at the indicated responder : stimulator cell ratios. Proliferation and cytokine production were monitored as in Fig. 2.2(A). Responder, freshly isolated $CD8^+$ splenic T cells from Balb/c mice; stimulator, irradiated WT or CD48 KO mice splenocytes. Results are pooled from three independent experiments in (A, B, C). **(D** and **E)** Same as Fig. 2.3(D and E), except that freshly isolated WT or CD48 KO OT-I splenic T cells stimulated with peptide-loaded splenocytes from WT or CD48 KO mice were analyzed. (F) Same as Fig. 2.3(F), except that WT or CD48 KO OT-II T cells stimulated with peptide-loaded splenocytes from WT or CD48 KO mice were analyzed. $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).


A
APCs + OVA 257-264 + IL-2
OT-1
Spleen
OT-1 cells
51Cr release
OVA 257-264
51Cr
EL-4, RMA or activated CD4+T cells
B
+ OVA
- OVA
Specific lysis (%)
E:T Ratio
WT
CD2 KO
C
+ OVA
- OVA
ns
WT
CD48 KO
D
WT
CD2 KO
Media
EL-4
EL-4 + OVA
CD8
CD107a
ΔCD107a (%)
EL-4 + OVA
E
WT
CD48 KO
Media
EL-4
EL-4 + OVA
CD8
CD107a
ΔCD107a (%)
EL-4 + pOVA

7

Fig 2.7 CD48 on APCs is needed for CD2-dependent cytotoxicity. **(A)** A depiction of the protocol used for Figure 2.7 is shown. Freshly isolated OT-I $CD8^+$ T cells from mice were primed with antigen + APCs and propagated in IL-2 (50 U ml^{-1}). Then, they were tested for cytotoxicity against EL-4, RMA or activated $CD4^+$ T cells as targets, loaded or not with peptide. **(B)** Previously activated WT or CD2 KO OT-I $CD8^+$ T cells were stimulated with EL-4, in the presence or the absence of OVA peptide N4 (1 µM). **(C)** Same as (B), except that previously activated WT or CD48 KO OT-I $CD8^+$ splenic T were analyzed. **(D)** Freshly isolated $CD8^+$ splenic T cells from WT or CD2 KO OT-I mice were first activated in vitro with OVA peptide (20 µM) and APCs. They were then incubated for 4 hours with medium alone, or EL-4 cells pulsed or not with OVA peptide (1 µM). CD107a represents the degranulation. $CD8^+$ OT-I T cells were identified by gating on $CD8^+$ cells. **(E)** Same as (D), except that WT or CD48 KO OT-I T cells were analyzed. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).


A
RMA vector
RMA CD48 KO2
RMA CD48 KO3
Normalized to mode
CD48
CD2
MHC I
B
+ OVA
- OVA
Specific lysis (%)
E:TRatio
OT-I
WT
CD2 KO
RMA
WT
CD48 KO
C
+ OVA
- OVA
Specific lysis (%)
E:TRatio
OT-I
WT
CD48 KO
RMA
WT
CD48 KO
D
WT
CD2 KO
Media
RMA
RMA CD48 KO
RMA + OVA
RMA CD48 KO + OVA
CD8
CD107a
ΔCD107a (%)
RMA + OVA
OT-I
WT
CD2 KO
RMA
WT
CD48 KO
E
WT
CD48 KO
Media
RMA
RMA CD48 KO
RMA + OVA
RMA CD48 KO + OVA
CD8
CD107a
ΔCD107a (%)
RMA + OVA
ns
OT-I
WT
CD48 KO
RMA
WT
CD48 KO



9

8

Fig 2.8 CD48 on APCs is required for maximal cytotoxicity. (A) The expression of CD48, CD2, and class I MHC (MHC I; H-2K^{b}) on scrambled control or CD48 KO RMA cells (red lines). Isotype control is shown by shaded histograms. **(B)** As in Fig. 2.7(B), except that previously activated WT or CD2 KO OT-I CD8^{+} splenic T cells were stimulated with RMA, with or without OVA peptide (1 µM). **(C)** Same as (B), except that previously activated WT or CD48 KO OT-I CD8^{+} splenic T were analyzed. **(D** and **E)** Same as Fig 2.7 (D, E), except that WT, CD2 KO and CD48 KO CD8^{+} OT-I T cells incubated with RMA cells were analyzed. **(F** and **G)** Same as (A and B), except that WT, CD2 KO or CD48 KO OT-I T cells activated with WT or CD48 KO CD4^{+} T cells were analyzed. **(H**) Same as Fig. 2.3(D), except that WT, CD2 KO or CD48 KO OT-I T cells activated with previously activated WT or CD48 KO CD4^{+} T cells were analyzed. Error bars represent means with s.d. in (B, C, D, E, F, G, H). *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).

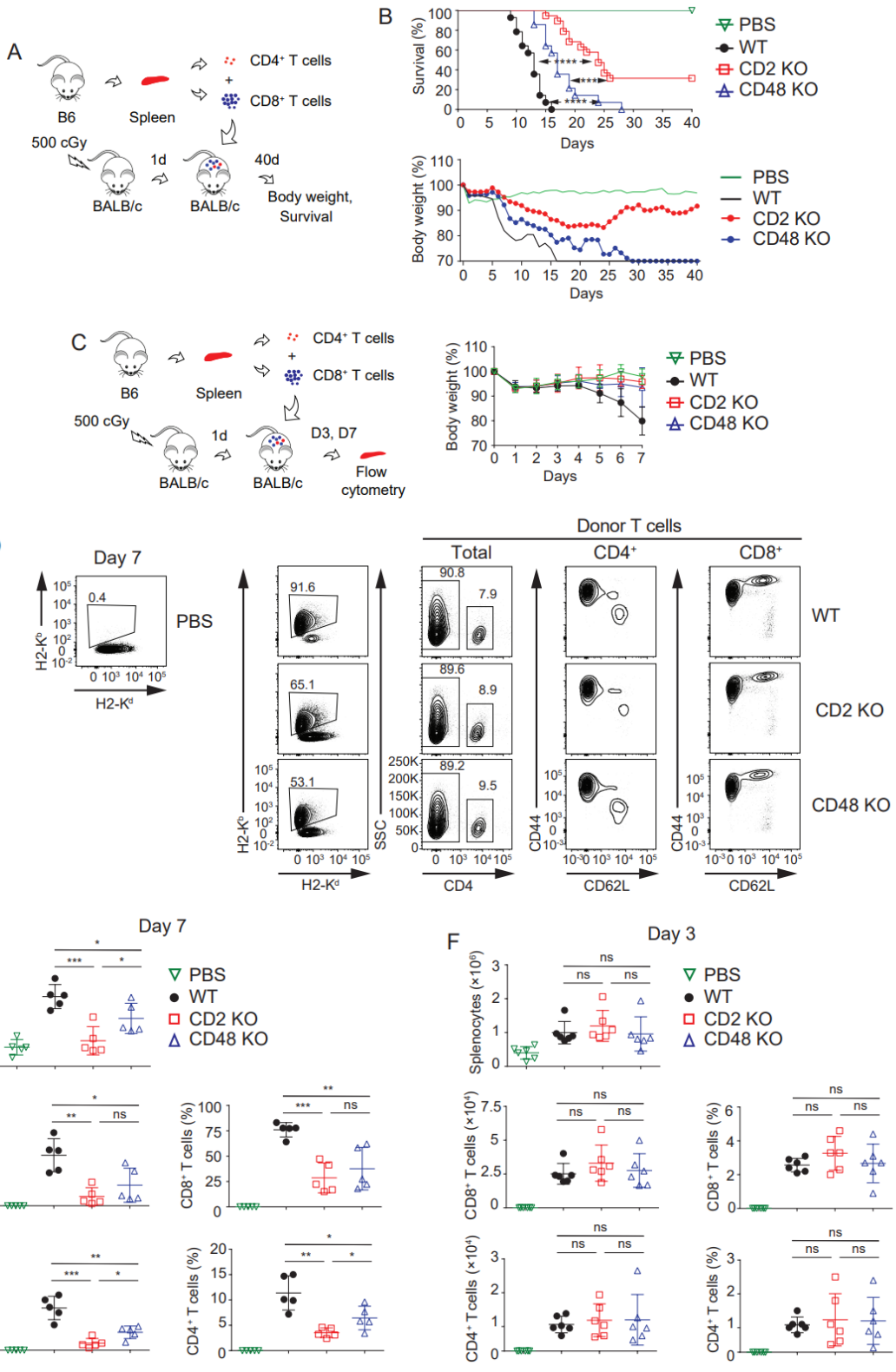

A
B6
Spleen
CD4+ T cells
+
CD8+ T cells
500 cGy
1d
40d
BALB/c
BALB/c
Body weight, Survival
B
Survival (%)
Days
PBS
WT
CD2 KO
CD48 KO
Body weight (%)
Days
C
B6
Spleen
CD4+ T cells
+
CD8+ T cells
500 cGy
1d
D3, D7
BALB/c
BALB/c
Flow cytometry
Body weight (%)
Days
D
Day 7
PBS
H2-Kb
H2-Kd
0.4
91.6
65.1
53.1
Donor T cells
Total
CD4+
CD8+
90.8
7.9
89.6
8.9
89.2
9.5
WT
CD2 KO
CD48 KO
SSC
CD4
CD44
CD62L
E
Day 7
Splenocytes (×10⁶)
CD8+ T cells (×10⁶)
CD8+ T cells (%)
CD4+ T cells (×10⁶)
CD4+ T cells (%)
PBS
WT
CD2 KO
CD48 KO
F
Day 3
Splenocytes (×10⁶)
CD8+ T cells (×10⁴)
CD8+ T cells (%)
CD4+ T cells (×10⁴)
CD4+ T cells (%)
ns

