Role of CD80 and CD86 Cosignaling Proteins Functional Domains in Molecular Structure and Adaptive Immune Responses

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Un PhD, c'est plus qu'un diplôme. C'est une expérience de vie, un cheminement qui a modelé la personne que je suis aujourd'hui. Un PhD, c'est aussi un fragile équilibre où l'on tente de balancer les déceptions et incompréhensions aux joies et réussites. Je désire exprimer ma sincère reconnaissance envers plusieurs personnes qui ont réussi à me faire maintenir cet équilibre à travers les années.

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Abstract

The initiation of adaptive immune responses requires the interactions of T cells with antigen presenting cells (APC) in the context of an immunological synapse (IS). Naïve T cell responses are dependent on the engagement of CD28 and CTLA-4 by CD86 and CD80, respectively amplifying and dampening the antigen specific signal. CD80 and CD86 cosignaling molecules display three major domains: a membrane distal IgV-like domain, a membrane proximal IgC-like domain and an intracellular domain. Crystallographic data has shown that only the IgV domain of CD80 and CD86 physically interacts with CTLA-4. However, extensive mutational analyses have also implicated the IgC domain in receptor binding and in the overall function of these molecules. The role of CD80 and CD86 within the IS and their exact molecular structure remains to be elucidated. The work presented in this thesis employs wild type, mutant, deleted and chimeric forms of CD80 and CD86 to characterize the role of their domains in molecular structure, receptor binding and overall cosignaling function in an antigen specific cellular interaction system. CD80 and CD86 are shown to be associated to the APC cytoskeleton. A highly conserved K4 motif within CD86 is shown to be a cytoskeletal association motif. Moreover, CD86 is shown to physically interact with ERM proteins. Only cytoskeleton-linked CD86 localizes at the IS and induce IL-2 production. CD80 and CD86 molecular organization is clearly established using cytometry-based fluorescence resonance energy transfer (FCET) and biochemical approaches. CD80 exists as a mixed monomeric and dimeric population and CD86 as a monomer in live cells. The crucial role of CD80 and CD86 IgC domain in multimerization is revealed. Importantly, the molecular structure of these molecules correlates with their binding properties and cosignaling function. A functional picture of CD80 and CD86 domains emerges where the IgV is responsible for receptor binding, the IgC domain impacts dimerization, and the intracellular domain functionally links these proteins to the cytoskeleton. The findings presented in this thesis certainly contribute to the general understanding of cosignaling protein interactions and functions and may facilitate the design of structure-based immunotherapeutics.

Résumé

L'initiation d'une réponse immunitaire adaptatrice requière l'interaction d'un lymphocyte T et d'une cellule présentatrice d'antigène (CPA) dans le contexte de la synapse immunologique (SI). L'interaction entre CD28 et CTLA-4 exprimées par les lymphocytes T avec CD86 et CD80 présentes à la surface des CPA, amplifie ou inhibe le signal antigène spécifique et est absolument nécessaire. Les molécules de co-stimulation CD80 et CD86 comportent trois principaux domaines, soit un domaine IgV, un domaine IgC et un domaine intracellulaire. Des études cristallographiques ont démontré que seul le domaine IgV lie CTLA-4. Cependant, de nombreuses études ont aussi souligné l'importance du domaine IgC dans la fonction co-stimulatrice de ces molécules. Le rôle de CD80 et CD86 au sein de la SI et leur structure moléculaire doivent être établis. Le travail présenté dans cette thèse utilise des molécules sauvages, mutées, tronquées et chimériques de CD80 et CD86 afin d'étudier le rôle de leurs domaines dans la configuration moléculaire, l'interaction de ces molécules avec leurs récepteurs et leur fonction co-stimulatrice. Nous avons démontré que CD80 et CD86 sont liés au cytosquelette des CPA. Une séquence conservée dans tous les domaines intracellulaires de CD86, le motif K4, est responsable de cette association. De plus, une interaction entre CD86 et les protéines ERM est révélée. Seules les molécules de CD86 associées au cytosquelette se retrouvent dans la SI et co-stimule la sécrétion d'IL-2. Des approches biochimiques et de transfert d'énergie de fluorescence par cytométrie démontrent que CD80 est monomérique et dimérique alors que CD86 est monomérique. Nous avons établi le rôle déterminant du domaine IgC dans la formation de ces multimères. De plus, la structure moléculaire de ces molécules corrèle avec leurs propriétés d'interactions et leur fonction co-stimulatrice. Nous proposons un modèle détaillant le rôle fonctionnel de chacun des domaines de CD80 et CD86; ainsi le domaine IgV est responsable de la liaison de récepteurs, le domaine IgC influence l'interface dimérique et le domaine intracellulaire lie ces molécules au cytosquelette. Les conclusions de cette thèse contribuent à la compréhension globale du phénomène de co-stimulation et pourraient faciliter le développement d'immunothérapies.

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Contributions of Authors

Chapter 2

CD80 and CD86 IgC Domains Are Important for Molecular Structure, Receptor Binding and Cosignaling Function

Tanya Girard, Mohamed El-Far, Denis Gaucher, Gaëlle Breton, Rafick-Pierre Sékaly

This manuscript will be submitted to the *Proceedings of the National Academy of Sciences* (PNAS) journal.

Tanya Girard and Dr Sékaly conceived the project. Tanya Girard cloned all CD80 and CD86 related constructs in pE-CFP and pE-YFP vectors and performed all experiments presented in this chapter. Dr Sékaly supervised the study. Tanya Girard amplified V80C86T86 and V86C80T80 by PCR and Dr El-Far cloned them in pE-CFP and pE-YFP. Dr Gaucher cloned the fusion, Fas and Lck constructs and Gaëlle Breton generated the mYFP construct, all used as controls in FCET experiments. Apart from supplementary figure 1 kindly prepared by Sylvain Gimmig, Tanya Girard made all figures presented in the manuscript. Tanya Girard wrote the article in collaboration with Dr Sékaly.

*Dr Sabbagh previously generated the CD4 and CD4K318E constructs used as controls in FCET experiments

Chapter 3

CD86 Cytoskeletal Association is Necessary for Immunological Synapse Localization and Effective Co-stimulation

Tanya Girard, Oreste Acuto, Geneviève Beaulé, Frédérique Michel, Rafick-Pierre Sékaly

This manuscript has been submitted to the *Journal of Immunology* in a *Cutting Edge* format and is under review.

Tanya Girard conceived this project. Tanya Girard performed all experiments presented in the manuscript. Dr Sékaly, Dr Acuto and Dr Michel supervised this study. Dr Michel generated the CD28neg and CD28pos cell lines used in this study. Tanya Girard cloned the CD86-K4 construct and generated all CD86 stable cell lines utilized in this study. Geneviève Beaulé contributed to the generation of primary human dendritic cells. Tanya Girard made all figures. Tanya Girard wrote the article in collaboration with Dr Sékaly. Dr Michel and Dr Acuto reviewed the article prior to submission.

*Dr Holterman, a previous post-doctoral fellow in Dr Sekaly's laboratory, had generated the CD80 and CD86 wild type, deletion and chimeric constructs used in both chapters. Tanya Girard used the constructs expressed in Srα neo vectors or as PCR templates for their cloning into different vectors.

Editing of the thesis

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Original Contributions to Scientific Knowledge

The results presented in this thesis contribute to the advancement of scientific knowledge in the field of immunology. The key findings presented in this thesis are summarized below.

-CD80 is present as a population of multimers and monomers in live cells

-CD86 is present as monomers in live cells

-The IgC domain of both CD80 and CD86 molecules negatively impacts their multimerization capacity

-The intracellular domain of both CD80 and CD86 molecules does not impact their molecular structure but are required for full T cell activation owing to their cytoskeletal linkage

-The molecular structure of CD80 and CD86 correlates with their receptor binding properties and cosignaling function

-CD80 and CD86 are associated to the APC cytoskeleton through their intracellular domains

-CD86 is present in lipid rafts microdomains

-Lysine residues 265-268 (K4 region) of CD86 is a cytoskeletal association motif

-CD86 is important for early T cell events such as conjugate formation

-CD86 cytoskeletal association is not required for initial T cell events such as conjugate formation

-CD86 is localized at the immunological synapse upon T cell interaction in a cytoskeleton-dependent fashion

-CD86 cytoskeletal association is of critical importance for the initiation of T cell responses

-The APC cytoskeleton is important in the initiation of T cell responses

-CD86 physically associates with ERM proteins

List of Abbreviations

Ala	Alanine
APC	Antigen Presenting Cells
β2m	Beta-2-microglobulin
BCR	B cell receptor
BTLA	B and T lymphocyte attenuator
CD80dC/CD80ΔC	CD80 deleted of its IgC domain
CD86dC/CD86ΔC	CD86 deleted of its IgC domain
CD80dT/CD80ΔT	CD80 deleted of its intracellular domain
CD86dT/CD86ΔT	CD86 deleted of its intracellular domain
CDR	Complementarity-determining region
CIITA	Class II transactivator molecule
CLIP	Class II associated invariant chain peptide
cSMAC	Central supramolecular activation cluster
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAG	Diacylglycerol
DC	Dendritic cells
DD	Death domains
DIG	Detergent-insoluble glycolipid-enriched
dSMAC	Distal supramolecular activation cluster
ER	Endoplasmic reticulum
Erk	Extracellular signal-related kinase
ERM proteins	Ezrin/radixin/moesin proteins
Fab	Fragment antigen binding
FCET	Cytometry-based FRET
FRET	Fluorescence energy transfer
GEM	Glycolipid-enriched membrane microdomains
GILT	Gamma-interferon lysosomal thiol reductase
GM-CSF	Granulocyte/Macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule 1
ICOS	Inducible costimulator
iDC	Immature dendritic cells
IDO	Indolamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IgC	Constant-like immunoglobulin domain

IgSF	Immunoglobulin superfamily
IgV	Variable-like immunoglobulin domain
Ii	Invariant chain
IL	Interleukin
InsP3/IP3	Inositol-1,4,5-trisphosphate
IRAK	IL-1 receptor-associated kinase
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
Kd	Equilibrium dissociation constants
kDa	Kilodaltons
LAT	Linker for activation of T cells
Leu	Leucine
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
Lys	Lysine
MALT	Mucosal associated lymphoid tissues
MAMPs	Microorganism-associated molecular patterns
MAP	Mitogen activated protein
mDC	Mature dendritic cells
Met	Methionine
MHC	Major histocompatibility complex
MIIC	MHC class II compartments
MTOC	Microtubule organizing center
MTX	Methotrexate
MyD88	Myeloid differentiation factor 88
NAR	New antigen receptor
NF-kB	Nuclear factor-kappa B
NK cells	Natural killer cells
NOD	Nonobese diabetic
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PD1	Programmed death 1
Phe	Phenylalanine
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
Pro	Proline
PRR	Pathogen recognition receptors
pSMAC	Peripheral supramolecular activation cluster

PtdIns(3,4,5)P3/PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4,5)P2/PIP2	Phosphatidylinositol-4,5-bisphosphate
PTK	Protein tyrosine kinase
RAG	Recombination activating gene
SAP	SLAM-associated protein
SLAM	Signaling lymphocytic activation molecule
SLP76	SRC-homology-2-domain-containing leukocyte protein
TAP	Transporters associated with antigen processing
TCR	T cell receptor
TEM	Tetraspanins enriched microdomain
Thr	Threonine
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor-associated factor
Tregs	Regulatory T cells
tRNA	Transfer RNA
Tyr	Tyrosine
Val	Valine
V80C86T86	CD80 IgV domain /CD86 IgC and intracellular domain
V86C80T80	CD86 IgV domain /CD80 IgC and intracellular domain
WAVE	Wiskott–Aldrich syndrome protein
xMHC	Extended MHC locus

Chapter 1

Introduction

1.1 Immunity

Living organisms do not live in closed environments and are continuously assaulted by a plethora of foreign material. In humans, foreign material can be various bacterial, fungal, protozoan, parasitic and viral pathogens and even their own material that can potentially present some danger such as malignant cells. Immunity refers to the integration of all defensive measures put in place to protect the organism from these dangers.

The vertebrates' immune system is made up of two subsystems, the innate and the adaptive immune systems. Innate immune receptors are promiscuous and mediate fast responses against dangers. In contrast, the adaptive immune system mediates highly specific responses in a slower fashion through antigen receptors. Distinctively, adaptive immunity is characterized by the generation of memory lymphocytes that can confer faster protection upon reencounter with the same antigen. Innate immune mechanisms provide sufficient time to the host to mobilize the more slowly developing mechanisms of adaptive immunity. Both immune systems dialogue and their interaction is essential to mount efficient immune responses for organism protection.

Organism protection is offered through the intricate actions of immune cells and lymphoid organs that make up the immune system. Lymphoid organs can be divided into two categories: the primary and the secondary lymphoid organs. The bone marrow and the thymus are the primary lymphoid organs and it is where lymphocytes are generated. Secondary lymphoid organs are the sites where immune responses occur; they are specialized to trap antigen and are also important in lymphocyte maintenance. Lymph nodes, the spleen and mucosal associated lymphoid tissues (MALT) are all secondary lymphoid organs.

Immune cells constantly patrol the organism from blood to tissues and from lymph to blood. Immune cells are either of myeloid or lymphoid origin. The myeloid lineage is composed of granulocytes, macrophages, dendritic cells and

mast cells. The lymphoid lineage consists of T and B lymphocytes as well as natural killer cells. Although B and T lymphocytes originate in the bone marrow, only B lymphocytes mature there while T lymphocyte precursors migrate to and mature in the thymus.

1.1.1 Evolution of the immune system

Immunity is believed to have evolved from generalized to highly specific reactions (1). 500 million years ago, a transposition event involving a recombination activating gene (RAG)-bearing element is believed to have given rise to the rearranging antigen binding receptors only present in jawed vertebrates (1). This transposition event conveyed a significant selective advantage since the greater the amount of genetic variation, the more adaptable the host will be. This transposition event is considered the defining point in the emergence of adaptive immunity (2, 3). The most recent ancestor of the rearranging antigen binding receptors would have been an immunoglobulin superfamily (IgSF) member. It could have either pre-existed or given rise to an immunoglobulin V-region type of innate immune cell surface receptor as suggested by studies of the shark new antigen receptor (NAR) (4, 5). Innate immunity is generally considered to be the more phylogenetically ancient and does not share the specificity and memory aspects of adaptive immune responses. However, recent advances suggest that jawless vertebrates, protochordates and invertebrates can probably mount RAG-independent immune responses (6) and some evidence for memory in invertebrates has also been presented (7).

1.1.2 Innate immunity

The innate immune system encompasses a collection of rapid defenses that do not depend on specific antigenic recognition. The simplest form of protection consists of mechanical barriers such as the skin epithelium or mucosal surfaces that prevent entry of most pathogens. The low surface pH of the epithelium prevents pathogen entry by inhibiting bacterial growth and also produces peptides, such as defensins, that have antimicrobial and immunoregulatory properties. Genomic

evidence has shown the possible existence of many yet uncharacterized antimicrobial peptides underlying their important contribution to innate immunity (8). The mucous secreted by gastrointestinal, respiratory and urogenital tract membranes traps invaders and represent another protective barrier. Moreover, the human body presents a normal microbial flora that competes with pathogenic microorganisms for nutrients. Another level of protection brought about by the innate immune system is the complement system. Activation of the complement cascade by one of three distinct pathways leads to protective mechanisms such as microbial opsonization, phagocyte recruitment and microbial lysis (9).

The cellular elements that compose the innate immune system are the eosinophils, that mainly respond to parasitic infections, the basophils and mast cells that contribute to hypersensitivity reactions through the release of granules, and the phagocytic cell types (monocytes, macrophages, neutrophils, dendritic cells) that kill engulfed material. Natural killer cells (NK cells) are also important in innate immunity through their cytocidal activity against infected and malignant cells that present deregulated major histocompatibility complex class I (MHC class I) molecules at their surface.

Through the use of germline-encoded receptors that are not clonally distributed (10), the innate immune cells recognize a limited number of evolutionary conserved motifs in pathogens. These diverse pathogen-associated molecular patterns (PAMPs) are also found in nonpathogenic microorganisms and the term microorganism-associated molecular patterns (MAMPs) has recently been suggested (11). PAMPs, such as lipopolysaccharides (LPS), mannans, teichoic acids, denatured DNA, and bacterial DNA, can be categorized into lipid, protein, or nucleic acids (10, 11). These PAMPs are recognized by pathogen-recognition receptors (PRR) (10).

Toll-like receptors (TLR) are a particularly important group of the PRR. Ten TLR have been identified in humans (10, 11). TLR are type I membrane proteins with

an extracellular domain presenting a structure called the leucine-rich repeat that is involved in ligand recognition. Intracellularly, most TLR contain a TIR (Toll/interleukin-1R) domain that interacts with the adaptor protein MyD88 (myeloid differentiation factor88) that couples with the serine/threonine IRAK (IL-1 receptor-associated kinases) leading to signal transduction. Each TLR activates similar signaling pathways, but some TLR trigger their specific pathways depending on which cytoplasmic adaptors they associate with. Regardless of the specific pathway induced, most TLR signals leads to the activation of a master switch in inflammation induction: transcription factor nuclear factor-kB (NF-kB) (12). NF-kB induction will trigger the release of pro-inflammatory cytokines including IL-6, IL-12 and TNF-α (11), antimicrobial peptide secretion and direct pathogen killing. The secreted pro-inflammatory cytokines will activate surrounding cells to produce chemokines or adhesion molecules helping in the recruitment of inflammatory cells into the infection sites.

1.1.3 Adaptive immunity

Unlike innate immunity that makes use of a fixed repertoire of inherited receptors, the potency of adaptive immunity resides in its capacity to generate billions of different antigen receptors from multiple gene segments assembled by somatic recombination to create unique antigen receptors capable of recognizing virtually any antigen. T and B lymphocytes are the cellular elements of the adaptive immune system. The generation of the T cell antigen receptor (TCR) and the B cell antigen receptor (BCR) is a complex process that creates an impressive repertoire through combinatorial joining. RAG enzymes initiate gene rearrangement. TCR are somatically rearranged from variable, diversity and joining gene segments to generate VαJα and VβDβJβ chains. A similar process than that used for the TCR brings about the BCR variability. Following rearrangement and selection, T and B cells leave the thymus and bone marrow and circulate within the body. Prior to antigen encounter, lymphocyte homeostasis involves short-lived serial contacts of low signal intensity with dendritic cells in the lymph node providing sub-threshold survival signals (13). Following antigen

exposure and response, some T and B cells will persist within the organism to provide rapid and specific responses upon antigen reencounter, a hallmark of adaptive immunity known as immunological memory.

1.1.3.1 T cell memory

Distinctively, adaptive immunity is characterized by its capacity to generate memory lymphocytes that have been clonally expanded following antigen encounter and that persist within an organism to provide rapid and specific responses to re-infection. Memory T cells can be divided into non-polarized or polarized phenotype, namely central memory and effector memory cells (14). Central memory T cells are non-polarized cells that express lymph node homing receptors such as CCR7 and that primarily migrate between blood and lymph nodes in a pattern similar to that of naïve T cells. Central memory T cells serve primarily as long-lived reservoirs of immunological memory. When stimulated with antigen, these cells give rise to additional central memory cells as well as effector memory T cells. Effector memory T cells are polarized, terminally differentiated T cells that are shorter lived and provide immediate protection to pathogens in peripheral tissues (14).

1.1.4 Dendritic cells: linking innate and adaptive immunity

Effective immune responses involve the concerted action of the innate and adaptive immune systems through the sentinel function of dendritic cells (DC). DC develop in the bone marrow and migrate in an immature form to the peripheral tissues where they will take up antigens from the environment. These cells express high levels of most TLR and dangers such as exposure to PAMPs and inflammatory mediators (15) will induce their migration to lymphoid organs via the blood or the lymph, a property which has not been described for other APC. Danger detection by DC will also lead to their maturation. DC maturation involves antigen processing and presentation in the context of major histocompatibility complex class II (MHC class II) molecules. DC have the capacity to present antigens encountered in peripheral tissues due to the dramatic

shift in MHC class II half-life that allows accumulation and persistence (over 100 h) of the peptide:MHC complexes formed (16, 17). Maturation also induces upregulation of costimulatory molecules expression and only costimulation-suited mature DC (mDC) can activate the two cellular elements (T cells and B cells) of the adaptive immune system (18). In secondary lymphoid organs, antigen-loaded DC will encounter antigen-specific T cells and initiate adaptive immune responses. DC are therefore crucial in linking innate to adaptive immunity (18) and this linkage allows proper protection of the organism.

1.2 The major histocompatibility complex

The MHC is the most important genetic region in the human genome with respect to both innate and adaptive immunity. Roughly, a third of the expressed transcripts identifiable within the MHC fall into the following immune functions: antigen processing, antigen presentation, immunoglobulin superfamily, inflammation, leukocyte maturation, complement cascade, non-classical MHC class I receptor family, immune regulation and stress response (19). The variety of immune functions encoded at that region might explain its association with hundreds of immune related diseases (20). The MHC region is located on the short arm of the human chromosome 6 (6p21.3). The first gene map of human MHC (classical MHC map) was published in 1999 and was shown to cover approximately 4 Mbp of DNA within the human genome (21). The presence of MHC relevant genes beyond the set MHC boundaries led to the idea of an extended MHC. The MHC locus as now been extended (xMHC) and spans 7,6 Mbp (19, 22, 23). The main gene clusters associated with the MHC are tRNAs (157 genes), histones (66 genes), zinc fingers (36 genes), olfactory receptors (33 genes), class I genes (26 genes) and class II genes (24 genes) (19). The fact that tRNAs and histones are the two largest clusters in the MHC indicates that this genome region is an expression hotspot. Because class I and class II molecules were first identified on the surface of human leukocytes, they are also referred to as human leukocyte antigen (HLA). HLA class I and HLA class II regions encode for cell surface glycoproteins responsible for antigen processing and presentation.

1.2.1 HLA class I gene cluster

The HLA class I cluster comprises the highly polymorphic classical class I genes (*HLA-A, -B and -C*), the less polymorphic non-classical class I genes (*HLA-E, -F, -G* and 12 pseudogenes) and the class I-like genes (*MICA, MICB*, and 5 pseudogenes) (19). MHC class I α chain alleles are co-dominantly expressed; individuals homozygous for all three class I loci (HLA-A, B and C) express three different MHC class I while heterozygous individuals can express six.

1.2.1.1 MHC class I molecule

The classical MHC class I molecules are expressed at the surface of all nucleated cells. The main function of MHC class I molecules is to present cytosolic antigens to CD8 T cells. MHC class I proteins are heterodimeric in structure. They consist of the non-covalent association between a polymorphic α chain and a small monomorphic soluble protein called β2-microglobulin (β2m). The α chain is composed of three extracellular domains, α1, α2 and α3. It is the fold between the α1 and α2 domains that creates a cleft in which the antigenic peptide lies. Polymorphism is mainly concentrated in the peptide-binding cleft thereby affecting the array of peptides that can be presented to T cells. The MHC class I cleft can accommodate peptides of 8 to 10 residues although nonamers have been shown to be favored (24, 25). Peptides binding to a specific class I allele show some conserved anchor residues.

1.2.1.2 Peptide processing and loading of MHC class I molecules

MHC class I molecules usually display self-peptides derived from self-proteins that were targeted to proteasome-mediated degradation. The 26S proteosomal complex is composed of a 20S catalytic subunit and fifteen regulatory subunits known as the 19S regulatory complex (26). In the presence of inflammatory cytokines such as TNFα and IFNγ, three proteolytic subunits are replaced to form the immunoproteasome (27). The formation of such a complex enhances antigen

processing efficiency (27). The peptides generated in the cytoplasm from the proteasome are picked up from an ER (endoplasmic reticulum) resident transmembrane heterodimeric complex called TAP (transporters associated with antigen processing) complex. This complex drives peptide translocation from the cytosol to the ER lumen where newly synthesized MHC class I molecules await loading. Within the ER lumen, MHC class I complexes are bound to a chaperone named calnexin. Once the α chain binds to β2m, two other chaperones called calreticulin and tapasin bind to the newly formed heterodimeric complex. Tapasin links the TAP transporter to the MHC class I complex, catalyzing peptide loading in the MHC peptide binding groove (28, 29). The peptide loaded MHC class I molecules can then exit the ER through the Golgi to the plasma membrane following the default secretory pathway. The MHC class I: peptide complexes are now ready to interact with CD8 T lymphocytes.

1.2.1.3 Non-classical MHC class I molecules

The non-classical MHC class I molecules have variable function, expression and distribution. HLA-E and HLA-G bind to NK specific receptors. This MHC class I innate immunity linkage is also shown by molecules such as MICA and MICB, which are upregulated by stress signals and that activate cells through the NKG2D receptor (30).

1.2.1.4 CD1 molecules

CD1 molecules are MHC-like molecules that bind β2m. In contrast to MHC class I molecules CD1 molecules (CD1 a to e) bind to lipid-based molecules thereby diversifying immune recognition.

1.2.2 Antigen presenting cells

APC are cells that collect and cleave antigens to present them in the context of MHC class II and that constitutively or inducibly express cosignaling molecules. The three types of APC, dendritic cells, B cells, and macrophages, differ in many aspects such as their morphology, their actin cytoskeleton structure and their cell

surface molecules expression. APC also produce different amounts of pro-migratory chemokines and although expression-profiling studies do not allow direct comparison of chemokine production by different types of APC, it is clear that activated DC and macrophages, but not B cells, can generate a pro-migratory environment that attracts passing leukocytes (31).

1.2.2.1 Dendritic cells

DC have diverse shapes with long actin-rich dendrites. Immature DC (iDC) engulf pathogens and deliver them to the nearest lymph node for T cell presentation. iDC express intermediate levels of MHC class II molecules and cosignaling molecules and upon maturation, upregulate the cell surface expression of both types of molecules by three to tenfold (17). As shown by inhibition studies, actin dynamics in DC, but not in other APC, are essential for immune response initiation (32-35).

1.2.2.2 Macrophages

Macrophages are round, phagocytic cells that rearrange their actin cytoskeleton upon the crosslink of their innate receptors initiating signaling that leads to pathogen engulfment. The ingestion of pathogens also activates antimicrobial killing mechanisms and stimulates the production of inflammatory mediators. Macrophages express low levels of MHC class II and cosignaling molecules prior to activation, an expression that is strongly upregulated after maturation in the presence of IFNγ and GM-CSF (36, 37).

1.2.2.3 B cells

Naïve B cells are round cells with a simple cortical actin cytoskeleton but activation leads to a more complex cytoskeleton (38). B cells principally function as APC in secondary lymphoid organs where class II restricted antigen presentation enables the cognate B:T cell interactions required to elicit T cell dependent humoral immunity (39). Naïve B cells express low levels of MHC and cosignaling molecules at the surface, but this expression is strongly upregulated

after maturation. Contrary to the two other APC types, B cells are not actively phagocytic. Rather, they use their BCR for antigen recognition and engulfment. The BCR is a membrane bound immunoglobulin (Ig) coupled to a non covalent Igα and Igβ heterodimer. Igα and Igβ signaling leads to B cell activation and triggers antigen internalization via clathrin coated pits (40).

1.2.3 The HLA class II gene cluster

The HLA class II cluster comprises the highly polymorphic, classical class II genes (*HLA-DP, -DQ, -DR* and pseudogenes) and the less polymorphic non-classical class II genes (*HLA-DM* and *-DO).*

1.2.3.1 MHC class II molecules

Unlike MHC class I, which is present on all nucleated cells, MHC class II molecules are mainly found on APC. They can also be found constitutively on other cell types such as the thymic medulla or cortical epithelial cells, following IFNγ induction on fibroblasts, mast cells, and endothelial cells or following activation on T cells (41). The main function of MHC class II molecules is to present exogenous antigens to CD4 T cells. MHC class II proteins are heterodimeric in structure. They consist of the non-covalent association between an α chain and a β chain. In contrast to the monomorphic α chain, β chain alleles of each locus (HLA-DR, DP, DQ) are polymorphic and co-dominantly expressed. Both the α and β chains are composed of two extracellular domains, transmembrane domains and short cytoplasmic tails. It is the membrane distal domains of both chains (α1 and β1) that form the peptide-binding groove. In contrast to MHC class I binding cleft, the peptide binding groove of MHC class II is open at both end and can accommodate peptides of 12 to 30 amino acids, often encompassing a core sequence (24), with a preferred size of 15 amino acids (24, 25, 42).

1.2.3.2 Non-classical MHC class II molecules

The less polymorphic non-classical class II genes are not expressed on the cell surface but are involved in peptide exchange and loading on class II molecules (43). HLA-DM is expressed by the same cell types as the classical MHC II molecules while the expression of HLA-DO appears to be restricted to B lymphocytes, subsets of DC, and cortical and medullary epithelial cells of the thymus (44).

1.2.3.3 Peptide processing and loading of MHC class II molecules

MHC class II molecules present extracellular antigens degraded through the endocytic route. Access to the endocytic route can occur in multiple ways depending on which APC engulfed antigen (39). The endocytic route can be divided in three main compartments: early endosomes, late endosomes and lysosomes. Reduction and cleavage of antigen in early and late endosomes involves enzymes such as gamma-interferon lysosomal thiol reductase (GILT), endopeptidases and exopeptidases (41). In lysosomes, antigen processing occurs through cysteine proteases called cathepsins. MHC class II transactivator molecule (CIITA) regulates the expression of MHC class II molecules. The α and β chains are co-translationally inserted into the membrane of the ER where they will form heterodimers. These heterodimers will interact with the nonpolymorphic invariant chain (Ii) predominantly through its CLIP (class II associated invariant chain peptide) domain (45) that binds to MHC class II molecules in a similar manner than antigenic peptides (46). This binding favors proper folding of class II molecules and prevents the binding of endogenous peptides to class II molecules. Properly folded nonameric complexes composed of three heterodimers bound to an Ii trimer will be exported from the ER to the Golgi. Golgi resident nonameric complexes will then be targeted to endosomal organelles due to sorting signals present in the cytoplasmic domain of Ii (47). These endosomal organelles are referred to MHC class II compartments (MIIC). In MIIC, Ii will be cleaved resulting in the release of MHC class II heterodimers bound to CLIP. The MHC class II/CLIP complexes will then be transported to other organelles where HLA-

DM is present. HLA-DM catalyzes the dissociation of CLIP, stabilizes the now empty MHC class II and assist in peptide selection favoring peptides with high affinity (48). HLA-DO can assist the peptide editing function of HLA-DM in B cells, although its exact role is still debate matter (44). Finally, peptide loaded MHC Class II molecules exit and are transported to the cell surface where they can fulfill their function of presenting antigen to CD4+ T cells.

1.2.3.4 MHC class II signaling

Apart from their main antigen presentation function, growing evidence demonstrate a signaling role for MHC class II proteins. Indeed, upon MHC class II ligation, intracellular signaling pathways impacting cellular adhesion (49), cytokine gene expression (50, 51), and proliferation, maturation and apoptosis of APC are induced (52). MHC class II localization in specific plasma membrane compartments and its interaction with given receptor-associated molecules are key in this signal transduction function (53). Although no known signaling motifs have been found in the short cytoplasmic tail of MHC II molecules, the transmembrane and cytoplasmic regions of these proteins have been shown to signal by coupling to other receptor-associated effector molecules for signal transduction (54). Moreover, the intracellular domains of HLA-DR molecules are important for cytoskeletal association following oligomerization (55). In B cells, MHC class II molecules associate with CD79a/CD79b, CD20, and CD19, and in monocytes, a CD18/MHC class II complex has been identified (54, 56, 57). Recently MHC class II associations with other cell receptors were reported in DC (53). Moreover, several studies have shown the importance of lipid rafts in regulating MHC class II signaling and APC activity and raft-associated MHC class II molecules were shown to accumulate at the IS and facilitate T cell activation (58-60). Disruption of lipid rafts impairs MHC II peptide presentation. The proportion of MHC II molecules constitutively localized to lipid rafts appears to vary depending on the APC type and its activation status (53). The translocation of MHC II molecules into lipid rafts occurs independently of their intracytoplasmic domains (60). MHC class II triggering activates intracellular

tyrosine phosphorylation through Src family tyrosine kinases. In addition, MHC class II signaling regulates the activity of the MAP (mitogen activated protein) kinases, such as Erk (extracellular signal-related kinase) in human monocytic and B cell lines (56, 60) and p38 in monocytes (61). PKC (protein kinase C) mediated signaling is also important for MHC class II mediated actin polymerization and cytoskeletal organization (53, 62). MHC class II signaling causes actin reorganization in mature DC (63), contributing to the formation of the immunological synapse (IS) during DC:T cell interactions. Finally, MHC class II signals appears to maximize the delivery of MHC class II: peptide complexes to the surface during the DC:T cell interaction (32).

1.2.3.5 αβ T cells recognize MHC:peptide complexes

Two populations of T cells distinguished on the basis of TCR expression exist. γδ T cells represent about 5% of all circulating T cells and are particularly enriched in epithelial-rich tissues such as the skin, intestine and reproductive tract (64). These cells can undergo gene rearrangement in the thymus but most of them do so in an extrathymic compartment (64). In contrast to the other type of T cells, γδ T cells are not MHC restricted and recognize soluble proteins and non-protein antigens of endogenous origin. The major class of T cells recognizing peptide antigens in complex with class I or class II MHC proteins are T cells expressing an αβ TCR. These cells can be further divided into two main classes, i.e. CD4 expressing T cells that regulate the cellular and humoral immune responses and cytotoxic CD8 T cells that are responsible for killing of cells infected intracellularly and that present peptide:MHC class I complexes at the cell surface (65). CD4 T lymphocytes and CD8 T lymphocytes have different but complementary functions during immune responses. CD4 constitute roughly 60% of T cells present in the blood and secondary lymphoid organs while CD8 represent around 40%. The work presented in this thesis is concentrated on αβ T cells of the CD4 lineage.