Fig 2.9 CD2 and CD48 expressed on T cells are critical for graft-versus-host disease. (**A)** Protocol of GVHD model. Freshly isolated $CD8^+$ and $CD4^+$ T cells (3:1) from WT, CD2 KO or CD48 KO mice (C57BL/6; B6) were injected in sub-lethally irradiated Balb/c mice (as recipient mice). Survival and body weight were monitored for 40 days. (**B)** Survival of recipients after transplantation of donor T cells from WT, CD2 KO or CD48 KO mice (B6). Control mice were injected with phosphatase-buffered saline (PBS) alone. *n* = 14 (WT donor T cells), 15 (CD2 KO donor T cells), 14 (CD48 KO donor T cells) and 6 (PBS). The means values of body weight of the surviving recipients are shown. **(C** to **F)** Same as (B), except that spleens of recipient mice were harvested on day 7 (D and E) or day 3 (F) after transplantation. (C), Protocol is depicted on the left, whereas body weight for the day 7-experiment is shown on the right. (D-F), Enumeration of donor T cells in spleens of recipient mice at day 7 (D, E) or day 3 (F) post-transplantation. Splenocytes were stained with anti-class I MHC H-$2K^b$ (B6-specific), anti-class I MHC H-$2K^d$; Balb/c-specific), anti-CD4, anti-CD62L and anti-CD44. Donor $CD4^+$ T cells were identified as $CD4^+$H-$2K^{b+}$, while donor $CD8^+$ T cells were identified as $CD4^-$H-$2K^{b+}$. Representative flow cytometry analyses at day 7 are shown in (D). Specific populations with percentages are boxed. Data for cell numbers and percentages of multiple mice are represented in (E and F). *n*=5 (WT donor T cells), 5 (CD2 KO donor T cells), 5 (CD48 KO donor T cells) and 5 (PBS) in (E). *n*=6 (WT donor T cells), 6 (CD2 KO donor T cells), 6 (CD48 KO donor T cells) and 6 (PBS) in (F). Results are pooled from three independent experiments. Each symbol represents a mouse in (E, F). Error bars represent means with s.d. For survival analyses, a log-rank (Mantel-Cox) test was performed. Otherwise, two-tailed unpaired Student's t test were used. $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).

Chapter 3 Ability of CD2 to promote T cell activation correlates with binding to kinase Lck

3.1 Summary

While CD2 and its ligands are critical for T cell activation, the scientific mechanism of CD2 controls TCR signalling needs to be elucidated. We confirmed that the activating function of CD2 requires its cytoplasmic domain, in particular, two of its proline-rich motifs. We also demonstrated that the proline-rich motifs recruit of kinase Lck to enhance the TCR signaling. We also found cis interactions of CD2 and CD58 in human Jurkat cells., this cis interaction can promote human T cell activation. CD2-CD58 provided a similar function in human T cells.

3.2 Introduction

The molecules which contain the SH3 domain can bind to the proline-rich domain of the CD2 molecule. It has been reported that CD2-associated protein (CD2-AP)[370], lymphocyte-specific protein tyrosine kinase (Lck)[371], Fyn[372], CD2-binding protein 1 (CD2-BP1)[373], CD2-BP2[374], CD2-BP3, Cbl-interacting protein of 85 kDa (CIN85)[375] bind to the cytoplasmic domain of CD2.

Firstly, we examined whether CD2, CD48, or both were the signaling receptor(s) promoting T cell activation. By retrovirally transducing WT CD2 or a cytoplasmic domain-truncated variant (Dcyto) of CD2 in previously activated CD2 KO T cells, we found that WT CD2, but not Dcyto CD2, restores T cell responses. These data (Fig. 3.6) indicated that the activating function of CD2 requires its cytoplasmic domain, and implied that CD2, rather than CD48, is the primary signaling receptor. To elucidate how CD2-CD48 cis interactions enhance T cell activation, the impact of CD2 or CD48 deficiency on TCR signaling has been examined. Freshly isolated T cells ($CD4^+$ or $CD8^+$) from WT, CD2 KO, or CD48 KO mice has been stimulated with a range of concentrations of TCR Abs followed by a secondary cross-linking Ab. To identify effectors interacting with the CD2 cytoplasmic domain, we obtained the CD2-OST mice from our collaborators, Dr. R. Roncagalli and Dr. B. Malissen. The CD2-OST mouse is a knock-in mouse in which an OST tag is fused to the C-terminus of CD2. The

addition of the OST tag does not interfere with the expression of CD2 at the T cell surface or with T cell development. The genes coding for CD2 and CD48 are located on different chromosomes. T cells from CD2-OST mice, expressing or not CD48, have been stimulated or not for various times with TCR Abs, and CD2-associated proteins have been recovered using streptavidin beads, which recognize with high affinity the OST tag. WT or CD48 KO mice in which CD2 lacks the tag have been used as controls. Interacting proteins have been identified by mass spectrometry and analyzed using bioinformatics tools for protein-protein interactions. Potential associations have been confirmed in WT T cells, by immunoprecipitation with a CD2 Abs followed by immunoblotting with relevant Abs and by confocal microscopy. Then, the impact of genetic deficiency of the putative effectors of CD2 on T cell activation has been studied. The necessary KO mice were generated using CRISPR-Cas, or obtained from commercial sources or collaborators, as needed. We also examined which sequences in the CD2 cytoplasmic domain are needed to promote T cell activation during cis interactions and ascertain their impact on the associations with CD2 effectors. In its cytoplasmic domain, mouse CD2 possesses 5 proline-rich sequences. These motifs were proposed to mediate binding to Src homology 3 (SH3) domain-containing effectors, such as Lck, Itk, CD2-AP, and CD2-BP, although much confusion exists regarding the functional relevance of these interactions. Serial truncations of the cytoplasmic domain have been created and tested to identify other potential critical sequences. The ability of transduced T cells to proliferate and produce cytokines in response to TCR Abs has been assessed. Moreover, TCR-triggered signals have been evaluated. The impact of the mutations/truncations on the associations of CD2 with the partners uncovered above have been tested. We also examined the role of CD2 and its ligands in human Jurkat T cells. A similar function was documented for CD2-CD58 in human T cells. Therefore, *cis* interactions between CD2 and its ligands in T cells are required for TCR signaling and T cell activation.

3.3 Methods

3.3.1 Cells and retroviral infection

Thymocytes or splenocytes were harvested from the indicated mice. To lysis the red blood cells (RBCs) with RBC lysis buffer (Sigma-Aldrich, R7757) at room temperature for 5 min. $CD4^+$ or $CD8^+$ T cells, were purified by negative selection from spleen. EL-4 and RMA (T-cell lymphoma), and RMA lacking CD48, were described[376-378]. Jurkat (clone E6-1; T lymphoblast) was obtained from American Type Culture Collection (Manassas, Virginia, USA). Jurkat T cells deficient for CD2, CD48 or CD58 were generated by the CRISPR-Cas9 system using the plasmid pSpCas9(BB)-2A-GFP (Addgene, plasmid #48138) and the guide RNA sequences 5'- CACCGAAGCTGGCTACAAATTTACA -3' and 5'- CACCGCTTGGGTCAGGACATCAACT -3' (for CD2), 5'- CACCGTCACTTGGTACATATGACCG -3' and 5'- CACCGCTGGTCGAAAGTATAAAACC -3' (for CD48), 5'- CACCGTGGTTGCTGGGAGCGACGCG -3' and 5'- CACCGAGACCACGCTGAGGACCCCC -3' (for CD58). Plasmids were transfected into Jurkat cells by electroporation (Gene Pulser Xcell, Bio-Rad). Cells having lost the expression of targeted genes were sorted using the BD FACSAria III Cell Sorter (BD Biosciences). Lack of expression was confirmed by flow cytometry. Constructs encoding wild-type, cytoplasmic domain-deleted (Δcyto) or proline (P)-to-alanine (A) mutated (M1: P276A, P277A, P278A, P279A; M2: P286A, P290A, P292A, P293A; M3: P306A, P307A, P308A; M4: P319A, P320A, P322A, P324A; M5: P328A, P330A, P331A) versions of mouse CD2 were generated by PCR and cloned into the pMSCV-MIGR-GFP retroviral vector. Infectious retroviral particles were recovered from the supernatant of Phoenix-Eco packaging cells transfected with the indicated retroviral constructs. Freshly harvested supernatants were added to the T cells and cells were infected by "spinfection" (1000 × g for 60 min at 32°C). Cells infected with empty vector retroviruses were used as control. At day 4 post-infection, GFP-positive cells were sorted, expanded in IL-2 (50 U ml^{-1}) and then used for experimentation.

3.3.2 Biochemical studies

To study TCR-triggered protein tyrosine phosphorylation or Erk activation, 10×10^6 freshly isolated $CD4^+$ splenic T cells (in 100 µl PBS) were stimulated with biotinylated anti-TCRβ (5 µg ml^{-1}) and avidin (10 µg ml^{-1}). Cells were then lysed by the addition of a twice-concentrated lysis buffer (100mM Tris, pH 7.5, 300mM NaCl, 40mM EDTA, 2% n-dodecyl β-D-maltoside) supplemented with protease and phosphatase inhibitors [356, 379, 380]. After 15 min of incubation on ice, cell lysates were centrifuged at 14,000 rpm for 5 min at 4°C to remove the nuclei. Equivalent amounts of cellular proteins were then separated by 8% SDS-PAGE and transferred to PVDF membranes. After overnight incubation of the membranes with primary antibodies at 4°C, proteins were visualized using a horseradish peroxidase (HRP)-coupled secondary antibody and enhanced chemiluminescence (GE Health)[356]. Briefly, purified $CD4^+$ T cells or Jurkat cells (2.5×10^6cells per ml^{-1}) were loaded with the calcium indicator dye Indo-1 (Thermo Fisher) for 30 min at 37°C. After washing, they were stimulated at 37°C with biotinylated anti-mouse TCR MAb H57-597 (0.5 µg ml^{-1}) or anti-human TCR MAb IP26 (1 µg ml^{-1}), and avidin, Con A (4 µg ml^{-1}) or phytohemagglutinin (PHA; 10 µg ml^{-1}; Sigma-Aldrich). For the mixing experiments, CD2 KO T cell (CD45.1) and CD48 KO T cell (CD45.2) were labelled with anti-mouse CD45.1 and mixed at 1:1 ratio; mixed cells were then stimulated with Con A, anti-mouse TCR MAb H57-597 or ionomycin. Changes in intracellular calcium were monitored over time by a BD LSR Fortessa flow cytometer (BD Biosciences), using the UV450/UV530 fluorescence ratio. As control, cells were stimulated with the calcium ionophore ionomycin (1 µg ml^{-1}; Sigma-Aldrich).

3.4 Results

3.4.1 CD2 promotes T cell activation via proline-rich motifs in its cytoplasmic domain

Whereas CD48 is GPI-linked and lacks a cytoplasmic domain, CD2 is a transmembrane receptor bearing a cytoplasmic domain that has a conserved sequence across species (Fig. 3.1A). To determine if CD2 mediates the signals promoting T cell activation during *cis* interactions, structure-function analyses of CD2 were performed. Mutants carrying proline-to-alanine substitutions in 5 conserved proline-rich motifs in the CD2 cytoplasmic segment, or a deletion of most of the CD2 cytoplasmic domain, were engineered (Fig. 3.1A). Then, constructs were retrovirally transduced in CD2 KO T cells. All mutants, in addition to wild-type CD2, rescued expression of CD2 on CD2 KO T cells (Fig. 3.1B). Upon stimulation with anti-CD3 Abs, CD2 KO T cells expressing wild-type CD2, but not GFP alone, displayed a rescue of T cell activation responses, either proliferation or cytokine production (Fig. 3.1C). CD2 KO T cells expressing the CD2 variants having mutations in the first (P1), second (P2) or fifth (P5) proline-rich region were also rescued. In contrast, though, cells that contained the CD2 mutants lacking the cytoplasmic domain, or having mutations in the third (P3) or fourth (P4) proline-rich region, were not rescued.

Hence, the cytoplasmic domain of CD2, in particular two of its proline-rich motifs, was critical for the ability of CD2 to promote T cell activation.

3.4.2 Ability of CD2 to promote T cell activation correlates with binding to kinase Lck

To elucidate further the mechanism of CD2 signaling, we generated a gene-targeted knock-in mouse in which the CD2 protein was tagged at its carboxy-terminus with a One-STrEP-tag (OST) tag, which enables affinity purification with streptavidin[381, 382]. Heterozygous knock-in mice were used for our studies. Compared to wild-type mice, T cells from $CD2^{OST}$ mice displayed normal development and expression of CD2, with the exception that the variant CD2, but not endogenous CD2, was recognized by anti-OST Abs (Fig. 3.2A).

Then, previously activated $CD4^+$ T cells from $CD2^{OST}$ mice were left unstimulated, or stimulated for 1 or 5 minutes with anti-CD2 Abs in an attempt to enhance engagement

of CD2. After lysing cells in maltoside-containing buffer, OST-tagged CD2 was recovered with streptavidin beads, and associated proteins were detected and quantified by mass spectrometry. We identified 68 high-confidence CD2-interacting proteins that showed a greater than 3-fold enrichment, with a p value below 0.05 in at least 2 of the 3 conditions of stimulation. As expected, the CD2-CD48 interaction (p=0.00015, in unstimulated cells) was detected regardless of the stimulation condition. No other GPI-linked protein was detected using these stringency criteria. However, when the enrichment-fold was reduced to 2, GPI-linked Thy1 was also noted, although the statistical significance was lower (p=0.017) and was significant only in unstimulated cells. Among the high-confidence interactors, we also identified the kinase protein tyrosine kinase Lck, as well as the CD3ζ and CD3γ chains of the TCR complex (Fig. 3.2B). As with CD48, these interactions were seen whether cells were treated or not with anti-CD2 Abs. Previously described CD2-interacting proteins such as Fyn, Itk, CD2AP and CD2BP1 were not observed.

To examine if one or more of the proline-rich motifs of CD2 might recruit Lck, which bears a Src homology 3 (SH3) domain capable of binding proline-rich motifs, pull-down assays were performed. Biotinylated peptides corresponding to each of the 5 proline-rich sequences of CD2 were synthesized and coupled to streptavidin beads. They were incubated with T cell lysates, and the presence of Lck was detected by immunoblotting. The peptide encompassing the fourth proline-rich region (P4), but not the other peptides or beads alone, was able to recover Lck (Fig. 3.2C). No difference in expression of Lck was noted between wild-type, CD2 KO and CD48 KO T cells (Fig. 3.2D).

Therefore, CD2 interacted with several proteins, including Lck and components of the TCR complex. These interactions were observed in unstimulated cells, in keeping with the idea that CD2 and CD48 were interacting prior to TCR stimulation. We also found that one of the proline-rich regions of CD2 necessary to promote T cell activation, P4, bound to Lck *in vitro*.

3.4.3 *cis* interactions between CD2 and CD48 are required for TCR signaling

Engagement of the TCR triggers multiple signals initiated by the protein tyrosine kinases Lck, Fyn and ZAP-70[345-347]. To determine how CD2-CD48 *cis* interactions enabled proper T cell activation, TCR-mediated signals were analyzed. Engagement of the TCR by anti-TCR Abs on CD2 KO or CD48 KO T cells resulted in compromised protein tyrosine phosphorylation, compared to wild-type T cells (Fig. 3.3A). This change mostly affected substrates of 36, 76 and 120 kilodaltons (kDas). There was also a reduction in TCR-evoked activation of the kinase Erk and calcium fluxes, two proximal responses downstream of protein tyrosine phosphorylation, and in TCR-triggered expression of CD25, a more distal feature of T cell activation (Fig. 3.3B-D). Similar calcium flux and CD25 expression defects were seen with Con A. For all signals, the impacts of CD2 or CD48 deficiency were equivalent (Fig. 3.3A-D).

3.4.4 *cis* interactions between CD2 and CD48 are required for TCR signaling

To ascertain if interactions between CD2 and CD48 expressed on adjacent T cells ($cis^{T\text{-}T}$ interactions) could rescue the defects in TCR signaling seen in CD2 KO or CD48 KO T cells, these two cell populations were mixed 1:1 prior to cell activation. To distinguish the two cell populations, CD2 KO mice were first bred with B6.SJL mice, which express CD45.1 instead of CD45.2. Expression of CD45.1 was used to distinguish CD2 KO ($CD45.1^{+}$) and CD48 KO ($CD45.1^{-}$) T cells (Fig. 3.4A). When CD2 KO T cells and CD48 T cells were mixed, there was no correction of the defect in calcium fluxes triggered by anti-TCR Ab or Con A in either cell type (Fig. 3.4A).

Thus, the CD2-CD48 *cis* interactions were required for proximal TCR signals. This effect required co-expression of CD2 and CD48 on the same cell.

3.4.5 *cis* interactions of CD2 and CD58 promote human T cell activation

In humans, CD2 interacts with CD58, which, like mouse CD48, is GPI-linked and expressed not only on APCs, but also on T cells[349, 350]. Although human CD2 can interact with human CD48, this interaction was described to be of much lower affinity[332]. To ascertain if a function analogous to that CD2-CD48 in mouse T cells was provided by CD2-CD58 in human T cells, the impact of loss of CD2 or CD58 (or CD48, as control) was evaluated, using the human T cell line Jurkat, which normally expresses CD2, CD58 and CD48. Variants of Jurkat lacking CD2, CD58 or CD48 were generated by CRISPR-Cas, using two different guide RNAs for each molecule (Fig. 3.5A).

In binding assays using soluble Fc fusion proteins, CD2 KO cells failed to bind CD58-Fc, whereas CD58 KO cells did not react with CD2-Fc, when compared to control Jurkat cells (Fig. 3.5B; Fig. 3.5C). Additionally, as was the case for mouse cells, CD2 KO cells had augmented (~10-fold) binding to CD2-Fc, whereas CD58 KO cells showed increased (~3-fold) staining with CD58-Fc. Cells minimally bound to CD48-Fc, with the exception of CD48 KO cells, which displayed augmented staining.

From a functional point-of-view, CD2 KO and CD58 KO cells, but not CD48 KO cells, displayed markedly reduced protein tyrosine phosphorylation and calcium fluxes in response to anti-TCR Abs or the lectin phytohemagglutinin (PHA) (Fig. 3.5D, E). Additionally, CD2 KO and CD58 KO cells, but not CD48 KO cells, had a pronounced decrease in anti-CD3 Ab-induced production of IL-2, compared to control cells (Fig. 3.5F). No difference was seen with PMA plus ionomycin.

Hence, CD2 and CD58 formed an exclusive receptor-ligand pair in human T cells. Co-expression of CD2 and CD58, but not of CD48, was critical for TCR signaling and T cell activation.

3.5 Discussion

When the functional study of CD2 was very conflicting, attempts were switched to explore the role of the intracellular segment of CD2 for TCR signaling. The fact that we

were able to retrieve so many conflicting articles underscores the fact that this research really needs to be elucidated.

Our data showed that CD2 is the molecule that transmits signals to intracellular, not CD48 or CD58 (data not shown). In its cytoplasmic domain, mouse CD2 possesses 5 proline-rich sequences. These motifs were proposed to mediate binding to Src homology 3 (SH3) domain-containing effectors, such as Lck, Itk, CD2-AP and CD2-BP (Fig. 3.6), although much confusion exists regarding the functional relevance of these interactions.

It was reported that CD2-mediated downstream signaling is dependent on protein tyrosine kinases such as Lck and Fyn. For Lck, using truncated mutants of CD2, they identified two proline-rich regions of the CD2 cytoplasmic structure involved in binding to Lck[383]. The proline-rich sequences in the cytoplasmic structural domain of CD2 bind to the SH3 structural domain of Lck. However, they lack functional evidence in T cell activation. The only in vitro functional assay used PMA, which bypasses TCR signaling to activate T cells, so the question is which molecule does Lck act on after it is recruited to the intracellular domain of CD2, and the same problem exists for Fyn, and different studies have found inconsistent binding sites for Fyn to the intracellular segment of CD2[370, 384]. Itk is a member of the Tec family of cytoplasmic tyrosine kinases and has also been shown to be involved in signal transduction of CD2 molecules[385].