1.3 Naïve T cell activation

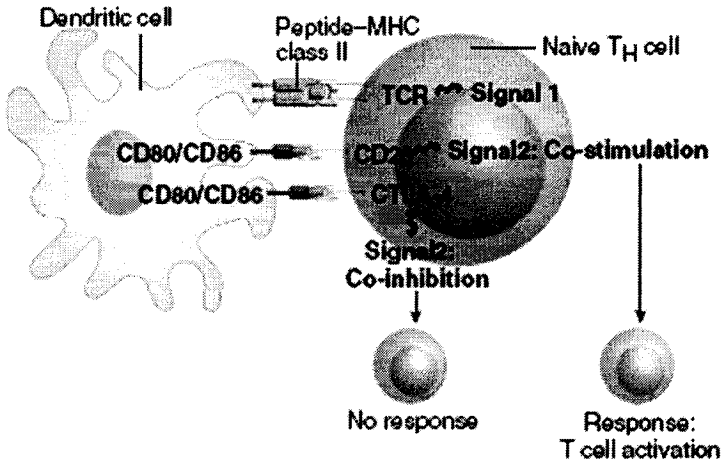

Figure 1 The Two Signal Model (from (1)) TCR Interaction with peptide-MHC class II presented by dendritic cells acts as signal 1 for T cell activation. Signal two consists of co-receptor and co-ligand interactions and can be either positive, leading to cell activation, or negative, leading to co-inhibition.

1.3.1 Two signal theory of T cell activation

The 'two signal' concept of lymphocyte activation was first proposed by Bretscher and Cohn and refined by many scientists (reviewed in (66)) trying to explain discrimination of self from nonself (67, 68). When the theory was first proposed, neither the TCR nor any cosignaling receptor–ligand interaction had been identified. As depicted in figure 1, the two signal theory postulates that two signals are required for activation of naïve T cells. Because T cell activation by costimulation is more complex than originally envisioned, the two signal model somewhat oversimplifies the contribution of each signal; however the essence of this model remains suited for the understanding of T cell activation.

1.3.1.1 Signal 1: the antigen specific signal

Signal 1 is an antigen specific signal that occurs when the TCR binds to antigenic peptides presented by MHC molecules. A single TCR can recognize structurally distinct MHC peptide complexes (69). The overall structure of the αβ TCR is composed of two chains, the α and β chains, that exhibit an IgV-like and IgC-like domain architecture reminiscent of the IgSF and are linked by a disulfide bond in

the hinge region, close to the membrane. Upon binding to a peptide/MHC, conformational changes are induced in the hypervariable loops (CDR loops) of the TCR (70). CD4 and CD8 co-receptors also bind to MHC/peptide along with the TCR and affect the qualitative nature of the binding (70). Differences in binding affinity, kinetics, or surface density, can lead to very different T cell responses. T cells have the capacity to detect as few as 10 specific peptide–MHC ligands on cells (71, 72) and even when presented with very low levels of peptide–MHC, a substantial proportion of TCR are ligated in the process of T cell activation (73). Delivered alone, signal one does not results in T cell activation; rather it leads to a state of unresponsiveness called anergy (74).

1.3.1.2 Signal 2: cosignaling

Signal 2 involves the interaction of a cosignaling receptor at the T cell surface with its counter receptor at the APC surface. Cosignaling molecules are cell surface molecules that cannot functionally activate T cells on their own, but rather amplify or counteract signals provided by signal 1 (75, 76). While prolonged TCR signaling is necessary for T cell activation, a recent report has shown that persistent cosignaling is also required to allow naïve T cells activation (77). Cosignaling molecules either belong to the IgSF (including the CD28, the B7 and the SLAM/SAP sub-families) or the TNF superfamily. Numerous receptor-ligand pairs in each of these two families can play a co-activating or co-inhibiting role at various T cell activation stages (78). The balance between negative signals from co-inhibitory receptors and positive signals from co-stimulatory receptors therefore dictates the ensuing response. Two interacting pairs are key in naïve T cell responses: the co-activating pair CD28:CD86 and the key co-inhibiting pair CTLA-4:CD80.

1.3.2 Primed T cells activation

In contrast to naïve T cells, primed T cells have a low activation threshold (79). A short TCR stimulation equivalent to about 30 minutes of receptor occupancy in the absence of CD28 engagement is sufficient to trigger proliferation and cytokine production; an even shorter stimulation triggers cytotoxic CD8 T cell responses (79, 80).

1.3.3 Positive and negative selection

During the recombination process that generates the antigen receptors, many unfit TCR and BCR are generated. During T cell development in the thymus, thymocytes move across antigen-presenting thymic epithelial cells in the cortical epithelium of the thymus where they are tested for their affinity for self-MHC molecules; this process is known as positive selection (81). During positive selection, CD4/CD8 lineage choice is determined by the duration of TCR engagement (82, 83). Cosignaling via CD80 and CD86 molecules expressed on thymal dendritic and medullary epithelial cells was also suggested to regulate CD4 and CD8 T cell differentiation since CD80/CD86 deficient animals showed CD4 skewing while CD80 or CD86 transgenic animals showed CD8 T cell skewing (84). Positively selected cells then go through a negative selection round by DC residing in the thymic medulla which removes self-specific T cells (85). B cells differentiation takes place in the bone marrow where somatic recombination occurs. This process takes place in the absence of antigen; it is only in peripheral tissues that B cells can further mature under the influence of T cell help and antigen where they can isotype switch and affinity mature by acquiring somatic hypermutations (86).

1.3.4 Central and peripheral tolerance

The elimination of autoreactive B and T cells in the thymus and bone marrow through negative selection is called central tolerance. Some self reactive cells can escape this negative selection if their MHC:self-peptide recognition is not of high enough affinity and also because not all self-antigens are presented in the thymus

or bone marrow. Tolerance in the periphery complements central tolerance and helps in autoimmunity prevention. Peripheral tolerance can occur due to the lack of costimulatory signaling that leads to deletion or anergy of the responding T cell (87) or through additional subsets of cells including DC and regulatory T cells (Tregs) (88). Two main categories of CD4+ Tregs exist (89). The first type is the naturally occurring Foxp3-dependent CD4+ CD25+ that are produced in the thymus and expresses high levels of CTLA-4 (89). CD28/B7 interactions are of crucial importance in the control of CD4+ CD25+ Tregs homeostasis (90). Studies have also indicated that CTLA-4 has a crucial role in regulating peripheral tolerance as a consequence of CTLA-4–CD80/CD86 interactions (91, 92). The second type of Tregs are the inducible Tregs and are Foxp3-independent, secrete IL-10 and occur following tolerogenic encounters in the periphery (89). Such tolerogenic encounters might involve bi-directional CD80/CD86:CTLA-4 signaling (from DC: Treg interactions), inducing tolerogenic tryptophan catabolism (93, 94).

1.3.5 The immunological synapse

Cellular interactions between migrating T cells and mDC in lymphoid organs leads to the onset of morphological changes, cell surface receptors recruitment at the interaction interface, remodeling of the cytoskeleton and signaling, ultimately leading to the transcription of specific genes. During the interaction, receptors at the interacting APC surface also become engaged and transmit signals that lead to APC maturation or death. The term immunological synapse (IS) refers to the contact site between an interacting T cell and an APC since signal exchange between the two immune cells is reminiscent of neuronal synapses (95). In original descriptions (96, 97) only contact interfaces exhibiting specific rearrangement patterns were termed IS. Nowadays, molecular segregation within a contact interface is not a prerequisite for it to be referred to as IS and several unsegregated IS have been shown to transmit a full range of signals such as the non-specific IS, the early IS and the IS formed between naïve T cells and DC (98).

The interaction mode is determined, in part, by the type and activation state of the APC and where the IS is formed. The type and duration of the cellular interaction seems to be inversely correlated with the APC activation potency and cytoskeletal activity. As previously stated, the most recognized form of synapse consists of receptor enrichment at the contact site. This pattern represents the mature stage of an IS. In a mature synapse, a central structure called the central supramolecular activation cluster (cSMAC) is enriched with TCR and CD28 and other signaling receptors (99). Surrounding the cSMAC is the peripheral SMAC (pSMAC), mainly enriched with adhesion molecules (99). Recent reports have characterized other markers for the pSMACs such as VLA4 (100), ADAP (101) and the transferrin receptor (102). The distal zone of the synapse where no cellular interface is formed is referred to as the distal SMAC (dSMAC) and is known to be enriched in CD45 (103).

In original descriptions, the main function of the IS was believed to be signaling initiation. It now appears clear that signaling is actually initiated prior to IS formation (104) and the cSMAC main function is to favor a cosignaling environment. The synapse generates a microenvironment favoring secondary events such as costimulatory and cytokine signaling and plays a central role in the delivery of full effector function via directed secretion (105, 106). However, some reports indicate that the cSMAC may enhance TCR signaling induced by low affinity agonists (107). Clearly, the role of the synapse might differ depending on what type of antigen is being presented. The IS is dynamic in nature as shown by studies demonstrating that a T cell will detach from an APC when presented with an alternative APC presenting higher antigen amounts (108).

Although the IS has mainly been studied between APC and CD4 T cells, synapse structures have also been described for CD8 T cells (109-111) and for natural killer cells, and in some cases in the absence of antigen (98). During cytolytic synapses, the narrow cleft formed between the membranes of interacting cells,

allows for the directional secretion of granules containing granzymes, perforin, and lysosomal proteins.

1.3.5.1 The cytoskeleton and the IS

As in neuronal synapses, the cytoskeleton plays a crucial role in cellular communication (95). At the IS, the cytoskeleton plays two main function. It first helps in its formation by regulating movement of molecules and membrane domains and secondly, it serves as a scaffold for signaling platforms to build on (103, 112). The contact between T cells and APC leads to actin and microtubule cytoskeleton polarization and transport of intracellular vesicles, cell surface receptors and signaling molecules to the contact site (113, 114). The portion of the actin cytoskeleton and its associated proteins that lies just beneath the plasma membrane is referred to as the cortical cytoskeleton and is important in IS formation and maintenance.

1.3.5.1.1 ERM proteins

ERM (ezrin, radixin, moesin) proteins are a major component of the cortical cytoskeleton and are involved in membrane-cytoskeletal associations (figure 2) (115). These proteins are particularly important in IS formation and maintenance (116-120). Following antigen recognition, ERM proteins have been shown to be rapidly and transiently dephosphorylated in T cells disconnecting the cortical actin cytoskeleton from the plasma membrane. Such dephosphorylation favors T cell and APC conjugation due to the decreased rigidity of the interacting T cell. This dephosphorylation was observed maximally within the first minute of TCR engagement followed by rephosphorylation of ERM proteins within 3 minutes (121). ERM proteins have been shown to be clustered at the IS in their active serine/threonine phosphorylated form and to localize in lipid rafts following TCR and/or CD28 triggering (117). ERM proteins have been suggested to form an anchor for lipid raft associated signalosomes (117).

Two conformational states have been described for ERM proteins and are shown in figure 2: a folded dormant form, soluble in the cytoplasm, and an unfolded active state that link transmembrane receptors to the cortical actin cytoskeleton. These conformation changes are regulated by phosphorylation of a conserved threonine residue in the C-terminal domain of the proteins. This phosphorylation disrupts the intramolecular association between the N- and the C-terminal domains leading to unfolding of the proteins. The C-terminal domain contains an actin-binding site, whereas the N-terminal domain interacts with the cytoplasmic domains of different molecules, including CD44, CD43, ICAM-1, ICAM-2, ICAM-3, and VCAM-1 (115). Most of the ERM interacting proteins contain a positively charged amino acid cluster at a juxtamembrane position that determines their association with ERM proteins. ERM molecules have also been implicated in cell signaling upon tyrosine phosphorylation of their intracellular domain (117, 122-126).

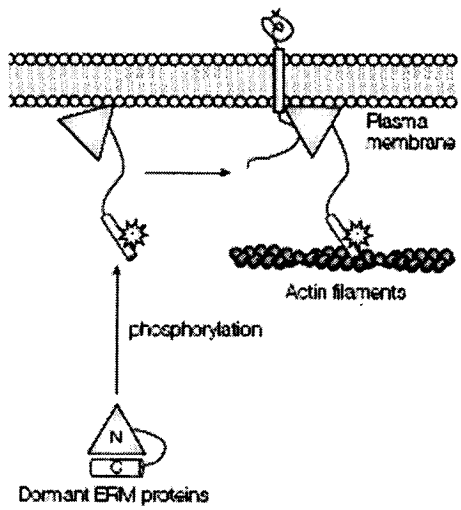

Figure 2 ERM Proteins Link Transmembrane Receptors to the Cytoskeleton **(adapted from (127))** ERM proteins exist in a dormant form in the cytoplasm. Phosphorylation of their C terminal portions recruits ERM proteins to the plasma membrane. Activated ERM proteins can then associate with transmembrane proteins linking them to the cortical cytoskeleton.

1.3.5.2 Membrane microdomains in the IS

The plasma membrane is not a homogenous lipid bilayer and contains various microdomains such as lipid rafts and tetraspans that can impact immune cell function. Lipid rafts (also known as GEMs and DIGs) are areas of the plasma membrane that are rich in cholesterol, glycosphingolipids, signaling proteins and GPI (glycosylphosphatidylinositol)-anchored proteins. These lipid microdomains are thought to play an important role in the localization of signaling proteins to the IS. For example, the TCR, its coreceptor CD4, and the Src kinases Lck and Fyn are recruited to these microdomains during T cell activation (128-130). Cosignaling events also mediate raft aggregation to the site of the synapse (131). APC molecules such as MHC class II and CD86 have also been shown to localize to rafts (132, 133). Tetraspan proteins (e.g. CD81, CD82, CD9, CD20, CD63) are ubiquitous membrane proteins that have the ability to interact with themselves, allowing them to organize a functional microdomain named the tetraspanin web or tetraspanin-enriched microdomain (TEM). Several proteins, including MHC class II, CD80 and CD86 have been shown to localize in tetraspans (134-136). An interplay has even been shown between tetraspans and lipid rafts in APC (137). Tetraspans can indeed serve as links between membrane receptor complexes, lipid rafts and the actin cytoskeleton (138, 139) Plasma membrane compartmentalization certainly increases antigen presentation, signaling activity and binding avidity of resident proteins and impact immune cell function.

1.3.6 Steps in immunological synapse formation

Five main steps leading to IS formation can be distinguished (98, 140). Progression through all of these phases requires sustained MHC:peptide-TCR interaction, sustained cosignaling, sustained membrane-proximal signaling and an intact cytoskeleton. An overview of the IS and its key receptor interactions and signaling events is presented in figure 3.

1.3.6.1 First step: cellular scanning, contact acquisition and adhesive arrest

In lymphoid organs, naïve CD4 T cells undergo a series of short-lived dynamic interactions with different DC, a process known as scanning (16, 87). During the scanning process, the cortical actin cytoskeleton of T cells is less tethered to the plasma membrane, allowing more efficient conjugation between the two cell membranes (121). When moving T cells recognize cognate peptide: MHC complexes, a transient arrest in migration, known as the stop signal, occurs (141) and leads to the arrest of adhesion-based interactions.

1.3.6.2 Second step: TCR signaling and early IS assembly

TCR signaling precedes IS formation (104) and TCR induced signaling leads to early IS assembly. The TCR is associated to the CD3 complex invariant accessory chain complex (CD3γ, CD3δ, and CD3ε and CD3ξ chains) that is responsible for signal transduction upon binding of the MHC: peptide complex at the APC surface. Within the first few seconds of contact, calcium signaling is detected (142) and its duration and intensity is a direct function of the strength of TCR signaling (143). PI3K activation and signaling is also an early event of TCR signaling (144-146). The cytoplasmic domains of the CD3 chains contain immunoreceptor tyrosine-based activation (ITAM) motifs that are phosphorylated by Lck and Fyn leading to an activation cascade involving Zap70, LAT and SLP76 (104). Activation of these proteins leads to the stimulation of PLC (phospholipase C) and activation of PKC and MAPK (99, 147). Activation of the small GTPases Ras and Rho promote actin dynamics in the contact zone (148). Altogether, T cell signaling will lead to the activation of genes important for lymphocyte proliferation and differentiation through the action of transcription factors NF-kB, AP1 and NFAT. Interactions with agonist peptide: MHC complexes, but not antagonist or null peptide favors stable and prolonged T cell-APC interactions (58, 147).