In addition, studies have also reported the identification of an intracellular protein known as CD2BP2that binds to two proline-rich compartments within the cytoplasmic region of CD2 and claim that this binding is essential for T cell activation[386]. However, to date, it has not been demonstrated how CD2BP2 goes about influencing T-cell activation.

In addition, CD2AP, CD2BP3,CIN85 have also been reported to bind to #4 in order to attach CD2 to the actin cytoskeleton, which has been used to explain the fact that CD2 mobility at the T cell plasma membrane has been shown to be reduced upon T cell activation[373, 387]. Immobilization of CD2 may be caused by cross-linking of the

cytoplasmic tail to the actin cytoskeleton. However, recent research evidence shows that upon T cell activation, CD2 molecules form a corolla-like structure at the periphery of the TCR complex, which we prefer to believe is responsible for the reduced mobility on the cell membrane upon CD2 activation[350]. It has also been proposed that the interaction between the intracellular domain of CD2 and CD2AP promotes the polarization of the actin cytoskeleton, which can affect the assembly of IS.

The proline-rich intracellular structural domain of CD2 is highly conserved in mammals. It can be inferred from previous studies that the intracellular tail of CD2 contains five potential SH3 binding sites that may bind to different SH3 domain-containing molecules and form a CD2-induced signaling network, a signaling network that affects IS assembly as well as T cell activation. However, the identification of exactly which downstream signaling molecule affects T cell activation, especially functionally, remains to be elucidated.

Structure-function analyses of CD2 indicated that the cytoplasmic domain of CD2 was essential for its capacity to enhance T cell activation. Moreover, mutation of either of two proline-rich motifs, M3 and M4, abolished the activating function. One of these proline- rich regions, M4, was able to bind to Lck *in vitro*. Coupled with the observations that CD2 co-immunoprecipitated with components of the TCR complex in the $CD2^{OST}$ mouse

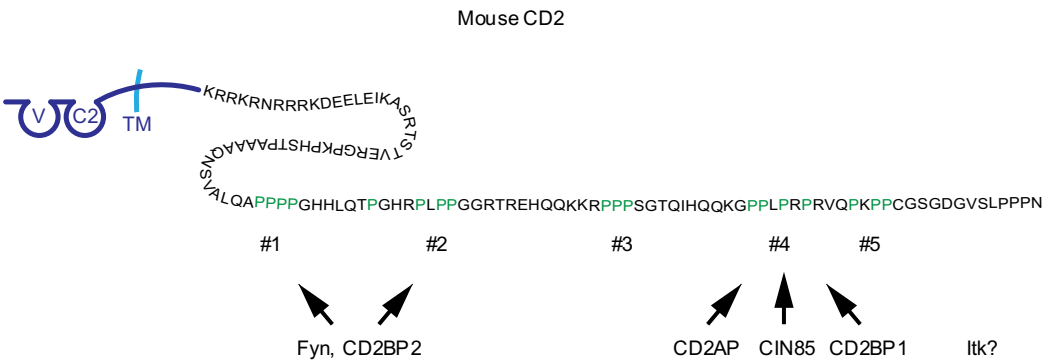


Fig 3.6 Potential adaptor molecules bound to the intracellular domain of mouse CD2. The intracellular segment of mouse CD2 contains five proline-rich binding domains (#1-#5) that can bind to the SH3 structural domains of other proteins. Fyn kinase has been shown to bind to #1, #2, #4, and #5. CD2 binding protein 2 (CD2BP2) has been shown to bind to #1 and #2. CD2BP1, the 85 kDa Cbl-interacting protein (CIN85) and CD2AP bind to #4.

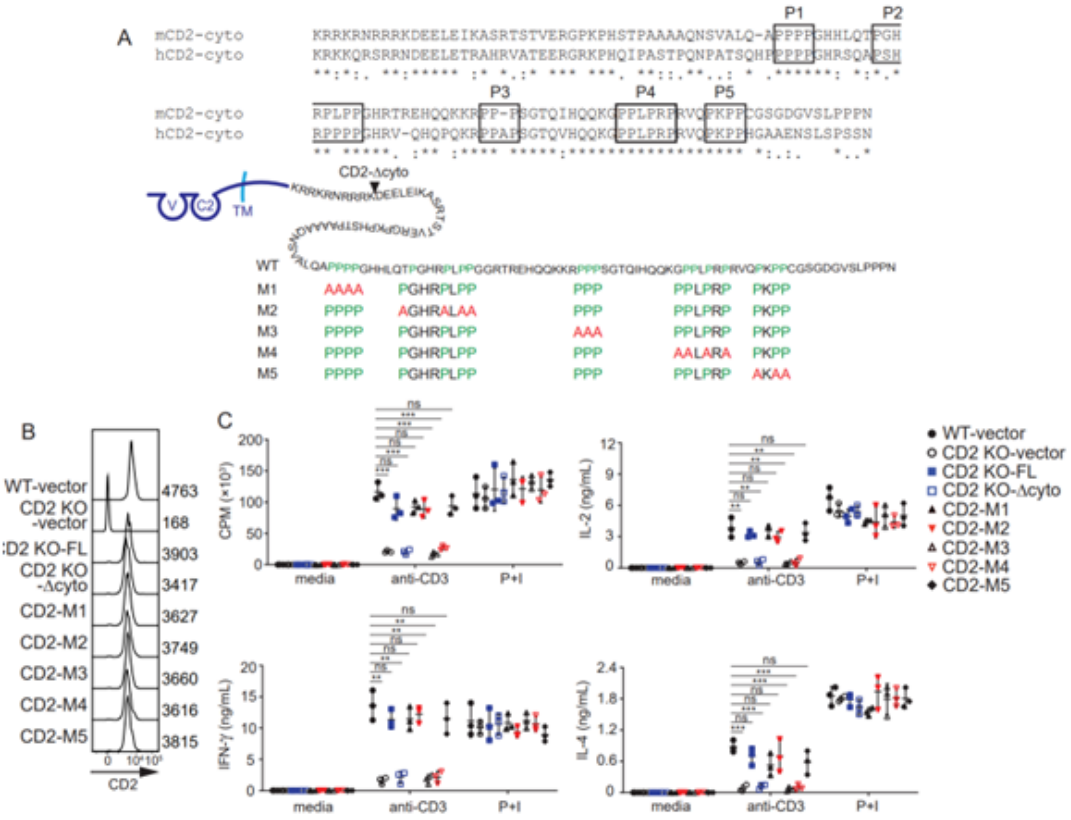
and that CD2-CD48 *cis* interactions increased TCR-elicited protein tyrosine phosphorylation, these data suggested that CD2 enhanced T cell activation by recruiting Lck in the vicinity of the TCR complex.

Further support for this idea was provided by our biochemical studies showing that CD2 and CD48 augmented TCR-triggered protein tyrosine phosphorylation, the most proximal TCR-induced signal. This effect was not global, but rather selectively affected tyrosine phosphorylation substrates of 36, 76 and 120 kDa, which possibly represented LAT, SLP-76 and c-Cbl. CD2-CD48 also augmented TCR-elicited calcium fluxes and Erk activation, two downstream effectors of phospholipase C (PLC)-γ1, which is activated in response to TCR stimulation. Although proximal TCR signaling was enhanced by CD2-CD48 *cis* interactions, it is unclear why TCR-evoked protein tyrosine phosphorylation was not globally augmented. Perhaps, only part of the TCR signalosome was influenced by CD2-CD48.

Based on our mass spectrometry data using previously activated T cells from $CD2^{OST}$ mice, the interactions of CD2, CD48 and TCR seemed to be constitutive and to

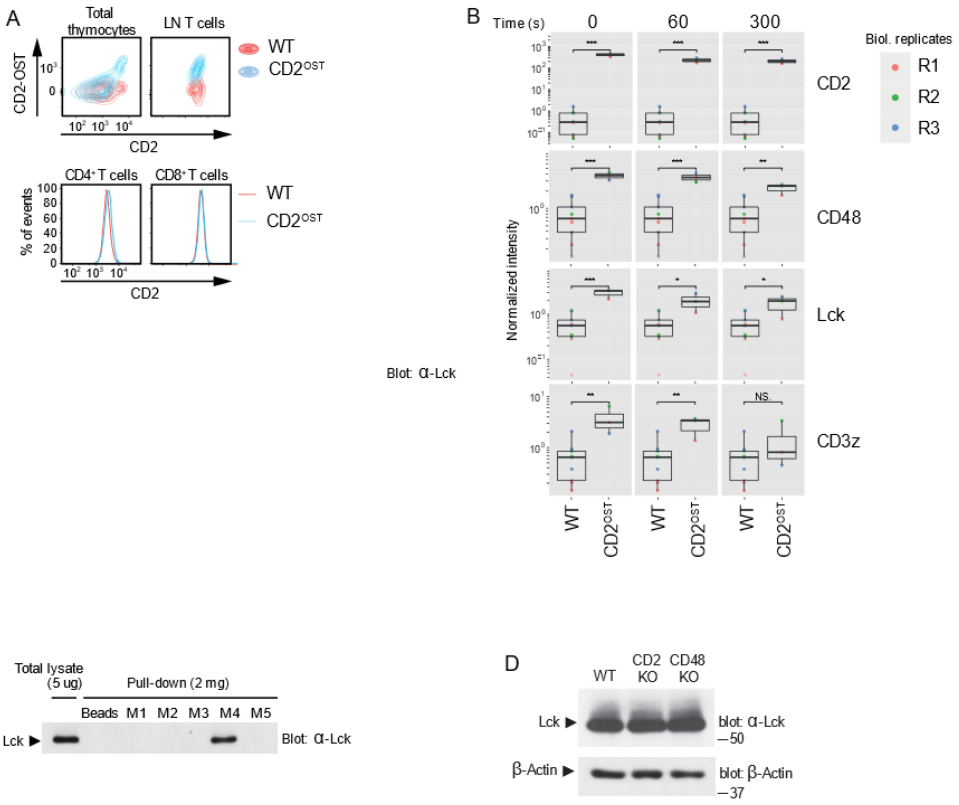
precede engagement of TCR by ligands. Whether this is only a feature of previously activated T cells or whether this is also seen with naïve T cells remains to be clarified. In any case, we presume that active CD2 signaling was prevented in our experimental setting, because of the high activity of protein tyrosine phosphatases, in particular CD45, in the vicinity of TCR. TCR engagement is known to sequester the TCR complex from CD45 to facilitate TCR signaling.

3.6 Figures and figure legends



10

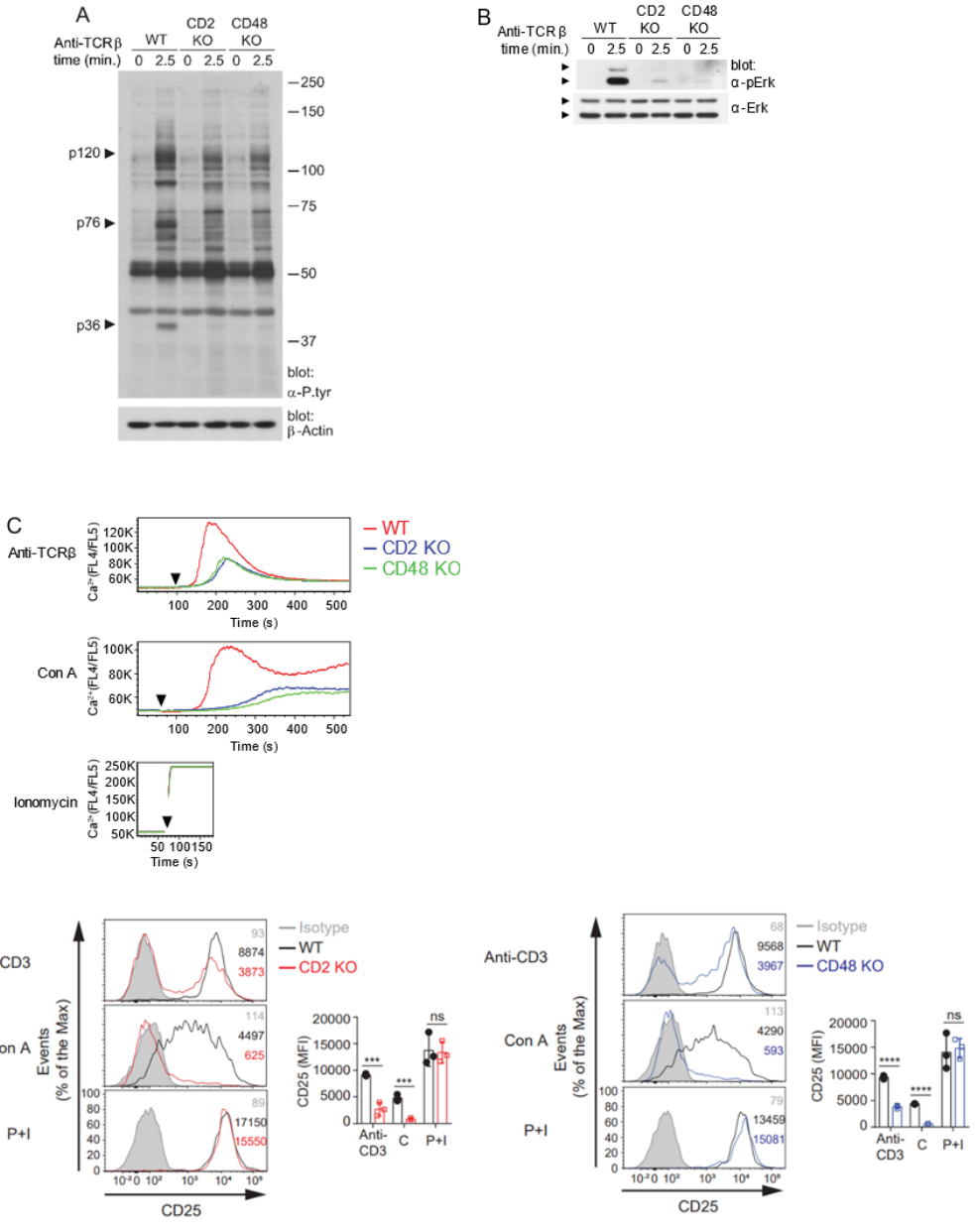
Fig 3.1 Proline-rich regions in the CD2 cytoplasmic domain are necessary to promote T cell activation. **(A)** The sequences of the intracellular domain of mouse and human CD2, in addition to the positions of the conserved proline-rich sequences, are shown at the top. Identical residues are shown as "*", whereas conserved residues with strongly similar properties are depicted as ":" and semi-conserved residues with weakly similar properties are shown as ".". A schematic representation of the primary structure of CD2, the site of the truncation for the cytoplasmic domain-truncated mutant (Δcyto) and the locations of the 5 proline (P)-to-alanine (A) mutants (M1 to M5) is found at the bottom. P1, P2, P3, P4 and P5 depict the five proline rich regions from CD2 cytoplasmic domain. TM, transmembrane domain. V, Ig-like V-type domain. C2, Ig-like C2-type domain. **(B** and **C)** Previously activated $CD4^+$ splenic T cells from the indicated mice were infected with full-length or mutated CD2. (B) After sorting of GFP-positive cells, expression of CD2 was verified by flow cytometry. FL, full-length. Representative of $n = 3$. **(C)** Sorted cells were stimulated with anti-CD3 alone or P+I. Activation was monitored as Fig. 2.2(A). Each symbol represents a mouse. $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests, unless specified otherwise).


A
Total thymocytes
LN T cells
WT
CD2OST
CD2-OST
CD2
CD4+ T cells
CD8+ T cells
% of events
B
Time (s)
0
60
300
Biol. replicates
R1
R2
R3
CD2
CD48
Lck
CD3z
Normalized intensity
NS.
WT
CD2OST
C
Total lysate (5 ug)
Pull-down (2 mg)
Beads M1 M2 M3 M4 M5
Lck
Blot: α-Lck
D
WT
CD2 KO
CD48 KO
Lck
blot: α-Lck
50
β-Actin
blot: β-Actin
37

Blot: α-Lck

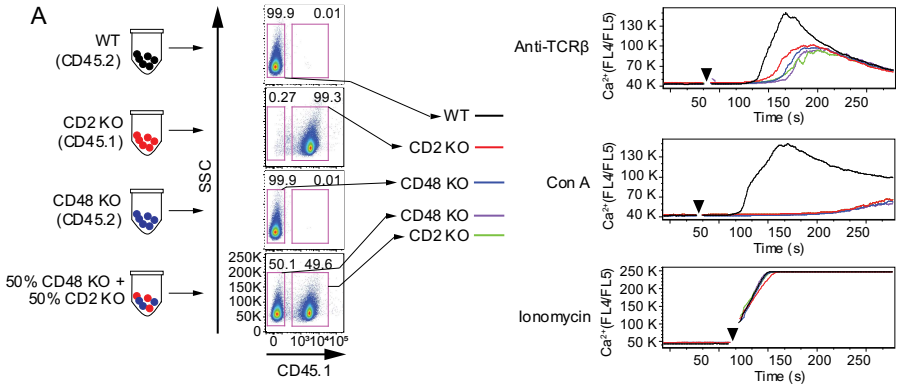
11

Fig 3.2 Proline-rich regions in the CD2 cytoplasmic domain are necessary to promote T cell activation. **(A)** Total thymocytes and lymph node (LN) T cells (gated on TCR^+) were analyzed (top). Expression of CD2 in $CD4^+$ and $CD8^+$ T cells from LN of wild-type (WT) and $CD2^{OST}$ mice (bottom). **(B)** The abundances of the CD2 interactors specified on the right were estimated for each time points and biological replicates (R1, R2 and R3). Normalized intensities (see Methods) for WT and $CD2^{OST}$ cells were compared using a two-sided Welch t-test. Imputed missing values are represented with lighter shaded dots. **(C)** Pull-down assay. Biotinylated peptides encompassing each of the 5 proline-rich sequences of CD2 were synthesized and coupled to streptavidin beads. They were then incubated with previously activated $CD4^+$ splenic T cell lysates. After several washes, the presence of Lck was detected by immunoblotting. Representative of $n = 3$. **(D)** Isolation of $CD4^+$ splenic T cells from WT, CD2 KO or CD48 KO mice, after lysis, basal level Lck was probed by blotting with our homemade anti-Lck antibody (top), β-actin as the loading control(bottom). Representative of $n = 3$. Results are pooled from three independent experiments in (C). Each symbol represents a mouse. $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests, unless specified otherwise).