1.3.6.3 Third step: IS maturation and receptor segregation

Upon continuous T cell:APC interaction, a mature IS forms at the cellular junction (99, 144) leading to the enrichment of given receptors such as the TCR, CD28 and cytokine receptors in the cSMAC. A pSMAC composed of adhesion molecules, and a more distal zone, the dSMAC, can also be observed (103).

1.3.6.4 Fourth step: TCR internalization

After sustained signaling, the TCR is internalized from the cSMAC into cytoplasmic vesicles, limiting its availability at the interaction interface. Along with co-inhibitory signaling, this results in a reduction of signaling intensity and, ultimately leads to the fifth phase of the IS.

1.3.6.5 Fifth step: IS dissolution

The processes that control IS resolution and T cell detachment are unclear. The proposed mechanisms include activation-related TCR internalization (149); upregulation and recruitment of coinhibitory receptors such as CTLA-4 (150, 151) expression of chemokines or chemokine receptors that reduce adhesiveness and initiate T cell motility (152) and redistribution of competing adhesion molecules, such as CD43 into the contact zone. During detachment, portions of the T cell membrane can remain attached to the APC (and vice versa), possibly causing focal zones of continued signaling (153).

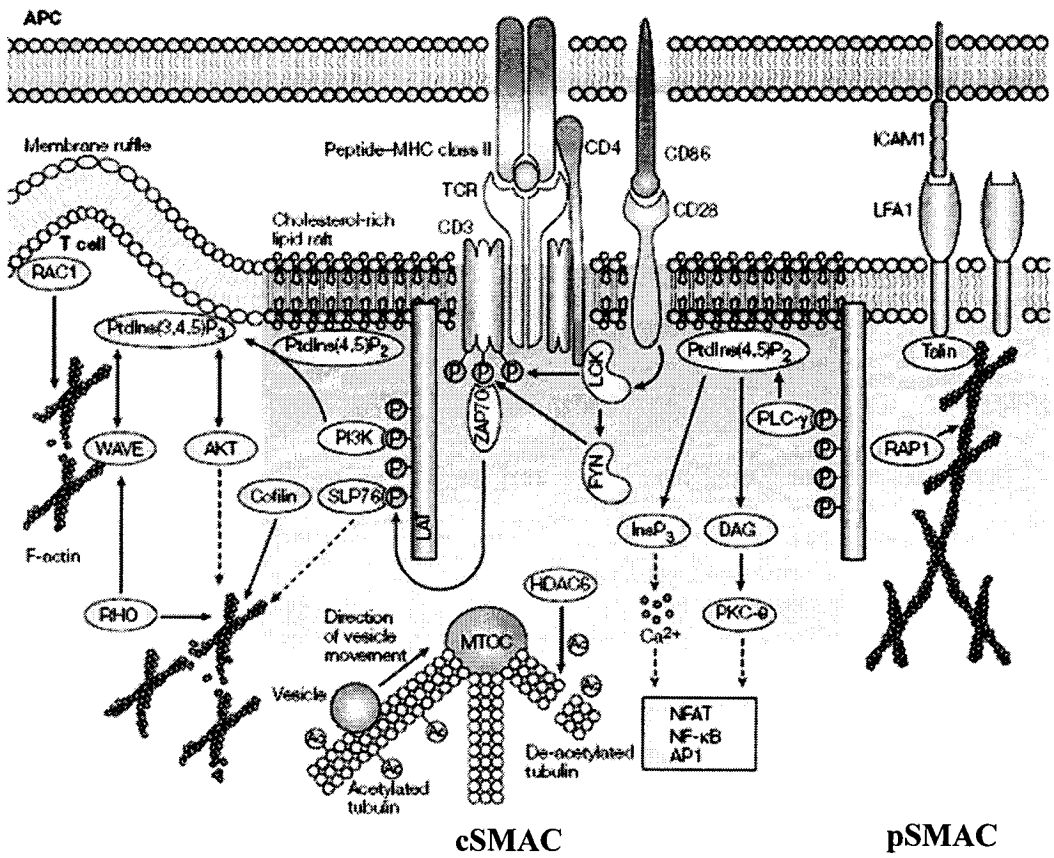

Figure 3 Immunological Synapse and Signaling (adapted from (98)) Following TCR triggering by peptide–MHC complexes, Lck relocalizes to lipid rafts and becomes activated. Lck activation is enhanced through co-stimulation by interactions between CD28 and CD86. Activated Lck engages Fyn through tyrosine phosphorylation, and together, Lck and Fyn phosphorylate ITAMs present in various molecules such as the CD3 chains and ZAP70 that will function as docking sites for adaptor and signaling proteins. ZAP70 phosphorylates the raft-associated adaptor protein LAT, which will serve as a platform for signaling molecules, including PLC, PI3K and SLP76. PLC-liberates inositol-1,4,5-trisphosphate (InsP3) from membrane phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). DAG activates protein PKC-which is involved in the activation of the downstream transcription factors NF-κB and AP1. In regions of more stringent adhesion, such as the pSMAC, talin links interactions between LFA1 and ICAM-1 to the actin cytoskeleton. As well as actin filaments, microtubules and the MTOC polarize towards the immunological synapse and form a scaffold for vesicle transport and signaling molecules.

1.3.7 The IS from an APC point of view

The organization of proteins and the cytoskeleton on the APC side of the IS is less well defined. However, increasing evidence show that APC play an active role in IS formation and maintenance (154). APC provide multiple signals for T cells that are dependent on the APC cytoskeletal activity (34, 35). Although CD28 accumulates at the IS without any interaction, *Pentcheva-Hoang et al.* (155) have shown that its counter-receptor at the APC surface, CD86, stabilizes it at the IS. On the other hand, CD80 preferentially recruits and stabilizes CTLA-4 to the synapse. Moreover, *Tseng et al.* (156) showed that ligand stability on the APC side can also affect receptor recruitment since CD80 deleted of its intracellular domain fails to recruit CD28, CTLA-4 and PKCθ to the synapse. *Tseng et al.* have suggested that cosignaling molecules such as CD80 may function to segregate CD28 and CTLA-4 away from the TCR creating a distinct costimulation zone in the IS, near the boundary between the cSMAC and the pSMAC, a zone dependent on cytoskeletal integrity.

1.3.8 The IS directs T cell differentiation

A phase of sustained signaling is crucial in supporting the commitment of activated T cells to full effector potential (144, 157). Early after activation, CD4 cells secrete IL-2 and are designated Th0 cells. Depending on the nature of the cytokines present at the site of activation, Th0 cells will go down one of two major differentiation pathways: Th1 or Th2. Th1 cells secrete Th1-type cytokines including IFN-γ and TNF-β and are important in inducing cell-mediated immunity. Th2 cells secrete Th2-type cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13 and are efficient at inducing humoral immune responses. T cell differentiation is established and maintained via the action of lineage specific transcriptional regulators induced by these cytokines. The Th1 specific transcription factor is T-bet while the Th2 subset specific transcription factor is GATA-3 (158). Apart from their different functions, effector cells express partially different sets of receptors specific for inflammatory chemokines that are required for migration to peripheral sites in contrast to naïve T cells that express

the lymph node homing CCR7 receptor (159). It appears that the IS serves as a platform for 'cytokine presentation' from DC to naïve T cells (157). Strong TCR signaling as been shown to lead to IFNγ receptor (157) IS polarization and the assembly of a Th1 signalosome further stabilized by the subsequent secretion of IFNγ (160). If IL-4 is delivered, an inhibitory signal prevents polarization, implying that the Th1 pathway may be the default response of the Th0. Parallel engagement of TCR and IL-4 receptor might then lead to assembly of a Th2 signalosome (161).

1.4 Cosignaling

Cosignaling molecules govern the functional outcome of the TCR signal (162) by modulating large sets of genes also modulated by TCR signaling, and only exceptionally induce specific genes such as IL-2 (75). Cosignaling requires a cascade of complex interactions (75). Cosignaling molecules can be co-activators or co-inhibitors. The relative contribution of these co-signaling molecules during different phases of the immune response is possible because of their temporal, spatial and functional separation. The cosignaling molecules are cell surface glycoproteins and are divided in two major groups: the immunoglobulin superfamily (IgSF; that includes the CD28, the B7 and the SLAM families) and the tumor-necrosis factor (TNF) superfamily. Recently, several proteins have been identified as new members of the IgSF or the TNF cosignaling superfamily. These new members appear to differ from the conventional pathways and from each other based on their expression patterns and differential effects on individual lymphocyte subsets. The work presented in this thesis focuses on the CD80 and CD86 cosignaling molecules important for naïve CD4 T cell activation. A brief overview of the newly identified cosignaling molecules is presented.

1.4.1 Ig superfamily

The IgSF is one of the largest in vertebrates' genomes. Significantly, the increase in IgSF numbers from invertebrates to vertebrates parallels the evolution of the adaptive immune system (163). The presence of a single exon coding for most

IgSF domains provided the genetic basis for duplication and diversification that gave rise to this large family of proteins. IgSF domains can be classified as IgV, IgC1, IgC2, or IgI according to their primary sequence and overall length (164). These domains all present the characteristic Ig fold of antiparallel beta strands arranged in two sheets linked by a disulfide bond (165) as shown in figure 4. This disulfide bond is not essential for the domain structure and IgSF proteins lacking this disulphide have been reported (166).

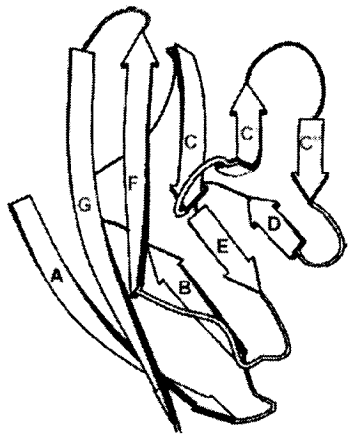

Figure 4 The Typical Ig Fold (adapted from (166)) Ig domains all present the characteristic Ig fold of antiparallel beta strands arranged in two sheets linked by a disulfide bond (between strands B and F). The front face is composed of GFCC'C'' strands and the back sheet is composed of the ABED strands.

The 'variable' (IgV) domains of immunoglobulins contain their antigen-binding properties and the 'constant' (IgC) domains mediate their effector functions. IgC domains are shorter than IgV domains as they lack strands C' and C'' within the Ig fold. Domains showing similarity to overall IgV domain sequences are referred to as IgV-like. However, although IgV-like domains show sequence similarity to the immunoglobulins variable domains, they do not present the variability that characterize antibody molecules. Domains with sequence patterns more similar to IgV but more of the size of IgC domains are called IgC2 domains and the classic IgC domains, found almost exclusively in Ig, MHC antigens and β2M, are referred to as IgC1. I set domains are intermediate between those of V- and C-set domains, in agreement with the suggestion that I set domains are the ancestral family. *Evans et al.* (167) recently proposed that IgV domains be subdivided into IgV1 and IgV2 domains to reflect structural differences between

IgV typical of antigen receptors and those of adhesion molecules. Hallmarks of antigen receptor IgV domains are the substantial beta strand A and B interactions and C'- and G-strand β-bulges, rather than the limited A'-G contacts and the β-bulges located immediately after C'- and G-strand of adhesion molecule IgV.

The majority of proteins with Ig domains are type I membrane proteins with a single transmembrane region although secreted and cytosolic IgSF proteins exist. About 33% of the molecules at the surface of human leukocytes are IgSF proteins and of these, half present an IgV-IgC2 domain organization (166). Cytoplasmic regions of IgSF proteins vary in length and many have recognizable motifs for signaling such as ITAM and ITIM (immunoreceptor tyrosine-based inhibititory motif) and regions that interact with adaptor proteins and cytoskeletal components. In many cases the IgV domain contains the binding specificity and although antibodies bind molecules with high affinity, most proteins containing IgSF domains have weak interactions such as MHC peptide antigen recognition by the TCR (70). The interactions usually involve a single Ig domain even if the other domains are most probably necessary to maintain the orientation of the binding site and the optimal distance from the membrane. Ig domains are glycosylated at various extents and since N-linked carbohydrates are almost as big as Ig domains, glycosylation represent a major feature of these proteins. The attached carbohydrates can restrict the movement of these membrane proteins and hence optimize their binding to receptors of opposing cells. Cosignaling proteins belonging to the IgSF can be further divided in three subfamilies, namely, the CD28 family, the B7 family and the SLAM/SAP family.

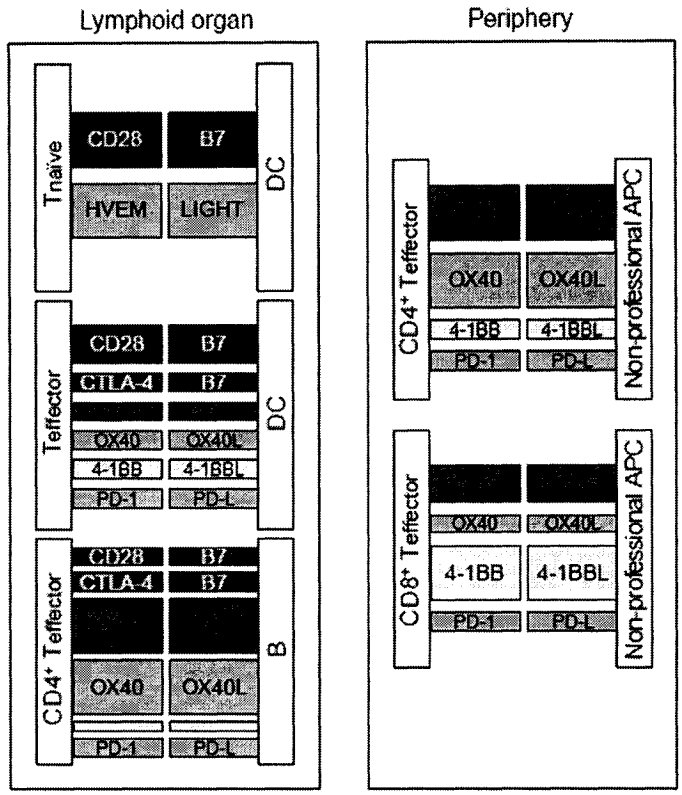

Figure 5 Cosignaling Molecules (adapted from (75)) This diagram depicts the multitude of cosignaling interactions that occur both in lymphoid organs and in the periphery. Only CD28 and HVEM and their counter receptors are involved in the initial T cell activation by professional APC in lymphoid organs. The CD28/CTLA-4: CD86/CD80 (B7) interactions are the focus of this thesis. T cells in a state of progressed activation utilize a multitude of cosignaling molecules such as CD28, ICOS, OX40, 4-1BB, CTLA-4 and PD-1 while interacting with professional APC. The relative importance of these various cosignaling molecules will change depending on local requirements. Most of the newly identified cosignaling molecules function in later stages of T cell activation and some in the periphery.

1.4.1.1 The CD28 family

CD28 was the first cell surface molecule identified as a costimulatory receptor (168). The CD28 family is composed of 2 co-activators (CD28 and ICOS -*inducible costimulator*-) and 3 co-inhibitors (CTLA-4/CD152 -*cytotoxic T lymphocyte-associated antigen 4*-, PD1 -*programmed death 1*- and BTLA -*B and T lymphocyte attenuator*-) that share 23 to 30% homology to CD28 (92). Figure 5

displays CD28 members, their counter-receptors and their expression sites. CD28 counter receptors belong to the B7 cosignaling protein family.

1.4.1.1.1 Structure and gene localization of the CD28 family

Members of the CD28 family are type 1 transmembrane proteins generally expressed on T lymphocytes and present one extracellular IgV domain and a short intracellular domain that contain motifs important for signaling. CD28, ICOS, and CTLA-4 are clustered in close proximity on chromosome 2q33 and have an unpaired cysteine that allows them to homodimerize on the T cell surface (169) in contrast to PD1 and BTLA that are located in distinct locations in the human genome (2q37 and 3q13, respectively) and that do not homodimerize. N-glycosylation has also been shown to be required for CTLA-4 dimerization (170).

1.4.1.1.2 Expression patterns of CD28 family members

CD28 is constitutively present on >90% of human CD4 T cells and 50% of human CD8 T cells but is constitutive on all murine T cells. CD28 costimulation is necessary for the initiation of most T cell responses and blockade of CD28 signaling results in ineffective T cell activation. ICOS is induced following T cell activation and expressed by effector T cells. Resting T cells express very low levels of CTLA-4 and its expression levels are kept very low even on activated cells (151). CTLA-4 has a 30- to 50-fold lower surface density than CD28 (171). CTLA-4 is stored in intracellular vesicles (172) and undergoes complex intracellular trafficking mediated by binding to the clathrin-adaptor molecules AP-1 and AP-2. However, CTLA-4 is constitutively expressed in the CD4+CD25+ Treg subset (173). BTLA is found on activated but not naïve T cells. The co-inhibitory receptor PD1 is only expressed on activated T cells and exerts its main function in the periphery.