A
Anti-TCRβ
time (min.)
WT
CD2 KO
CD48 KO
0 2.5 0 2.5 0 2.5
p120
p76
p36
250
150
100
75
50
37
blot: α-P.tyr
blot: β-Actin
B
Anti-TCRβ
time (min.)
WT
CD2 KO
CD48 KO
0 2.5 0 2.5 0 2.5
blot: α-pErk
α-Erk
C
Anti-TCRβ
Con A
Ionomycin
Ca²⁺(FL4/FL5)
Time (s)
WT
CD2 KO
CD48 KO
D
Anti-CD3
Con A
P+I
Events (% of the Max)
CD25
Isotype
WT
CD2 KO
93
8874
3873
114
4497
625
89
17150
15550
CD25 (MFI)
Anti-CD3
C
P+I
ns
Isotype
WT
CD48 KO
68
9568
3967
113
4290
593
79
13459
15081

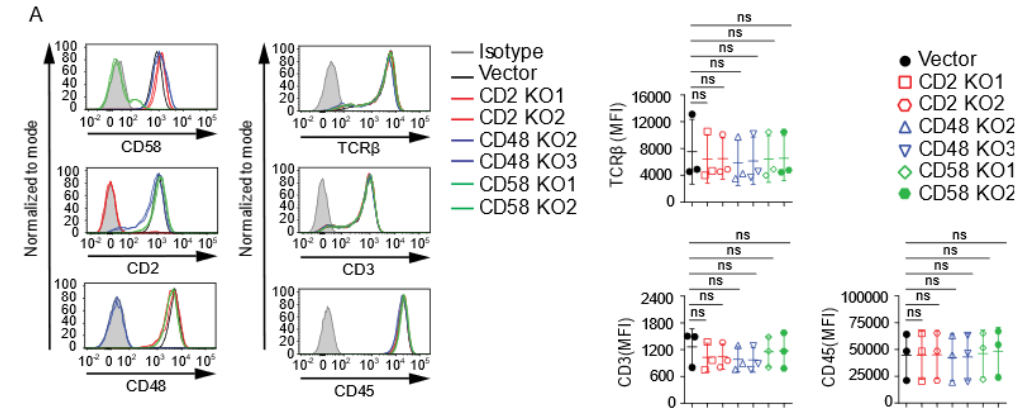
12

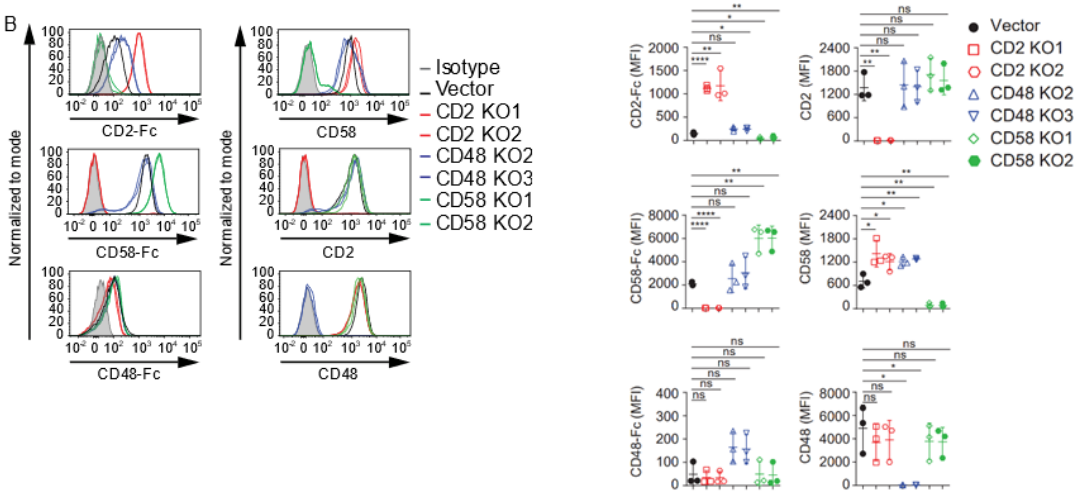
Fig 3.3 *cis* interactions between CD2 and CD48 are required for maximal TCR signaling. **(A** and **B)** Freshly isolated $CD4^+$ T cells were incubated for 0 min and 2.5 min with biotinylated anti-TCRβ (5 μg ml^{-1}), then add avidin (10 μg ml^{-1}). Phosphotyrosine (P.tyr)-containing proteins were monitored(A) while activation of Erk was detected(B). β-*actin* and total Erk were studied in parallel as loading controls. **(C)** Changes in intracellular calcium were detected. **(D)** Flow cytometry analyses showing CD25 expression, isotype-matched rat IgG1, λ as staining control. Representatives of three experiments are shown in (A, B, C). Each symbol represents a mouse in (D). $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests, unless specified otherwise).


A
WT
(CD45.2)
CD2 KO
(CD45.1)
CD48 KO
(CD45.2)
50% CD48 KO +
50% CD2 KO
SSC
CD45.1
99.9 0.01
0.27 99.3
99.9 0.01
50.1 49.6
WT
CD2 KO
CD48 KO
CD48 KO
CD2 KO
Anti-TCRβ
Con A
Ionomycin
Ca2+(FL4/FL5)
Time (s)

13

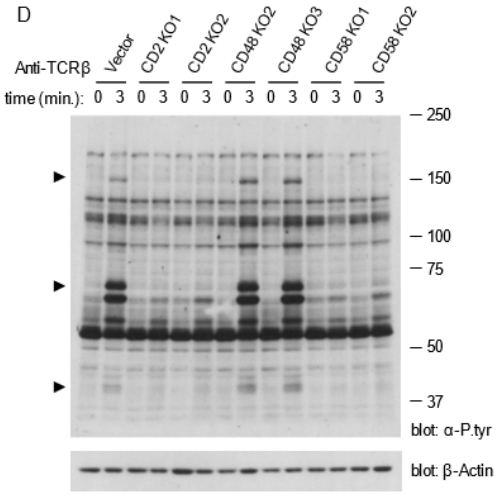
Fig 3.4 *cis* interactions between CD2 and CD48 are required for maximal TCR signaling. **(A)** Protocol is depicted on the left, whereas changes in intracellular calcium were monitored for the indicated periods is shown on the right. The leftmost column of the cartoon represents freshly isolated $CD4^+$ T cells. On the immediate right, the pseudocolor blots depicts cells were labelled with CD45.1 to distinguish the CD2 KO cells from CD48 KO cells in the mixed tube (the bottom row). The rightmost histograms show the changes in intracellular calcium, same as Fig 3.3 (C). Representatives of three experiments are shown in (A).





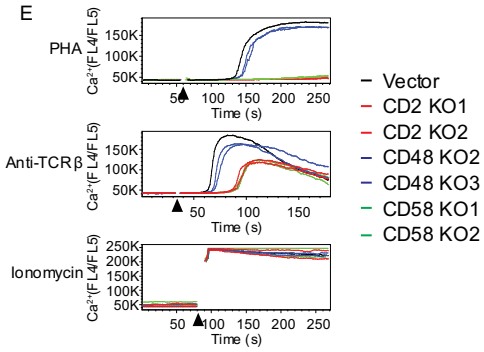
C

	WT	CD2 KO	CD58 KO
CD2-Fc / CD58	1.0	4.3	NA
CD58-Fc / CD2	1.0	NA	2.5



14

E


PHA
Anti-TCRβ
Ionomycin
Ca²⁺(FL4/FL5)
150K
100K
50K
250K
200K
50
100
150
200
250
Time (s)
Vector
CD2 KO1
CD2 KO2
CD48 KO2
CD48 KO3
CD58 KO1
CD58 KO2

F

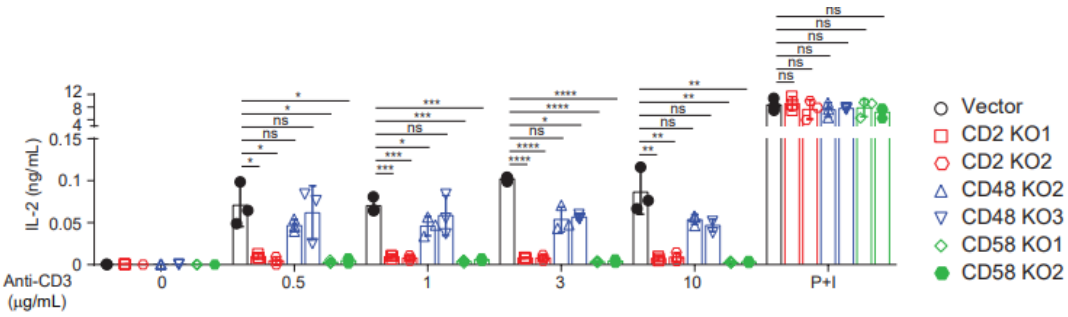

IL-2 (ng/mL)
12
8
4
0.15
0.1
0.05
0
Anti-CD3 (μg/mL)
0
0.5
1
3
10
P+I
ns
Vector
CD2 KO1
CD2 KO2
CD48 KO2
CD48 KO3
CD58 KO1
CD58 KO2

Fig. 3.5 *cis* interactions of CD2 and CD58 promote human T cell activation. Polyclonal populations of Jurkat cells lacking CD2, CD58 or CD48 were generated for each of these molecules. Jurkat cells transfected with empty vector alone were used as control. **(A)** Flow cytometry analyses of expression of markers on the variants of Jurkat cells. Shaded histograms represent isotype control staining. **(B)** Cells were stained with Fc portion of CD2, CD58 or CD48, followed by a secondary antibody directed against the Fc segment of the fusion proteins. Expression of CD2, CD58 and CD48 was analyzed with Abs directed against these molecules (red lines). Shaded histograms, isotype control. Representative histograms are shown at the top, while data from multiple independent experiments are shown at the bottom. Each symbol represents an individual experiment. (**C)** Same as Fig. 2.4(B), except that staining with human CD2-Fc and human CD58-Fc to Jurkat cells was analyzed. Data are from (B). **(D)** Same as Fig. 3.3(A), except that variants of Jurkat cells were determined. A representative of three experiments is shown. (**E**) Same as Fig. 3.3(C), except that variants of Jurkat cells stimulated with anti-TCRβ (top, 1μg ml^{-1}), the lectin phytohemagglutinin (PHA, middle, 10 μg ml^{-1}), or ionomycin (Iono, bottom, 1 μg ml^{-1}) were studied. A representative of three experiments is shown. **(F)** Variants of Jurkat cells were activated. After 48 hours, production of IL-2 was determined by ELISA. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, NS $P > 0.05$, not significant (two-sided unpaired Student's t-tests).

Chapter 4 Discussion

TCR recognition of specific peptide/MHC complexes usually triggers T-cell activation; in addition to this chief signaling, co-stimulatory signals are also required to achieve full T-cell activation. In the absence of co-stimulation, T cells have been shown to be induced into an exhausted state and unable to exert their functions. *In vitro*, when we use antibodies to activate T cells, T cell activation is better in the presence of anti-CD28 antibodies, which act independently of anti-CD3 antibodies. This suggests that certain stimulation pathways may have unique functions that cannot be compensated for by other pathways involved in co-stimulation. Thus, blocking individual costimulatory pathways has the potential to elicit unique immunomodulatory effects and intentionally direct T-cell activation to achieve the desired effect, which is a necessary condition for immunology to be available for clinical treatment. Now, to achieve robust activation and expansion of human T cells *in vitro*, CD3, CD28 and CD2 cell surface ligands are bound and cross-linked, thus providing the primary and co-stimulatory signals required for T cell activation [388].

We demonstrated that CD2 and its ligands interact in *cis* on the T cell surface to promote TCR signaling through Lck recruitment. Lck is one of the most important members of the Src family of protein tyrosine kinases and is essential for early T cell signaling [271]. The Lck molecule consists of consecutive SH4, SH3, SH2 and tyrosine kinase (SH1) structural domains. When the TCR interacts with peptide-MHC, CD4 or CD8 molecules bind to the MHC molecules, and Lck is then activated. Lck directly phosphorylates ITAMs on the CD3ζ chains of the TCR complex, and this phosphorylation leads to the recruitment and activation of the Syk family kinase ZAP-70. Activated ZAP-70 will initiate a cascade of signaling events (Fig. 4.1). The amount of Lck associated with CD4 molecules has been reported to be 10-20-fold higher than that associated with CD8 molecules [271]. According to our experimental results (Fig. 2.3D-F), in *in vitro* activation assays, in the presence of MHC molecules, we can see that the response of OT-I cells is more dependent on CD2 molecules, perhaps as compensation for the relatively weak

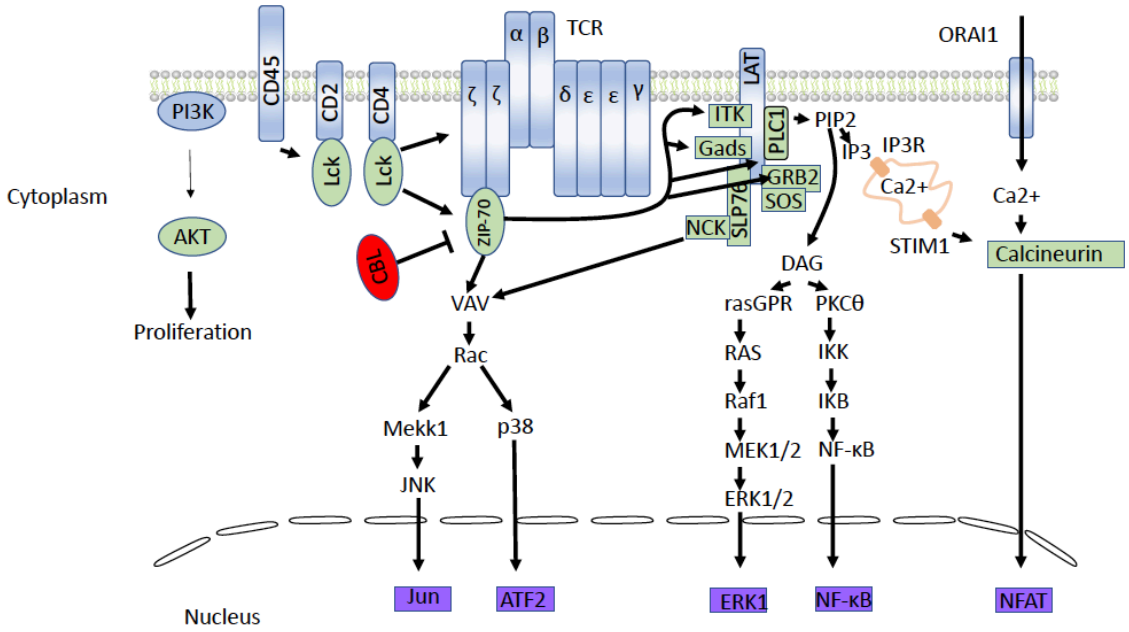


Fig 4.1 T cell receptor signaling pathway. An early event in TCR activation is the phosphorylation of ITAMs on the cell membrane of the TCR/CD3 complex by Lck. CD45 receptor tyrosine phosphatases regulate the phosphorylation and activation of Lck and other Src family tyrosine kinases. ZAP-70 is recruited into the TCR/CD3 complex, where it is activated to promote recruitment and phosphorylation of downstream adaptor or scaffold proteins. Phosphorylation of SLP-76 by ZAP-70 promotes recruitment of Vav, the adaptor proteins NCK and GADS, and inducible T-cell kinase (Itk). Phosphorylation of phospholipase Cγ1 (PLCγ1) by Itk leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates the PKCθ and MAPK/Erk pathways, both of which promote activation of the transcription factor NF-κB. IP_3 triggers Ca^{2+} release from the ER, promoting cellular entry of extracellular Ca^{2+} through calcium-release-activated Ca^{2+} (CRAC) channels. Calcium-bound calmodulin activates the phosphatase calcineurin, which promotes IL-2 gene transcription via the transcription factor NFAT. Feedback regulation at several points in these pathways allows for different outcomes depending on the cell type and environment.

recruitment of Lck by CD8 molecules. Also, especially at low concentrations of peptide-MHC stimulation, T cells lacking CD2 would have a greater activation defect, which we speculate is due to the fact that when there are few peptide-MHC molecules, CD4 or CD8 molecules are also recruited less, and CD2 signaling would be very critical. This is compensation for CD4 or CD8 signaling that is not redundant and would be very important, especially when antigen stimulation is weak or early in the immune response.

CD2 antigen has been identified as a marker of T cells at a very early stage. Although the sequence of the gene encoding CD2 is highly conserved in the mouse and human genomes, it was found that the pattern of CD2 in mouse immune system may be different from that in the human. Mice lack the expression of CD58, which is the primary ligand for CD2 in humans. Mice express CD48, while it was found that CD48 has a relatively low affinity for CD2 and can bind to both CD2 and 2B4 [389]. In addition, CD2 expression is lacking in human B cells, while CD2 is widely expressed in mouse B cells (data not shown). All these factors interfere with the study of CD2.

Previous studies had provided evidence for a mitigated defect of CD2 and CD48 in T cell activation that was primarily limited to low-affinity antigens or low concentrations of antigens [366]. They crossed CD2-deficient mice with transgenic mice expressing the LCMV-derived peptide p33-specific TCR, and they used splenocytes rather than purified T cells as responders to study the activation of CD2-deficient T cells. The CD2-deficient mice they used were from another research group; the group that produced this CD2-deficient mouse concluded that CD2 is dispensable for the function of T cells [336]. However, our data showed that CD2 and CD48 have a broad key role in normal T cell activation, including in response to high-affinity antigens. Perhaps, the differences between the previous results and ours related to the fact that, in the earlier studies, mice in a mixed 129/B6 background were used. Furthermore, we used purified T cells as responders. We used irradiated splenocytes as APCs: this also eliminates the possibility of macrophages engulfing T cells. We used CRISPR-Cas gene editing technology to make mouse models.

CRISPR-Cas gene editing technology has the potential of off-targeting. To ensure that our conclusions are solid, as described in our experimental methods, two different CD2 knockout strains were made as well as three CD48 knockout strains, and heterozygous KO mice were backcrossed to the C57BL/6J background for ten generations and subsequently bred to homozygosity for experimentation. To ensure the credibility of the experimental results, each experiment used littermates of the same sex and we performed three independent experiments. And we used different experimental approaches to verify our conclusions. Furthermore, one group argued for the presence of *cis* and *trans* interactions between CD2 and CD48, which we believe may be a result of their use of macrophages as APCs [366]. Another study showed that the CD2-deficient mice displayed normal or only minimally compromised T cell responses to anti-CD3 Abs or antigenic peptides, although CD2 deficiency accentuated the defects caused by lack of CD28 [390]; the authors used the same 129/B6 background mouse model.

Even though CD48 also exists in humans, it is not the primary ligand of CD2. The dominant ligand of CD2 in humans is CD58 [332, 349]. We confirmed these findings herein. Using Jurkat as a human T cell model, we observed that CD2 and CD58 in Jurkat enhanced TCR-evoked signals and T cell activation, leading to cytokine production. CD48 had no impact on these responses. Hence, like CD2 and CD48 in mice, CD2 and CD58 in humans interact in *cis* at the surface of T cells to promote TCR signaling and T cell activation. Even if the identity of the CD2 ligands is not conserved across species, the importance of *cis* interactions for the function of CD2 is preserved. Given that CD58 is more highly expressed in human effector-memory and innate-like T cells, compared to naïve T cells, the role of the CD2-CD58 *cis* interactions may be especially prominent in in the former cell types.

In published models, receptor-ligand interactions are typically depicted as occurring in *trans*. However, many receptors and their ligands are co-expressed on the same cell, raising the possibility that *cis* interactions can also take place. Firm evidence

for the existence of *cis* interactions within individual cells has been provided for several receptor-ligand pairs in immune cells. Examples include PD-1 and its ligand PD-L1, which are co-expressed on APCs [369]. In this case, the *cis* interactions interfere with the *trans* interactions, thereby suppressing the function of PD-1. Likewise, inhibitory Ly49 receptors and class I MHC can interact in *cis* on NK cells, thus attenuating the function of Ly49 in NK cells [391]. Lastly, CD8 was shown to interact in *cis* with class I MHC at the surface of T cells, thereby compromising the function of CD8 in T cells [392].

In all these cases, the *trans* interactions were believed to predominate functionally over the *cis* interactions. Moreover, the *cis* interactions were found to suppress the impact of the *trans* interactions. However, these features do not apply to the *cis* interactions between CD2 and its ligands in T cells. Our data suggested that the CD2-CD48 *cis* interactions functionally predominated over the *trans* interactions for most T cell activation responses. In addition, either *cis* or *trans* interactions promoted T cell activation, and, in the case of cytotoxicity, these effects were synergistic. Whether these distinctive characteristics of the CD2-mediated *cis* interactions apply to *cis* interactions involving other molecules should be assessed. As a corollary, the possibility of targeting *cis* interactions to alter the functions of immune cell receptors for therapeutic purposes deserves consideration.