1.4.1.1.3 Knockout models of the CD28 family members

In CD28 knock-out mice, T cell differentiation appears normal but show strongly impaired T cell and B cell responses due to the lack of B7 mediated cosignaling.

The critical role of CTLA-4 as a negative regulator of T cell activation is dramatically illustrated in CTLA-4-deficient mice, which die from massive lymphocytic infiltration and tissue destruction in critical organs (174, 175). Mice expressing only the extracellular domain of CTLA-4 show lymphadenopathy but are spared from the massive lymphoproliferation illustrating the importance of CTLA-4 intracellular domain for its function (176). The ICOS pathway appears to play a critical role in humoral immunity since ICOS deficient mice have decreased serum IgG levels and show defects in isotype switching and germinal center formation (177). PD1 knock-out animals suffer from a phenotype similar to CTLA-4 knock-out mice with lymphocyte infiltration in tissues although disease severity is different (178). BTLA deficient mice present enhanced immune responses (179).

1.4.1.1.4 Structure and function of CD28 and CTLA-4

CD28 and CTLA-4 are the major regulators of TCR engagement in naïve T cells. CD28 (168, 180) and CTLA-4 (180, 181) were cloned in 1987. Both are obligate homodimers due to their interchain disulphide linkage (180). Interestingly, both the transmembrane and intracellular region of CTLA-4 are required for homodimer formation (182). CTLA-4 dimerization is also dependent on N-glycosylation (170). Approximately 25% of the residues are rigorously conserved between CD28 and CTLA-4 and conservation of surface residues in CD28/CD152 is essentially limited to the CD80/CD86 binding site located in their CDR3-like regions (183). A key surface plasmon resonance study from *Collins et al.* (184) established that CTLA-4 is a bivalent homodimer and CD28 is a monovalent homodimer, a finding confirmed in the crystal studies of both molecules (167, 185, 186). Recent studies have described a CTLA-4 molecule that can exert inhibitory functions independent of CD80 and CD86 binding (187).

1.4.1.1.5 CTLA-4 solution study

In 1997, *Metzler et al* (188) reported the solution structure of CTLA-4 obtained by NMR spectroscopy (figure 6). The structure of the extracellular domain of

CTLA-4 consist of two β-strands, the ABED and A'GFCC' strands connected by the canonical Ig sulfide bond and an additional non-Ig sulfide bond. CTLA-4 extracellular contains two N-linked glycosylation sites. The conformation of the MYPPPY loop extends the A'GFCC' face by making the beta sheet surface flatter providing a large accessible surface suitable for binding interactions (188). Crystal studies of the CTLA-4 receptor bound by its two ligands have also been reported and are discussed in another section (185, 186).

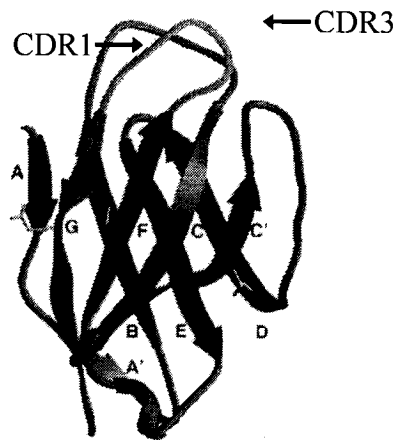

Figure 6 Ribbon Diagram of CTLA-4 Extracellular Domain (from (188)) Ribbon diagram depicting CTLA-4 secondary structure: beta strands A, A', B, C, C', D, E, F, G form two β-pleated sheets (front face A'GFCC' and back face ADEB) that adopt a β-sandwich configuration. The CDR1 region and CDR3 region are indicated while the disulfide bonds are shown in ball stick representations.

1.4.1.1.6 CD28 crystal

In 2005, almost 20 years after its original description (168), *Evans et al.* (167) reported the crystal structure of the extracellular region of CD28 in complex with a Fab (fragment antigen binding) fragment. As shown in the surface representations in figure 7, the structure of monomeric CD28 resembles that of monomeric CTLA-4 since the ligand binding faces of both monomers bear similarities, including the conserved MYPPPY loop. However, notable structural differences are also observed. The dimer interface of both molecules show differences that prevent the formation of CTLA-4/CD28 heterodimers consistent with the failure to detect such heterodimers. Importantly, CD28 and CTLA-4 show great difference in the orientation of their monomeric subunits relative to

CD80 and CD86 binding. In contrast to CTLA-4 IgV domains that are arranged in a 'V' structure, IgV domains of CD28 are arranged in a 'U' structure. Although both arms are available for ligand binding, simultaneous binding of separate monomers is prevented by a physical clash of the IgC of CD80 and CD86. Even if direct physical clashes may not completely prevent bivalent binding, occupancy at one site would reduce the 'on rate' for binding at the second site by about 86% (167). Therefore the CD28 molecule can only bind to monomeric forms of its ligands.

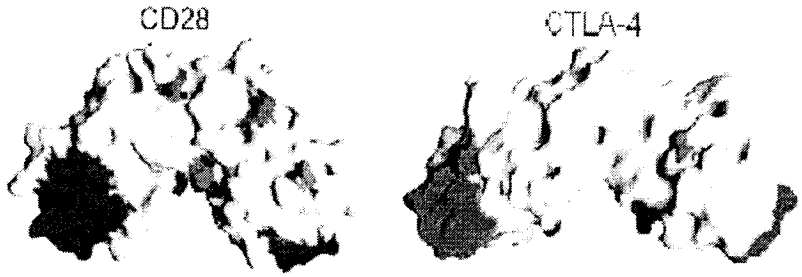

Figure 7 CD28 and CTLA-4 Molecules (adapted from (167)) Surface representations of the putative CD28 homodimer observed in the crystal lattice (left) and the native CTLA-4 homodimer (right). The ligand-binding surface of CD28 is in dark gray and the equivalent surface in CTLA-4 is light grey. Glycosylation sites in each protein are shown as punctual grey sites. The C termini (and the T cell surface) are toward the top. Of note, the arms of CD28 form more of a 'U' structure while CTLA-4 arms form more of an open 'V' conformation.

1.4.1.1.7 MYPPPY motif

As previously noted, the most notable conservation of amino acids between CD28 and CTLA-4 not including IgSF consensus residues is the hexapeptide MYPPPY sequence motif located in the IgV domains of both CD28 and CTLA-4 (183). The hexapeptide hydrophobic motif MYPPPY is located to the FG loop, that is, in the CDR3 analogous region. More specifically, this motif mediates CTLA-4 and CD28 binding to CD80 and CD86 and the high conservation of this motif can certainly explain why interactions between CD28 and B7 family proteins are detectable across species after 300 million years of divergent evolution. As expected from typical IgV domains, CD28 and CTLA-4 CDR1 and CDR3 regions are closely apposed in the membrane while the CDR2 region does not interact directly with the counterreceptor. Solvent exposed CDR3 regions on antibody

structures form a major component of the antigen recognition site (189) and parts of these loops are also important binding determinants in the CD8-MHC class I interaction (31, 32) and in the CD4-MHC class II interaction (33,34). Mutation of any of the MYPPPY residues in CTLA-4 or CD28 leads to reduced binding of CD80 or CD86 (183, 190-192). Results show that, although the same overall region on CD28 and CTLA-4 is involved in the interactions with CD80 and CD86, subtle but important differences in ligand recognition exist between the two molecules. Altogether, the MYPPPY motif is involved in differential, as well as common, recognition of the counter-receptors (192). The contacts between CD80 and the CTLA-4 MYPPPY motif are shown in figure 8.

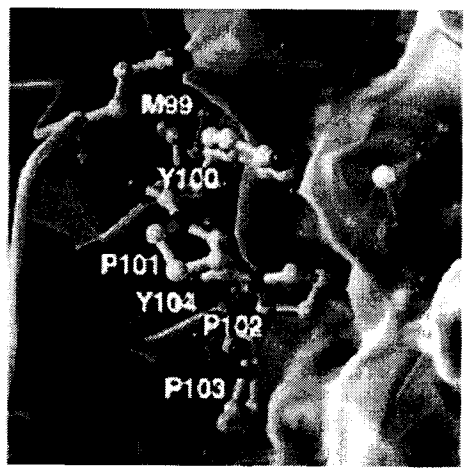

Figure 8 MYPPY Motif in CTLA-4:CD80 Interaction (adapted from (185)) The MYPPPY loop of CTLA-4 (shown on the left side) is buried in a shallow depression of the CD80 GFCC' surface (shown on the right side). Hydrogen bonds are formed across the β-sheets of the interacting proteins.

1.4.1.1.8 CD28 and CTLA-4 signaling

CD28 and CTLA-4 signaling takes place within the IS (150, 155, 193). Signaling via both the TCR and CD28 results (194, 195) in rearrangements of membrane and cytoskeletal components, MTOC (microtubule organizing center) reorientation, accelerated intracellular vesicle trafficking, activation of transcription factors, accumulation of lipid rafts in the IS, high levels of IL-2

cytokine and survival signals (194). CD28 lowers the threshold for T cell activation by decreasing the number of ligated TCR that is required for a given biological response (79). The unique function of CD28 resides in its ability to induce IL-2 in naïve T cells (75). Mutational analysis of the CD28 cytoplasmic tail indicates that induction of cytokines and control of cell survival are regulated by distinct domains within the CD28 cytoplasmic tail. On the other hand, CTLA-4 engagement selectively blocks augmentation of genes regulated by CD28-mediated co-stimulation, but does not ablate gene regulation induced by TCR triggering alone (196). CTLA-4 inhibition of T cell activation can occur by various mechanisms (reviewed in (151)) such as out-competing CD28 for binding to B7 proteins due to its higher affinity for them (184), by forming periodic structures with its ligand that could interfere with the IS (185, 186, 197), by inducing immunosuppressive cytokines and/or by promoting the assembly of inhibitory signaling complexes antagonizing TCR and CD28 signaling.

1.4.1.2 B7 family

In contrast to the CD28 family members that were discovered by their functional effects, most of the B7 family member ligands were discovered by homology searches that revealed many proteins resembling CD80 and CD86, the founding members of this family. Some of these newly identified molecules are not recognized by any of the known receptors for B7 molecules. Because this thesis focuses on the classical B7 proteins CD80 and CD86, a brief introduction to other B7 is given and CD80 and CD86 are discussed in a separate section. Figure 6 shows some B7 members, their counter receptors and expression sites.

1.4.1.2.1 Structure of the B7 family members

The genetic linkage of B7-related molecules to the MHC has been noted previously (198). Two or four extracellular Ig-like domains in a IgV/IgC order characterize the B7 family members. The B7 family consists of 4 co-activators (CD86, ICOS-L/B7H2, PDL2/B7DC, B7H3) and 4 co-inhibitors (CD80,

PDL1/B7H1, B7H4/B7x, B7H3) although studies are still clarifying the exact role of some of these molecules.

1.4.1.2.2 Expression of the B7 family members

B7 proteins are generally expressed on APC although they can be expressed on other cells. CD80 and CD86 are expressed mainly on APC with different expression kinetics. CD80 and CD86 expression on T cells has also been reported and may play a role in lymphocyte homeostasis (91). In general, co-activatory (co-stimulatory) molecules are constitutively expressed at low levels and rapidly upregulated upon receiving various inflammatory stimuli while co-inhibitory molecules expression is induced. ICOS-L is expressed on B cells, monocytes, iDC but not mDC. DC and macrophages express B7DC. T and B cells express B7H1 and mRNA can be found in various human tissues; however its expression requires exposure to inflammatory cytokines such as IFN γ. B7H3 mRNA is expressed in both lymphoid and non-lymphoid tissues. Both an inhibitory and stimulatory function for B7H3 has been suggested although studies in knockout mice support an inhibitory function for this molecule (199). B7H4 mRNA is detected in many tissues but the protein is not expressed in normal cells suggesting a post-translational control mechanism. B7H4 might have a role in the regulation of the early phases of immune responses during inflammation in peripheral tissues (92).

1.4.1.3 The SLAM/SAP family

Based on their sequence homology, the SLAM family is part of a broader subgroup of Ig-like receptors referred to as the CD2/SLAM family (200-202). The genes coding for the CD2/SLAM family are all encoded on human chromosome 1 except for the *sap* gene that is encoded on the X chromosome. All members of the SLAM family are composed of an extracellular domain formed of two or four Ig-like domains that include a N-terminal IgV domain without disulphide bonds and a IgC2 domain with 2 putative disulphide bonds. SLAM proteins also present an intracellular domain that contains tyrosine motifs. SLAM receptors are

expressed in a variety of immune cells and function at the IS. Ligand binding induces their interaction with adaptor molecules SAP (SLAM-associated protein) and/or EAT2 proteins and signaling that recruits and activates several SRC kinases. This signaling is believed to fine tune cell activation mediated by TCR and cosignaling molecules. Signals mediated by the SLAM receptors can also affect the function of APC (200).

1.4.2 TNFR/TNF family

Figure 6 shows some TNFR members, their counter receptors and expression sites. Apart from CD40 that plays a crucial role in immune responses initiation, members of the TNFR/TNF family can influence T cell responses in various ways (203). TNFR family members have a major role in T cell survival following CD28-B7 cosignaling. As reviewed in (203), TNFR family members are type I transmembrane proteins characterized by extracellular cysteine-rich motifs and fall into three groups (203):

(1) DD (Death domains)-containing receptors (such as Fas/CD95) that activate caspase cascades leading to apoptosis

(2) Decoy receptors

(3) TRAF (TNF receptor-associated factor) *binding receptors* that lack DD but contain TRAF recruitment motifs

TNFR ligands are type II cell surface glycoproteins (203). Knock-out mice show no drastic phenotype but show T cell survival impairments (203). TNFR clustering by trimeric TNF ligand leads to TRAF aggregation and their interaction with downstream signaling molecules mediating important downstream events such as survival, cellular proliferation and cytokine secretion (203). All costimulatory members of the TNFR family have the ability to recruit TRAF2 but differ in their recruitment of other TRAF proteins. Henceforward is a brief overview of some TNFR pairs that affect initial T cell activation. The CD27/CD70 pair is expressed on T cells and their ligands are induced soon after activation suggesting a function early after TCR/CD28 signals as seen with the

HVEM/LIGHT pair (203). The 4-1BB (CD137)/41BBL pair has been shown to have preferential effects on CD8 T cells costimulation in some models while the OX40 (CD134)/OX40L pair shows preferential effects on CD4 T cells (203).

1.4.2.1 CD40: A TNFR that acts as a major switch for T cell cosignaling

CD40 is important in both cell mediated and humoral immunity. CD40 is as a master switch for T cell cosignaling as it regulates the upregulation of costimulatory molecules and adhesion molecules important in the initiation of immune responses on APC (204). CD40 is a membrane glycoprotein expressed on B cells, DC, macrophages, epithelial cells, hematopoietic progenitors, and activated T cells. Its ligand, CD154 (CD40 ligand), is a type II integral membrane protein expressed on activated T cells, activated B cells, and activated platelets. CD40 function is dependent on several different structural motifs in its cytoplasmic domain. Moreover, an extracellular Cys residue has also been implicated in some CD40 functions by allowing dimer formation (205, 206). CD40- and CD154-deficient mice show phenotypes that are quite similar to each other implying an exclusive receptor–ligand pair (204) and revealed the importance of these molecules in humoral memory as their deficiency leads to defects in B cell isotype switching, B cell migration and germinal centers development.

1.4.3 Cosignaling superfamily crosstalk

Two recent studies (207, 208) have provided a new perspective in this already complex area by describing the unexpected interaction between the immunoglobulin family member BTLA and the TNFR family member HVEM. Not only does these studies show crosstalk between two distinct cosignaling superfamilies, they describe the interaction of a co-activating and a co-inhibiting receptor (209). The crystal structure of this interacting pair has been determined (210). Although HVEM is believed to transmit a positive cosignal in T cells upon

binding of its TNF ligand LIGHT, binding to BTLA has been suggested to induce negative signaling resulting in T cell proliferation inhibition (207, 208).

Major questions remain with respect to signal integration generated by the various cosignaling pairs. Studies involving mice lacking more than one receptor or ligand might prove useful in determining the interplay between these molecules. A model of T cell activity integrating the cosignaling function of both superfamilies could certainly aid in the understanding of how lymphocyte differentiation is specified, effector cells regulated, memory generated, and tolerance maintained (78).

1.5 CD80 and CD86

CD80 and CD86 (also known as B7-1 and B7-2; sometimes referred to as the B7) are the only functional ligands of CD28 and CTLA-4. Despite some structural similarities and common binding partners, different functional properties have been attributed to CD80 and CD86. These different biological properties emanate from different patterns of expression that mimic that of CTLA-4 and CD28 respectively, different signaling pathways, different binding properties and differences in molecular organization.