CD2 is largely described to interact with its ligands, CD48 in mice and CD58 in humans, that are present on antigen-presenting cells (APCs). However, both CD48 and CD58 are also highly expressed on T cells. Previous studies have also shown that CD48-deficient T cells have a significant defect in activation, especially in response to anti-CD3 stimulation [367, 368], which also suggests that CD48 on T cells is critical for T cell activation, which is consistent with our conclusions. However, in this literature [368], the argument that CD48 can deliver activation signals to the interior of T cells via lipid rafts was very tenuous. First, they do not have any direct microscopic evidence. Furthermore, the TCR complex is located in the lipid rafts, and showing the significance of CD48 for the

phosphorylation of ζ-chain by disrupting the lipid rafts needs to be carefully considered. Even though chimeric antigen receptors (CARs) can redirect T cells to target tumor cells in a TCR-independent manner, their activity is still limited by antigen sensitivity. The addition of CD58 to APCs to increase CAR-T cell activation using CD2-CD58 *trans* interactions has a very limited effect, which also shows that the *trans* interaction of CD2 with CD58 is not critical in T cell activation [393].

CD2 can also respond to ligands displayed on the APCs; these *trans* interactions are critical in limited situations, such as cytotoxicity (Fig. 2.7B,C; 2.8B,C). For example, in Fig. 2.7B,C, we found that WT T cells were significantly cytotoxic to EL-4 cells in the presence of antigen, and this response was significantly compromised in CD2 KO T cells. However, no defect was observed in CD48 KO T cells. This is due to the fact that CD48 molecules on EL-4 cells rescued CD2 signaling on T cells through *trans* interactions. In cytotoxicity assays, *cis*, as well as *trans* interactions of CD2 and his ligand, were present simultaneously (Fig. 2.8B,C). A recent study found that CD58 knockdown in human melanoma cell lines significantly reduced tumor cell lysis compared to wild-type melanoma cells. CD58-CD2 interactions between cancer cells and T cells [394] or CAR T cells [395], respectively, are essential for cell lysis. These results are consistent with our cell cytotoxicity data.

For CD2 downstream signaling molecules, previously described CD2-interacting proteins such as CMS, Itk and CD2BP1 were not observed in our study. The previous studies were mainly conducted using immunoprecipitation. However, functionally, at least for T-cell activation, we do not believe CD2 critically interacts with CD2AP. Because CD2AP-deficient mice show glomerular damage at 6-7 weeks of age due to severe renal lesions and proteinuria, and lead to death. However, neither of our two CD2-deficient models had such lesions, and we hypothesize that the interaction between these two molecules is no longer functionally linked during long-term evolution.

Although we determined that CD2 and its ligands interact in *cis* at the surface of T

cells to promote TCR signalling via Lck recruitment, details regarding the spatial-temporal organizations of CD2 and its ligands in the TCR complex remain unclear. Additionally, several studies have showed the location of CD2 in the TCR complex, with initial studies indicating that CD2 was localized in or near the central supramolecular activation complexes (cSMACs) of the IS on Jurkat cells [363]. However, recent studies reported that CD2-CD58 interactions were in the distal regions of SMACs [350]. Furthermore, these previous studies were all based on the experimental model that CD2 and its ligands interact in *trans*. Notably, our data indicate that Lck interacts with CD2 in naïve or activated T cells—even in the absence of CD48/CD58. This raises the following questions: How does the CD2-CD48/CD58 *cis* interaction contribute to T cell activation? What is the organization of CD2 and its ligands on T cells in a steady state and under activation? Lipid rafts have been implicated in signal transduction, TCR itself associates with lipid rafts, and the clustering of T cell activation-associated membrane proteins into lipid raft microdomains is known to serve a critical role in regulating T cell activation. Although CD2 is not a lipid raft protein, its CD48/CD58 ligands are classical lipid raft proteins. It has been suggested that the cross-linking of an antigen-loaded MHC molecule with TCR leads to the TCR-CD3 complex translocating to lipid rafts and being surrounded by an abundance of classical lipid raft proteins—including CD48 or CD58. One explanation for how the CD2-CD48/CD58 *cis* interaction contributes to TCR signalling is that after T cell priming, the clustering of TCR signalling molecules into lipid raft microdomains (e.g., CD48 or CD58) occurs. CD2 and its ligands have a low affinity for each other and dissociate very rapidly. However, after clustering in the lipid raft microdomains, numerous CD48/CD58 molecules may still result in considerable receptor-ligand avidity and pull CD2-Lck toward the TCR complex.

Siplizumab, a humanized monoclonal anti-CD2 antibody, has the potential for clinical application. It has been reported that (https://www.fiercebiotech.com), ten transplant patients received a conditioning regimen of antibodies. Seven of these patients

had been off immunosuppressive drugs for five years or longer. Three of the patients were later restarted on low-dose immunosuppressive drugs, but the remainder had been off the drugs for up to 11 years at the time the data were published. Siplizumab inhibits allogeneic mixed lymphocyte responses *in vitro* and primarily reduces the percentage of $CD4^+$ and $CD8^+$ effector memory T cells [396]. In addition, a recent study also found that siplizumab induces NK cell fratricide [397]. Siplizumab has been investigated for the treatment of plaque psoriasis [398], stem cell transplantation [399], and several other diseases. Overall, the clinical data regarding anti-CD2 agents suggest that this is a safe treatment for a wide range of therapeutic applications.

Recently, the significance of CD2 and its ligands in tumour immunotherapy has also been investigated. Chimeric antigen receptor (CAR) T-cell therapy has achieved significant success in treating a variety of hematologic malignancies, but many patients are resistant to this therapy. In these patients, it was found that loss of CD58 confers immune evasion of CAR T cells [400]. It has been found that many tumour cells lose CD58 expression [401, 402], and this downregulation is associated with immune evasion of the tumour. CD58 expression on cancer cells is required for an effective T cell-mediated antitumor response, and CD58-CD2 interactions between cancer cells and T cells are essential for T cell tumour killing [403]. This also reminds us of the previous report that a strain of HCMV has been shown to downregulate the cell surface expression of CD58 in host cells [404], thereby evading the cytotoxicity of CTL. These facts underscore the importance of the CD2-CD58 signaling pathway in human immunity.

Chapter 5 Conclusion and Future Directions

5.1 Conclusions

The overarching subject of our research was to explore the function of CD2 and its ligands in T cell activation by using our KO mice. Our study has proven the critical role of CD2 and its ligands in T cell activation through cis interaction. In summary, we have revealed the following: 1) in the absence of CD2 or CD48, conventional T cells—including $CD4^+$ T cells and $CD8^+$ T cells—showed normal development in the thymus and spleen. Furthermore, even the subpopulation of CD44+ "memory" T cells in CD4+ T cells or CD8+ T cells showed normal development. Molecules associated with TCR signalling were normally expressed on T cells from CD2 or CD48 KO mice; 2) In the absence of CD2 or CD48, we observed that the activation of mature T cells in response to various stimuli showed a very large defect, including responses to antigens, superantigens, alloantigens, mitogenic lectins and anti-TCR-CD3 antibodies without or with anti-CD28. In contrast, CD2 or CD48 is not required for the response to PMA and ionomycin; 3) According to the results of competing Fc binding, independent T cell confocal images or FRET assays, CD2 and CD48 have a physical cis interaction on the surface of T cells; 4) The loss of CD48 on APCs had no significant effect on T cell activation response. Furthermore, freshly isolated CD2 KO and CD48 KO T cells were mixed and the defects in calcium fluxes on each type of cell were not restored with anti-TCR or Con A. However, one exception was T cell cytotoxicity, which required CD48 on both T cells and APCs for maximal response; 5) The deletion of CD2 or—to a lesser extent—CD48 on T cells results in a significant GVHD reduction. The effect of CD48 KO was not as pronounced as in the CD2 KO group, which we believe is due to $CD8^+$ T cell-mediated cytotoxicity since recipient mouse cells act as APCs and the CD48 molecules on their surface can rescue the killing effect of donor CD48 KO T cells. The survival of CD2 KO or CD48 KO T cells in recipient mice was normal, which is comparable to WT donor T cells; 6) One proline-rich region of the CD2 cytoplasmic domain interacted with kinase Lck to promote T cell activation; 7) In humans, CD2 interacts with CD58 in cis on T cells, which is critical for T cell activation.

In summary, our results support the conclusion that cis interaction between CD2 and its ligands on T cells is essential for T cell signalling and T cell activation, which is a novel finding that can contribute to understanding how CD2 and its ligands harness T cell activation as well as for clinical applications. On the other hand, our studies also proved that anti-CD2 blocking antibodies can efficiently block the cis interaction between CD2 and CD48, which further demonstrates that this blocking treatment can phenocopy the functional defects of CD2 Ko T cells *in vitro*. As such, manipulating CD2-CD48 signalling in vivo could also be a promising clinical avenue for alleviating autoimmunity.

5.2 Future Directions

Despite this study shedding light on our understanding of cis interactions between CD2 and its ligands on T cells, as well as this cis interaction operating in GVHD, we still have some inquiries that need to be studied. Therefore, certain results leave us with some deep questions that will need to be addressed by extended studies in the future, for example, dissociation of cis-CD2–CD48 duplex on T cells to alleviate autoimmune disease symptoms.

Our study has established the essential role of CD2 and its ligands in GVHD. Additionally, WT T cells phenocopy the function defect of CD2 KO T cells, which were treated with anti-CD2 blocking antibody. Our study suggests that this anti-CD2 blocking antibody detaches the CD2-CD48 cis interaction. However, we did not test the effect of this anti-CD2 blocking antibody in GVHD. We presume that the anti-CD2 blocking antibody is also effective in alleviating autoimmune disease symptoms. To determine whether the anti-CD2 blocking antibody can be administered as an approach to treat autoimmune disease symptoms, experiments should be conducted to test the therapeutic potency of blocking antibodies against autoimmune disease models, such as experimental arthritis or EAE.

Chapter 6 References

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