1.5.1 Genomic organization of CD80 and CD86

CD80 and CD86 were identified in 1989 and 1993 respectively (211-213). Both human and murine CD80 genes were cloned by *Freeman et al.* (212, 214) and the human and murine CD86 genes were cloned by *Freeman et al.* (211, 215) and *Azuma et al.* (213). The human *cd80* gene (3q13.3-q21) is composed of six exons. Exon 1 is not translated. The second exon contains the initiation codon and encodes a signal peptide. The third and fourth exons correspond to the IgV and IgC domains. The fifth and sixth exons encode respectively the transmembrane portion and the cytoplasmic tail (216). The human *cd86* gene (3q21) has eight exons (217). Exons 1 and 2 contain the untranslated region. Exon 3 codes for the signal peptide. Exons 4 and 5 encode the IgC and the IgV domains. Exon 6

encodes the transmembrane domain and exon 7 and 8 encode the cytoplasmic domain. The close relationship between exons and functional domains is a characteristic feature of genes of the IgSF. CD80 and CD86 are believed to have arisen via duplication of a common precursor. The preservation of both genes in all mammals strongly suggests that they have been subjected to distinct selection pressures. Alternative splice variants have been described for both CD80 and CD86 (218-222). Mice deficient in CD80 and CD86 have significant abnormalities in both humoral (including Ig class switching and germinal center formation) and cellular immune responses suggesting considerable overlap in the cosignaling functions of CD80 and CD86 (223).

1.5.2 IgSF domain description of CD80 and CD86

CD80 and CD86 are type I membrane proteins of 60kDa and 80kDa respectively. They are members of the IgSF. As shown in figure 9, their extracellular regions consist of two anti-parallel β-sandwich IgSF domains (membrane distal IgV and membrane proximal IgC) joined by a short linker region. As with other IgSF members, IgV and IgC from CD80 and CD86 present front and back sheets composed of AGFCC'C'' and BED strands, respectively. Despite their common receptors the extracellular regions of human CD80 and CD86 share only ~25% sequence identity. An early study from *Bajorath et al.* (224) used a topological fingerprint method to compare CD80 and CD86 to other known IgSF proteins. The B7 IgV domains were shown to include some structural features that departed from currently known Ig folds. In contrast, the IgC domains displayed significant sequence compatibility with IgC structures and were best matched to β2m IgC1 suggesting that the B7 IgC region, like β2m, might be involved in protein-protein interactions perhaps with the N-terminal IgV domain or, alternatively, with other molecules. CD80 and CD86 are both glycosylated (213) and *N*-linked glycosylation sites in the IgV and IgC domains are localized to regions opposite to the receptor interaction site. Extensive glycosylation of CD80 and CD86 may aid in solubility since the extracellular domains contain a number of hydrophobic residues. CD80 and CD86 intracellular domains present 27 and 63 amino acids

respectively. CD86 presents three predicted PKC phosphorylation sites and CD80 is believed to be linked to the cytoskeleton via its intracellular domain (225, 226).

Figure 9 Ribbon diagram of CD80/CD86 (adapted from (227)) CD80/CD86 extracellular IgV and IgC domains consist of two anti-parallel β-sandwich IgSF domains joined by a short linker region. Glycosylation sites are shown in ball-stick representations.

1.5.3 CD80 and CD86 expression

CD80 and CD86 expression is tightly regulated at the APC surface. CD86 is expressed constitutively at low levels and is rapidly upregulated following T cell encounter and maximally expressed around 24h, whereas CD80 is expressed later after activation and is stable for a longer period of time (228, 229). The different expression patterns of CD80 and CD86 suggest distinct interactions with CD28 and CTLA-4. Indeed, CD28 and CD86 are present earlier on the cell surface compared to CD80 and CTLA-4. Therefore CD28:CD86 interaction is believed to play a pivotal role in the activation of naïve T cells whereas CD80:CTLA-4 plays a major role in terminating T cell responses. Apart from APC expression, CD80 and CD86 can be expressed on T cells; this proteins expression may be important for *in vivo* T cell homeostasis (91).

1.5.4 CD80 and CD86 signaling in APC

CD80 and CD86 binding to CD28 and CTLA-4 induces T cell signaling. However, many studies have also shown that CD28 and CTLA-4 binding to

CD80 and CD86 proteins delivers signals in APC. Binding of CD28 to CD80 and CD86 leads to IL-6 production by DC, resulting in an increased immunostimulatory activity (230). On the other hand, binding of CTLA-4 to CD80 and CD86 proteins induces IFNγ, which, in turn, up-regulates the expression of the IDO (indolamine 2,3-dioxygenase) enzyme resulting in tryptophan catabolism and T cell proliferation suppression and apoptosis (94, 231). These signaling cascades are dependent on the simultaneous expression of CD80 and CD86.

In accord with the predicted preferential receptor interaction (184), CD86 cross-linking up-regulates B cell proliferation, enhances the expression of antiapoptotic molecule Bcl-xL and stimulates the production of IgG1, IgG2a, and IgE (232, 233). In contrast, cross-linking of CD80 on B cells leads to reduced proliferation and up-regulation of proapoptotic molecules caspase-3, caspase-8, Fas, FasL, Bak, and Bax (233, 234). A recent study showed that CD86 induced signaling in activated B cells increased the activity of PI3K and the phosphorylation of PKC and IkB thereby modulating B cell gene expression and activity (235). It is interesting to note that 'reverse signaling' has also been described for other B7 family members such as B7DC (236-238). Crosslinking of B7DC on DC by pentameric IgM antibodies leads to the activation of DCs, which subsequently enhances their ability to stimulate T cells; interestingly B7DC has only one intracellular amino acid (239). Signaling within the APC might be an important feedback mechanism for the regulation of T cell: APC interactions (240). Finally, covalent receptor dimerization and counter receptor multimerization are two key features of the CD28/CTLA-4/CD86/CD80 interaction system that may regulate signal transduction by controlling the duration of receptor occupancy.

1.5.5 CD80 and CD86 binding data

Our understanding of CD80 and CD86 function is supported in part by knowledge of their affinity and binding kinetics to CD28 and CTLA-4 (241). The Kd

(equilibrium dissociation constant) values and interaction properties of CD80 and CD86 with their receptors are shown in figure 10. Both CD80 and CD86 show higher affinity for CTLA-4 than to CD28 with a difference of at least 10-fold (214). CTLA-4 covalent dimerization is required for its high binding avidity even if each monomeric subunit contains a binding site for CD80/CD86 (182). It has been suggested that binding of the first B7 molecule to CTLA-4 reduces the on-rate for binding of the second (184). The Kd of CD80 binding to CD28 is 4uM and that of CD80:CTLA-4 is of 0.2uM (242, 243). The Kd value of CD86 binding to CD28 is of 20uM and that of CD86:CTLA-4 is of 2.6uM (242, 243). CD86 shows faster on and off rates to CTLA-4 when compared to CD80 (241, 243). Earlier studies had emphasized the similarities in CD80 and CD86 binding properties. However, the CD80 and CD86 proteins used in these studies were multivalent biasing the results (191, 241). The interaction properties of the CD28/B7 system were clearly established in a key biacore study from *Collins et al.* (184). In this study, CD80 behaved as a homodimeric ligand in contrast to CD86 that behave as a monomeric ligand. The general conclusion of these findings showed that CD80 is the preferential ligand of CTLA-4 and CD86 is the preferential ligand for CD28. Compared to CD28:CD86 and CTLA-4:CD80 complexes, CD28:CD80 and CTLA-4:CD86 complexes are believed to be of intermediate strength since CD28 is monovalent and CD86 does not self-associate. Importantly, these results are in agreement with findings of *Pentcheva-Hoang et al.* (155) that showed, using APC deficient in either CD80 or CD86, that while CD80 favors binding to CTLA-4, CD86 shows a preference for CD28 in the context of the IS. The interaction hierarchy with initial engagement of CD86 by CD28 followed by engagement of CD80 by CTLA-4 appears to have evolved in such a way that weak interactions are sufficient to trigger T cell activation while much stronger interactions are required for attenuation, thereby providing an intrinsic mechanism for modulating T cell responses. The CD28:CD86 interaction has similar properties to TCR and adhesion molecule ligand interactions; i.e., binding is monovalent and has fast kinetics and similarly high Kd values (244, 245). Interactions with these properties are ideally suited for dynamic cellular

contacts, facilitating the "scanning" of cellular targets for antigens and adhesion molecules early in immune responses. The switch between monovalent and bivalent binding is likely to have had the largest single effect on the relative strength of these interactions. A structural change of this type could have initiated the functional diversification of this signaling system (184). Very interestingly, a recent report (246) shows that CD28 monovalency is essential for cosignaling. In this study, a chimeric bivalent CTLA-4 (extracellular)/CD28 (intracellular) protein could bypass the need for TCR co-engagement for signal initiation. Therefore CD28 monovalency impacts the autonomy of this receptor and only the combination of TCR and CD28 ligation can lead to full T cell activation.

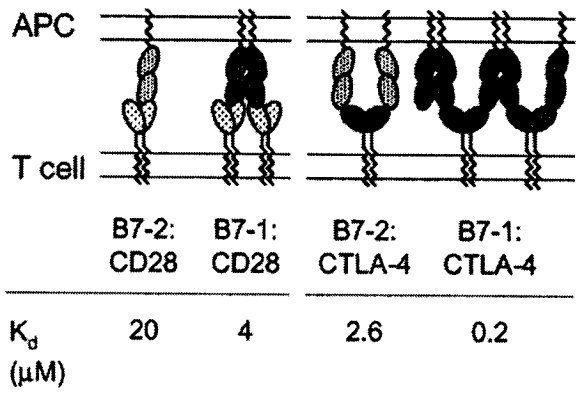

Figure 10 Interaction Properties of CD80/CD86 and CD28/CTLA-4 (adapted from (184)) Binding properties of human CD28, CTLA-4, CD80/B7-1 and CD86/B7-2 and their respective Kd values. The predicted binding properties are as follows: CD28 is a monovalent homodiner; CTLA-4 is a bivalent homodimer; CD80/B7-1 is a monovalent homodimer; CD86/B7-2 is a monovalent monomer.

1.5.6 CD80 and CD86 Ig domains in their binding properties

Many studies have addressed the role of CD80 and CD86 Ig like domains in receptor binding. A pioneer mutational analysis study from *Peach et al.* (247) identified conserved residues within the IgV domains of both CD80 and CD86 to be critical for binding to CTLA-4 and CD28. 11 hydrophobic residues within the IgV domain of CD80 were shown to be critical in receptor binding. The identified residues mapped to the GFCC'C'' front face of the IgV fold, the same face known to mediate interactions in other IgSF members (248, 249). Mutagenesis of

corresponding residues in CD86 established that some, but not all, of these residues also played a role in CD86 receptor binding. IgC domain deletion also differentially affected CD80 and CD86 binding to their receptors. CD80 IgC deletion completely abrogated receptor interaction (247) and a mutational study from *Guo et al.* (250) identified several amino acids in loops between strands B, C, D and E of the IgC to be important for CD80 interaction with CTLA-4/CD28. *Freeman et al.* (251) and *Rennert et al.* (252) also pointed to the importance of CD80 and CD86 IgC domains. Domain-specific constructs of human CD80 and CD86 revealed that CD80, CD86 and the IgV domain of CD86 bound to CTLA-4 and CD28 in contrast to CD80 IgV domain, the CD80 IgC domain and the CD86 IgC domain that were unable to bind CD28 nor CTLA-4. *Fargeas et al.* (253) not only identified IgV residues W84 and Y87 as being critical for CD28 and CTLA-4 binding, they also showed that mutations at conserved residues within the IgC domain of CD80 lead to a defect in CD28 and CTLA-4 binding (253). *Agadjanyan et al. (254)* also focused on the domain structure of these molecules by studying chimeric and deleted forms of CD80 and CD86 molecules. CTL responses against the HIV env glycoprotein when coinjected with the various forms of CD80 and CD86 were studied. Dramatic improvement in *in vivo* costimulation was observed after removal of the IgC of CD80. Moreover, the chimeric molecule expressing the IgV domain of CD80 and the remaining of CD86 enhanced T cell activation. These data showed the importance of CD86 IgC domain in T cell activation. *Vasu et al.* (255) further deepened the understanding of the role of IgC domains of CD80 and CD86 by preparing chimeric constructs in which the IgC domains of human CD80 and CD86 molecules where swapped. The cosignaling function of these chimeric molecules was assessed in CHO cells. Like CD86, the V80C86T80 chimera showed a substantial increase in T cell activation relative to CD80; in contrast, relative to CD86, V86C80T86 diminished T cell activation. Altogether, these studies show that both IgV and IgC domains are crucial for CD80 and CD86 function.

1.5.6.1 CD80ΔC

It is interesting to note that a naturally occurring CD80 splice variant lacking the IgC was observed. This splice variant was first described by *Inobe et al.* (221) in 1994 in LPS stimulated C57BL/6 mice splenic B cells by RT-PCR. Moreover, by analyzing B7 expression in a number of different cell lines by PCR, *Guo et al.* (250) also reported this alternatively spliced CD80 lacking the IgC domain. In contrast to the Inobe study, this alternatively spliced form had lost its binding to both CD28 and CTLA-4. These results are in accord with the *Peach et al.* (247) findings that showed that the absence of CD80 IgC domain resulted in a 10-fold reduced binding to both CTLA-4 and CD28.

1.5.7 Crystal studies of ligated CD80 and CD86

As suggested by earlier mutational studies, crystal studies (185, 186) showed the crucial importance of the IgV front faces of both CD80 (shown in figure 11) and CD86 (shown in figure 12) in interacting with CTLA-4. The binding interface is formed by packing interactions between the front sheets of each molecule. The AGFCC'C'' face forms a shallow concave surface that accommodates binding of the MYPPPY loop. The CTLA-4 FG loop that contains the MYPPPY motif makes hydrophobic contacts and five hydrogen bonds with a largely nonpolar surface of CD80 consisting of Tyr 31, Met 38, Thr 41, Met 43,Val 83, Leu 85, Ala 91, Phe 92 and Leu 97. At the core of the interface, Pro 102 of CTLA-4 and Tyr 31 of CD80 participate in a stacking interaction. The CTLA-4/CD86 interface is also stabilized by five hydrogen bonds and van der Waals contacts and the hydroxyl group of CTLA-4 Tyr-100 forms a hydrogen-bonding network with CD86 Glu-42 and Lys-49 that contributes to the stability of the interaction between CD86 and CTLA-4.

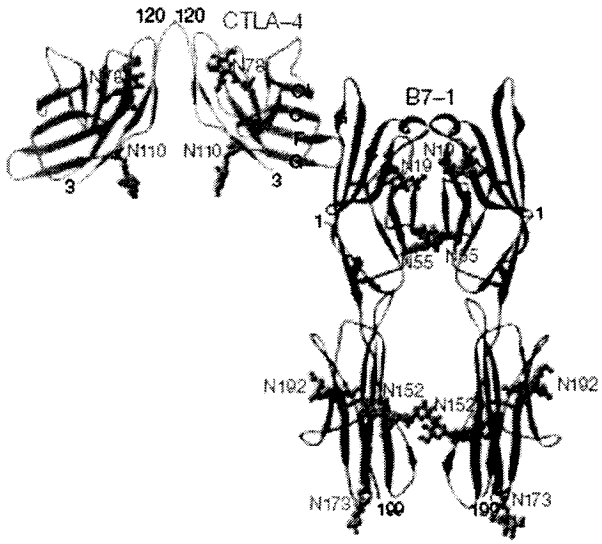

Figure 11 Ribbon Diagram of CD80 (B7-1) in Complex with CTLA-4 (from (185)) Ribbon diagram shows two CD80 and two CTLA-4 molecules as observed in the asymmetric unit of the crystal structure. N-linked carbohydrates are shown. β-sheets involved in the receptor-ligand interaction are labeled.

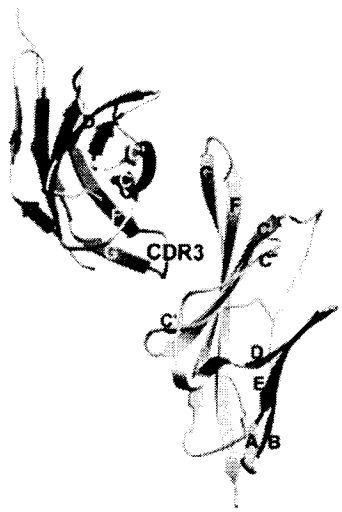

Figure 12 Ribbon Diagram of the CD86/CTLA-4 Binding Interface (from (186)) Ribbon diagram of the binding interface between CTLA-4 (left) and CD86 IgV domain (right). The CDR3 loop of CTLA-4 that contains the MYPPPY motif is labeled.

1.5.7.1 The IgC domains in the crystals

From the crystallographic studies, interesting differences in the IgC domains of CD80 and CD86 that may affect CTLA-4 binding were observed. Four extra amino acids in CD86 B-C loop (insertion 144–147 KKMS) and in CD86 C-D loop (insertion 150–153 LRTK) may change the overall conformation of the IgC domain in comparison to CD80. Because CTLA-4 does not bind directly to these residues, alterations in this area may affect CTLA-4 binding perhaps through altered dimerization capacity. Moreover, as shown in figure 13, numerous atomic contacts between the IgV and IgC domains of CD80 were uncovered, perhaps stabilizing the conformation of the variable domain (185, 227). These observations may explain earlier mutational studies that have highlighted the importance of IgC domains in CD80 and CD86 function. In addition, it was also proposed that glycosylation of Asn 173 located in CD80 IgC domain could have a profound effect on the stabilization of CD80 dimers (185).

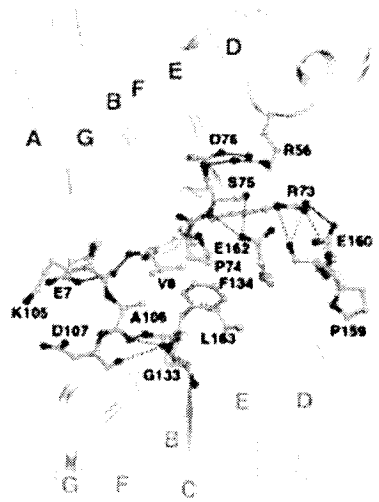

Figure 13 Ball-stick Representation of the IgC and IgV Interface in CD80 (from (227)) The interdomain region of CD80 is shown with the membrane distal IgV at the top and the membrane proximal IgC domain at the bottom. The network of residues mediating interdomain contacts is shown in ball-and-stick representation. A hydrophobic core (formed by Val-8, Pro-74, Ala-106, Phe-134, and Leu-163) in addition to electrostatic contacts and hydrogen bonds (between Ser-75 and Glu-162 and between Arg-73 and both Pro-159 and Glu-160) appear to stabilize the upright stature of the IgV domain of the molecule.

1.5.8 CD80 and CD86 dimers in CTLA-4 crystals

The disulphide-linked CTLA-4 dimer can support binding of two independent B7 molecules. The binding sites are distal to CTLA-4 dimer interface. Both CD80 and CD86 were crystallized as dimers when complexed with CTLA-4. The CD80 crystal lattice is dominated by a side-to-side molecular contact. This 'zipper arrangement' (shown in figure 14) is possible since CTLA-4 colligates CD80 around an axis orthogonal to the membrane. Thus maintains the 140Å intermembrane distance believed to be a critical feature of the IS (96). This type of binding is unique to CTLA-4/CD80 interactions. The biological relevance of the CTLA-4-B7 lattices was tested in a report from *Darlington et al.* (170) using CTLA-4 dimerization mutants. Interestingly, a monomeric CTLA-4 mutant still localized to the IS and inhibited T cell activation (170) in a CD80/CD86 dependent manner. Counter receptor binding therefore appears to ultimately determine the formation of CTLA-4 inhibitory lattices.

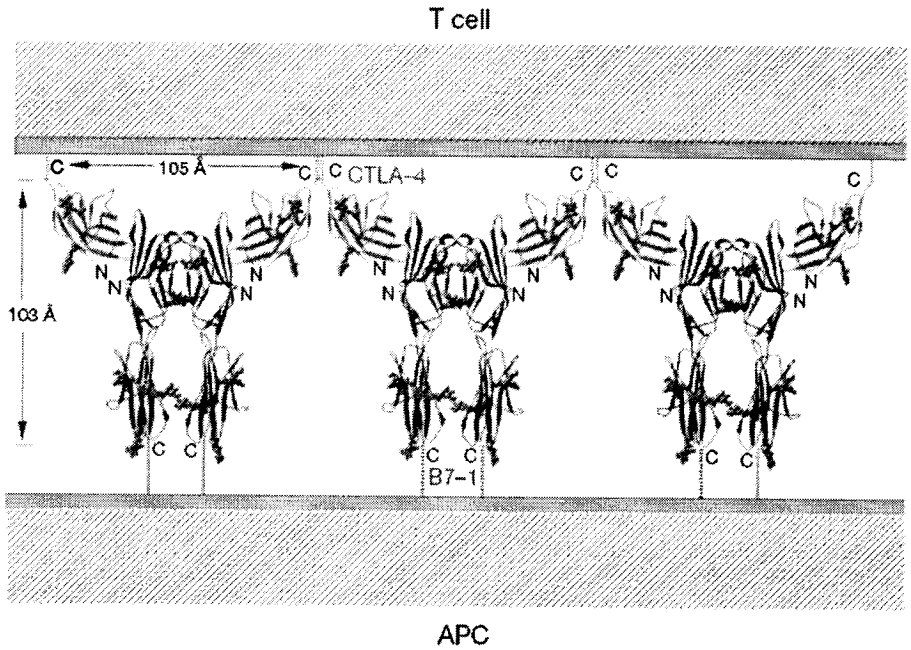

Figure 14 CD80 and CTLA-4 Molecular Association in the Crystal Lattice (from (185)) Shown are the 'zipper arrays' in which CTLA-4/CD80 complexes would be evenly spaced along membrane surfaces with a separation of 105Å. In the perpendicular direction, across membranes, ligated receptors would span 140Å. Geometrically, sugar chains (shown in ball-and-stick representation) attached at Asn 173 on CD80 (bottom) are close to the cell membrane perhaps stabilizing the orientation of the CD80 dimers.

In the crystal lattice, CD80 was shown to form parallel 2-fold rotationally symmetric homodimers. Moreover CD80 has been shown to dimerize by other experimental approaches in many studies. First, when crystallized alone, deglycosylated CD80 is detected as a homodimer, as is fully glycosylated CD80 in solution using analytical ultracentrifugation (227). The affinity of CD80 self-association is consistent with CD80 existing at the cell surface in a dynamic equilibrium dominated by the dimer. In contrast, in the CTLA-4/CD86 complex, the two CD86 monomers are not related by perfect twofold rotational symmetry. Moreover, there is no data supporting CD86 dimerization. Analytical ultracentrifugation and gel filtration studies have showed that CD86 is monomeric in solution and the IgV domain of CD86 was shown to be monomeric in the crystalline state (256). *Collins et al.* suggested (184) that the potential glycosylation at the dimer interface (Asn-8) of CD86 would interfere with its dimerization. This hypothesis was invalidated since unglycosylated bacterially-expressed CD86 did not form dimers. Moreover, CD80 crystal structure identified the dimer interface that involves V11, V22, G45, M47, I58, D60, I61, T62, and L70 contributed from the B, C'', D, and E strands on the back sheet of the IgV domain (227). The majority of residues contributing to the dimer interfaces in CD80 and CD86 occupy the same positions in their respective primary sequences. When comparing these residues, the chemical properties of the dimer interfaces of CD80 and CD86 are very different. As shown in figure 15, the majority of the residues mediating CD80 dimerization are hydrophobic (shown in light grey) and the majority of the residues mediating CD86 dimerization are hydrophilic (shown in dark grey) (256). This dimer interface difference provides a mechanism for preventing the formation of CD80:CD86 heterodimers. Finally, unligated and ligated structures demonstrate that CD86 does not undergo any significant conformational reorganization upon binding suggesting that CTLA-4 interaction could not have induced CD86 dimerization. Altogether, the deviation from 2-fold symmetry and the hydrophilic nature of the putative CD86 interface in addition to previous studies showing CD86 to be monomeric leads to believe that the observed CD86 dimers are the result of crystal packing effect. Moreover, free and

complexed CD80 and CD86 proteins exhibit high structural similarity suggesting that receptor binding could not promote nor enhance their dimerization.

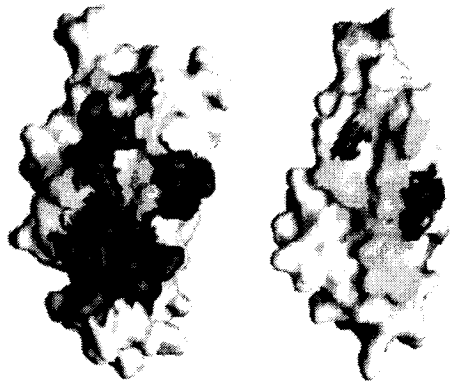

Figure 15 Surface Properties of CD80 and CD86 Dimer Interface (from (256)) Comparison of the surface properties dimer interfaces of CD86 (left) and CD80 (right). Hydrophilic residues are shown in dark grey while the hydrophobic residues are shown in light grey. It is obvious that hydrophilic residues dominate the CD86 dimer interface, whereas hydrophobic residues predominantly form that of CD80.

In a 2005 study, *Bhatia et al.* (257) used confocal microscopy-based fluorescence energy transfer (FRET) to demonstrate the different oligomeric states of CD80 and CD86. CD86 was shown to exist as a monomeric population in fixed HELA cells while CD80 was present as a mixed population of monomers and dimers, with dimers predominating. The dimer interface proposed by prior crystallographic analysis was validated by a series of mutations in CD80 resulting in the expression of a predominantly monomeric species on the cell surface. Moreover, as suggested, no heterodimers between CD80 and CD86 could be detected at the cell surface.

Altogether, CD28/CTLA-4/CD86/CD80 show great structural diversity that could account for the distinct functional properties of these interactions.

1.6 Cosignaling-based immunotherapeutics

Cosignaling is of therapeutic interest because its manipulation might provide means to enhance or terminate immune responses. Preclinical studies exploring the role of members of the CD28 and B7 families have supported the targeting of these pathways for new therapeutic approaches. Numerous animal studies (229, 258) and clinical trials (259) have shown that manipulating these interactions may hold promise for immunotherapy.

1.6.1 Targeting CTLA-4

Because of CTLA-4 negative regulation of T cell activation, reagents that block CTLA-4 could enhance T cell responses. CTLA-4Ig, a fusion protein containing the extracellular domain of CTLA-4 linked to an IgG1 Fc region, is the reagent that is the furthest in its therapeutic development and it appears likely that this reagent will be the first cosignaling therapeutic. CTLA-4Ig can block the interaction of CD28 with CD80 and CD86, and has been shown to suppress immune responses in multiple preclinical models of autoimmune and inflammatory disease (260). CTLA-4Ig usage was first reported in patients with *Psoriasis vulgaris* were the reagent showed some efficacy (261). More recently, CTLA-4Ig has been tested in combination with methotrexate (MTX) in clinical trials of rheumatoid arthritis (259). Patients receiving CTLA-4Ig in combination with MTX showed better clinical improvement when compared with those receiving MTX alone. However, exacerbations of disease were also reported following CTLA-4Ig usage complicating predictions for therapeutic use. It was suggested that exacerbations might result from the loss of regulatory T cell function (90, 262). Using a different approach, a clinical trial in stage IV melanoma patients delivered anti-human CTLA-4 antibody along with tumor antigen epitope peptides. Tumor regression was observed although patient numbers was limited and several patients developed manifestations of autoimmune responses that resolved after discontinuation of therapy. (263, 264) The anti-CTLA-4 antibody appeared to break tolerance to autoantigens. Further studies are needed to identify appropriate clinical strategies to maximize efficacy,

while minimizing autoimmune side effects. Definitely, despite some encouraging results, further studies will be needed to assess long-term efficacy and safety for CTLA-4Ig and anti human CTLA-4 antibody usage.

1.6.2 CD28 superagonists

Superagonistic CD28 antibodies generate a strong activating signal that bypasses the need for TCR signaling (265-267). CD28 superagonist administration *in vivo* has been shown to lead to preferential activation and expansion of naturally occurring CD4+CD25+CTLA-4+FoxP3+ Treg cells leading to the amelioration of autoimmune diseases in preclinical models (265-267). Moreover, treatments over a broad dose range were never accompanied by side effects. Because of the success in preclinical studies, superagonistic CD28 antibodies were believed to be a promising novel treatment option. Very recently, the first phase 1 clinical trial of these antibodies was reported (268). Administration of TGN1412, a superagonist humanized anti-CD28 monoclonal antibody yielded unexpected results. The volunteers receiving this treatment suffered from a massive systemic inflammatory response syndrome. The exact mechanism of this devastating reaction awaits clearer description and will certainly help in the global understanding of cosignaling.

1.6.3 Targeting CD80 and CD86

Strategies to block CD80 and CD86 using antibodies have been promising in preclinical studies, including primate studies in transplantation (269) and in autoimmune and inflammatory disease models (260). *In vivo* studies showing that CD80 and CD86 antibody blockade enhances and attenuates immune responses, respectively support the view that CD86 is largely activating and CD80 inhibitory (270-272). CD80 and CD86 antibodies are currently in early trials for the prevention of GVHD and psoriasis (273, 274). However, the therapeutic targeting of the costimulatory pathway by CD80 and CD86 antibodies may be complicated by expression of CD80 and CD86 on T cells. Downregulation of T cell responses through this expression may be a clinically desirable process (91). Moreover, the description of reverse signaling into CD80/CD86 expressing cells is a novel

aspect of CD80/CD86 function and may cause a variety of biological effects that have yet to be fully described (93, 94, 230).

1.6.4 CD80 and CD86 as vaccine adjuvants

Co-immunization of CD80 and CD86 has improved the efficacy of gene- and cell-based vaccines in animal models and has shown promising results in pre-clinical tumor models (275-278). Recent use of B7 knockout mice in vaccine studies has confirmed that induction of immune responses to a DNA encoded antigen is critically dependent on CD86, but not CD80 (279, 280). Moreover, the timing of expression of CD80 versus CD86 appeared important in this model system (279, 280). Because many tumors are poorly immunogenic due to the absence of B7 proteins at their surface, alternative clinical approaches have explored the use of vaccination strategies with tumor cells expressing high levels of CD80 and CD86 to enhance tumor immunity (281-284). These applications are at their early stages and will require robust clinical studies to assess their efficiency.

Although substantial progress has been made in the cosignaling field, investigators continue to discover important biological functions for these molecules such as their function in peripheral tissues, in non-lymphoid organs and lymphocyte homeostasis to name but a few. Understanding the physiological implications of all cosignaling molecules is necessary to comprehend T cell disregulation in autoimmune and allergic diseases, for modulating transplant and tumor immunity and for the design of therapeutics targeting these molecules. The ultimate clinical utility of cosignaling therapeutics remains dependent on a more complete understanding of the complex biology of the CD28 and B7 families.

1.7 Project rationale and thesis objectives

Adaptive immune response initiation is entirely dependent on the interaction of a T cell and an APC in the context of the IS. During this interaction, an antigen specific and a costimulatory signal is delivered allowing full T cell activation. The cosignaling molecules CD86 and/or CD80 at the APC surface are absolutely

required in the generation of effective naïve T cell responses by binding to their T cell counter receptors, CD28 and CTLA-4. CD80 and CD86 are the founding members of the B7 cosignaling family and are type I membrane proteins that belong to the IgSF presenting an IgV and a IgC domain in their extracellular portion. Despite their shared receptors, different properties have been observed for both molecules including different expression kinetics, different binding properties, different signaling pathways and different functional properties. Although having been discovered more than a decade ago, many structural and functional characteristics of CD80 and CD86 are still elusive and await clear descriptions. A better understanding of the structure and function of CD80 and CD86 will help in understanding their different properties. Because of CD80 and CD86 crucial role in immune response initiation and termination, these and other cosignaling molecules are of high therapeutic interest. However, it is clear that a better understanding of their function is required before they are successfully used as immunotherapeutics. Moreover, with the description of several new members in the B7 cosignaling family, analysis of the domain properties of CD80 and CD86 could certainly act as a paradigm.

An area of controversy in CD80 and CD86 function concerns the contribution of the different domains in receptor binding. While crystallographic data show that only the IgV domain of both molecules is responsible for receptor interaction, substantial mutational analysis data have pointed towards a dual IgV/IgC implication. Another controversial issue is the molecular structure of these proteins at the cell surface. Although CD80 and CD86 have crystallized as homodimers, biophysical studies have suggested otherwise. These findings certainly need verification in a cellular context. Moreover, although the initiation, maintenance and function of the IS have been the subject of many experimental studies in T cells, the role of the APC and the APC surface molecules within this structure remains to be established.

The work presented in this thesis intends to establish the role of CD80 and CD86 domains in their molecular structure and their role in naïve T cell activation. Specifically, the research objectives are to investigate the role of CD80 and CD86 domains in respect to (a) their effect in the molecular structure of CD80 and CD86 (b) their contribution to receptor binding and (c) their overall impact on the cosignaling function of these molecules.

Chapter 2

CD80 and CD86 IgC Domains Are Important for Molecular Structure, Receptor Binding and Cosignaling Function

Work presented in this chapter investigates the role of the CD80 and CD86 domains in their molecular structure, in their contribution to receptor binding and in their overall impact on the cosignaling function of these molecules. The experimental questions are answered through the use of wild type, deleted and chimeric CD80 and CD86 molecules. Methods used to answer the experimental questions are a peptide-specific cellular interaction system, a newly described cytometry-based fluorescence energy transfer method, non-denaturing gels and soluble receptors assays.

CD80 and CD86 IgC Domains Are Important for Molecular Structure, Receptor Binding and Cosignaling Function

Running Title: Distinct Functional Roles for CD80 and CD86 Ig Domains

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Non-standard abbreviations used :

Flow cytometric energy transfer (FCET)

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Abstract

CD80 and CD86 are prototypical members of the B7 cosignaling molecules family and play pivotal roles in naïve T cell activation. CD80 and CD86 present a membrane distal variable-like (IgV) domain, a membrane proximal constant-like (IgC) domain and an intracellular domain. CD80 and CD86 show several distinct features such as differential expression patterns, unique receptor affinities and distinctive oligomeric states. Crystallographic studies have clearly identified the IgV domain of both molecules as responsible for receptor interaction. However, many earlier studies suggested that both IgC and IgV domains are required for full cosignaling function. In this study, we characterized the role of CD80 and CD86 domains in molecular structure using cytometry-based fluorescence energy transfer (FCET) and non-denaturing gels, in receptor binding properties using soluble receptors assays, and in cosignaling function in a peptide-specific cellular interaction model. We report the first detection of CD80 dimers and CD86 monomers in live cells. Our FCET data combined to our biochemical results clearly show that the IgC domain inhibits multimer formation in both molecules. Indeed, CD80ΔC expressing cells showed greater dimer and multimer ratios when compared to CD80 wild type and CD86ΔC cells express CD86 as a mixed population of monomers and dimers in contrast to CD86 wild type that is only monomeric. The molecular structure of both molecules correlates with their CD28 and CTLA-4 binding properties. Finally, both IgC and intracellular domains are shown to be required for full CD80 and CD86 cosignaling. These findings reveal the distinct but coupled roles of CD80 and CD86 domains in naïve T cell activation.

Introduction

CD80 and CD86, also known as B7-1 and B7-2, are the prototypical members of the B7 cosignaling molecules family and are key players in the activation of naïve T cells. CD80 and CD86 are type 1 transmembrane proteins expressed mainly on antigen presenting cells (APC) and belong to the immunoglobulin superfamily (IgSF). They present two extracellular domains, with a membrane distal variable-like domain (IgV), a membrane proximal Ig constant-like domain (IgC) and an intracellular domain. As typical Ig domains, both IgV and IgC consist of anti-parallel beta sandwiches joined by a short linker region (224). These beta sandwiches are composed of an AGFCC'C'' front sheet and a BED back sheet (185, 186). Despite sharing only 25% sequence identity, CD80 and CD86 bind to common receptors through the MYPPPY motif present in the CDR3 region of CD28 and CTLA-4 expressed on naïve and activated T cells respectively. Recent studies have however demonstrated CD28 to be the preferential receptor for CD86 and CTLA-4 the preferential receptor for CD80 (155, 167). These cosignaling molecules are known to be present at the immunological synapse and their interaction leads to bi-directional signaling. Apart from different receptor affinities, CD80 and CD86 show several other distinct features such as differential expression patterns and unique oligomeric states. CD86 is expressed constitutively and is rapidly upregulated following T cell interaction while CD80 is upregulated later in the immune response (229). Many mutational and structural studies have implicated residues of both the IgV and IgC regions of CD80 and CD86 for CD28 and CTLA-4 receptor interaction (247, 250, 253, 285). However, co-crystal structures of CD80 and the isolated IgV domain of CD86 complexed with CTLA-4 have shown that it is the IgV, and more specifically the front face of these molecules that contacts this receptor. No direct interaction between the IgC domain and CTLA-4 has been noted (185, 186). However, (185, 227) atomic contacts between the IgV and IgC domains of CD80 are thought to help stabilize the conformation of the IgV domain. Solution, crystallographic, biochemical structural and imaging studies have shown that CD80 predominantly exists as a dimer in a mixed dimer/monomer population at the cell surface and CD86 exists

solely as a monomer (185, 186, 227, 256, 257). Sequence analysis of the dimer interface revealed that the majority of residues in CD80 are hydrophobic while those of CD86 dimer are hydrophilic (256). These differences not only support the difference in CD80 and CD86 multimeric state, but also provide a mechanism that prevents formation of CD80:CD86 heterodimers (256). In this study we aimed to better characterize the role of CD80 and CD86 domains in molecular structure, binding properties, and function using wild type, deletion and chimeric constructs. Molecular structure can be studied by measuring the fluorescence resonance energy transfer (FRET) between proteins coupled to CFP and YFP fluorescent proteins. FRET is the process by which an excited donor fluorophore transfers its non-radiative energy to an acceptor molecule when the emission spectrum of the donor overlays the absorption spectrum of the acceptor and when the distance between the donor and acceptor molecules is less than 100 angstroms (Å). When FRET occurs, there is an enhancement of the acceptor fluorescence and a quenching (attenuation) of the donor emission. FRET can be measured by different techniques such as spectrofluorimetry, confocal microscopy, and flow cytometry. Confocal microscopy FRET is an expensive and technically challenging technique that requires substantial acquisition and analysis time to achieve statistical significance. Moreover, it often relies on fixed cell samples. Flow cytometry based FRET (FCET) allows the quick analysis of a large number of live cell events making it a very good option to measure protein interactions. Using this method, we describe the first detection of CD80 homodimers and CD86 monomers in live cells and clearly identify an inhibitory role of IgC domains in multimer formation. This IgC-dependent molecular structure alteration impacts CD28 and CTLA-4 interaction. Finally, both IgC and IgT domains are shown to be required for full CD80 and CD86 cosignaling function.

Material and Methods

Constructs generation

cDNAs encoding for CD80 and CD86 wild type, deleted of their IgC domains (CD80ΔC, CD86ΔC) or deleted from their intracellular domain (CD80ΔT, CD86ΔT) as well as chimeric molecules V1C2T2 and V2C1T1 were previously described (254). These cDNAs were amplified with oligonucleotides containing a 5' XhoI and a 3' SacII restriction site for directional cloning into the pECFP-N1 and pEYFP-N1 expression vectors (Clontech). The sequence of the oligonucleotides were as follows: CD80Fwd CTCGAGGCCACCATGGGCCACACACGG; CD86Fwd CTCGAGGCCACCATGGGACTGAGTAAC; CD80Rev CCGCGGTACAGGGCGTAC; CD86Rev CCGCGGAAAACATGTATC; CD80ΔTRev CCGCGGTCTTGGGGCAAAGCA; CD86ΔTRev CCGCGGTTTCCATAGAATTAG. pECFP-Memb coding for a fusion protein consisting of the N-terminal 20 amino acids of neuromodulin that contains a signal that targets ECFP to cellular membranes was from Clontech and PEYFP-Memb was cloned by replacing the ECFP from pECFP-Memb by EYFP from the pEYFP vector. CD4 and CD4K318E in fusion with CFP and YFP were previously described (286). As a positive control for FRET, a CFP-YFP chimera (pECFPhYFP) plasmid was generated as follows. The pEYFP-N1 vector was linearized with *Nhe*I and the ends were blunted using T4 DNA polymerase. The DNA was then digested with *Xba*I to release the YFP insert, which was then inserted into the pBluescript II SK vector (Stratagene) that had been digested with *Eco*RV and *Xba*I, generating pSKYFP-N1. A hinge (h) sequence, encoding three repeats of Gly-Gly-Gly-Gly-Ser, was generated by annealing the complementary oligonucleotides 5'- CCG GTG GGA GGA GGA GGC AGC GGC GGC GGA GGA AGC GGC GGA GGC GGA TC -3' and 5'- CAT GGA TCC GCC TCC GCC GCT TCC TCC GCC GCC GCT GCC TCC TCC TCC CA -3', and was ligated into *Age*I/*Nco*I treated pSKYFP-N1, resulting in the plasmid pSKhYFP-N1. This plasmid was digested with *Eco*RI, the ends were blunted by treatment with T4 DNA polymerase, and the hYFP insert was digested out with *Xba*I. This

fragment was inserted into the *SmaI/XbaI* sites of pECFP-C1 yielding the fusion pECFPhYFP plasmid.

Cell lines and transfections

The human epithelial kidney cell line 293T was obtained from ATCC. These cells were maintained in DMEM media supplemented with 10% FCS and 1% penicillin-streptomycin. 293T cells were transfected using a calcium phosphate based method. Briefly, 1.2 million cells were seeded in 100mm plates, grown overnight, and the media was refreshed 1 hour prior to transfections. DNA and calcium phosphate solution was added to HBS solution drop by drop and the mixture was added to the cells. Twenty-four hours following transfections, cells were washed and detached in PBS containing 3mM EDTA. The murine mastocytoma B2D cell line stably expressing HLA-DR0101 was previously described (287). These cells were transfected with 25ug DNA by electroporation at 260V and 950uF. Human CD28 positive Jurkat T cell line (A14 Jurkat T cells) derived from the CH7C17 Jurkat T cell line expressing a Vα1.2/Vβ3.1 TCR specific for $HA_{306-318}$ peptide restricted to HLA-DR0101 was previously described (195).

Antibodies and reagents

CD80 antibodies used for flow cytometry analysis included PeCy5-conjugated anti-human CD80 clone L307.1 from BD Pharmingen, PE-conjugated anti-human CD80 clone 37711 from R and D Systems and uncoupled anti-human CD80 antibody clone BB1 from ID Labs Canada. For immunoblots, anti human CD80 clone 9091 was from Santa Cruz Biotechnology. CD86 antibodies used for flow cytometry analysis included PE-conjugated anti-human CD86 clone IT2.2 and clone FUN1 from BD Pharmingen, unconjugated anti-human CD86 clone BU63 from ID Labs Canada, and PE-conjugated anti-human CD86 clone HA52B7 from Beckman Coulter. For immunoblots, anti-human CD86 clone BU63 was used. CD28Fc and CTLA4Fc were purchased from R and D Systems. All secondary antibodies were from Molecular Probes including. A polyclonal rabbit antibody

against CFP and YFP was from BD Pharmingen. Peptide corresponding to residues 306-318 (PKYVKQNTLKLAT) of the influenza hemagglutinin protein ($HA_{306-318}$) was synthesized with >95% purity at the Sheldon Biotechnology Center (Montreal, Canada). β-actin antibody and CHAPS detergent were from Sigma.

Flow cytometry Detection of FRET

All flow cytometry was performed on a BD™ Bioscience LSRII cytometer using a method adapted from (288). The Coherent Sapphire™ Solid state at 20mW power and the Coherent Vioflame™ Solid state 405-nm at 25mW power laser lines were used for YFP and CFP excitation respectively. The optical configuration is shown as supplementary data figure 1. Briefly, YFP signals were collected using a 524/10 bandpass filter in the primary laser pathway (laser 1). The CFP and FRET signals were collected using 460/20 and 585/42 bandpass filters, respectively, along with a 500 long-pass dichroic splitter filter inserted into Vioflame Solid state laser pathway (laser 2). Because FRET and YFP signals are detected on separate detectors in this configuration, the FRET process does not affect YFP signal. All FACS data were analyzed using FACS DIVA software (Becton Dickson, San Jose, CA).

Western Blotting

293T cells were lysed on ice for 30 minutes in 1% NP40, 5mM EDTA, 0.1% SDS and protease inhibitors (Roche) containing buffer. For non-denaturing experiments, transfected cells were lysed in 2% CHAPS buffer containing 10% glycerol and protease inhibitors (Roche). Lysates were cleared by centrifugation at 13000rpm for 10min. The amount of total protein in each sample was quantified with the Micro BCA assay (Pierce). Samples were then prepared with reducing (containing β-mercaptoethanol and SDS) or non-reducing loading buffer (buffer containing trypan blue and glycerol). The samples prepared with reducing loading buffer were boiled for 10 minutes and equal loads of protein were analyzed by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF)

membrane (Bio-Rad). The membranes were blocked for 1h at room temperature in PBS supplemented with 5% milk and probed with specific antibodies overnight at 4°C under agitation. Blots were developed using horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) and the ECL Chemiluminescent Detection System (Amersham Pharmacia). All quantification made use of the ImageQuant 5.1 program.

Biochemical Fractionation

This protocol was adapted from (289). $10x10^6$ transfected 293T cell lines were pelleted, washed in PBS, resuspended in hypotonic solution (10 mM HEPES pH 6.9, 10 mM KCl, protease inhibitors) and incubated on ice for 20 minutes. Cells were disrupted by pipetting up and down 20 times. Nuclei were pelleted at 3200 rpm for 10 minutes at 4°C. Supernatant from pelleted nuclei was centrifuged further at 35000 rpm for 30 minutes at 4°C. The supernatant (cytosolic fraction) was separated and the pellet (cytoskeletal plus membrane fractions) was resuspended in NTENT buffer (500 mM NaCl, 10 mM Tris–HCl pH 7.2, 1 mM EDTA, protease inhibitors and 1% Triton X-100). This fraction was centrifuged at 14000 rpm for 30 minutes at 4°C. The resulting pellet, resuspended again in NTENT buffer, comprised the cytoskeletal fraction, while the supernatant comprised the membrane fraction.

FRET Efficiency and Molecular Distance Calculations

As in (288), CFP quenching was measured by using two distinct FRET populations, a control negative FRET population in our case expressing pECFP-Memb and pEYFP-Memb and the tested FRET population. The positions of these two populations were adjusted in a plot of YFP versus FRET in order to equalize their YFP intensities. In the advent that the tested population was FRET positive, CFP quenching was measured by subtracting the CFP MFI value of the FRET negative population from the CFP MFI value of the FRET positive population with both populations expressing equal YFP MFI. The FRET efficiency by FACS (f_e^f) was calculated using the following formula:

$$f_e^f = (I_{(CFP,\ FRET\ -)} - I_{(CFP,\ FRET+)}) / I_{(CFP,\ FRET\ -)}$$

where $I_{[CFP,FRET-]}$ and $I_{[CFP,FRET+]}$ are the CFP intensities in the negative and positive FRET populations, respectively, when both populations have equal YFP intensities.

The relative molecular distance between two fluorophores (r) was calculated according to the formula:

$$f_e^f = R_0^6 / (R_0^6 + r^6)$$

where R_0, the Förster radius, is the distance corresponding to 50% FRET efficiency and is about 50 Å for a CFP-YFP pair (288).

CD28Fc and CTLA4Fc Stainings

Transfected 293T cells were incubated for 1 hour at 4°C with various concentrations of CD28Fc or CTLA4Fc. Cells were then washed and incubated for an additional 30 minutes with a secondary antibody specific for human Fc coupled to Alexa fluor. After washing, cells were fixed in 2% paraformaldehyde and analyzed at the cytometer. To control for expression levels of the various proteins, data was analyzed by gating on equal MFI CFP populations.

EC50 calculations

In brief, EC_{50} were determined as the amount of CD28Fc or CTLA4Fc resulting in 50% positive cells as determined by flow cytometry staining. These calculations were made using a shareware MS excel worksheet ED50v10. Briefly, the log concentrations of CD28Fc and CTLA4Fc were plotted on the *x*-axis and the corresponding % positive cells on the *y*-axis. The EC_{50} value was determined from the curve by reading out the x-axis value such that the y-axis value equals half of the peak value of the y axis.

ELISA

B2D cells were electroporated with various YFP-fusion constructs and positive cells were cell sorted. The sorted cells were co-cultured overnight with A14 Jurkat T cells at a 1:1 ratio at 37°C in round-bottom 96-well plates with various concentrations of HA peptide. Supernatants were assayed for human IL-2 according to manufacturer's instructions (BD Pharmingen).

Results

Wild Type, IgC and Intracellular Domain Deleted CD80 and CD86 Proteins Are Recognized at the Cell Surface

Wild type, IgC and IgT CD80 and CD86 constructs were cloned in frame with E-CFP and E-YFP to generate fusion constructs expressing the fluorescent molecules at the C terminus. To test the expression and conformational integrity of these constructs, equal amounts of wild type molecules (CD80 and CD86), IgC deleted molecules (CD80ΔC and CD86ΔC) and intracellular domain deleted molecules (CD80ΔT and CD86ΔT) (figure 1, panel A) were transfected in 293T cells. Their surface expression was tested using a number of different antibodies with unique binding specificities directed against the IgV domain of either CD80 (clone L3074, clone 37711 and clone BB1) or CD86 (clone IT2.2, clone BU63, clone FUN1 and clone HA52B7). As shown in figure 1, panel B (CD80 stainings) and panel C (CD86 stainings) all tested cells were 100% positive for all antibodies tested. A difference was observed in the mean fluorescence intensity (MFI) of these stainings, especially between wild type and IgC deleted molecules. It was not clear if this MFI difference was due to poor antibody binding resulting from a conformational change introduced by the domain deletion or rather, if the constructs were expressed at different levels at the membrane. We therefore separated the cytoplasmic, membrane and cytoskeletal fraction of 293T transfected cells (figure 1, panel D). From these experiments, it is evident that the membrane expression levels of IgC deleted molecules were significantly lower than wild type and intracellular deleted molecules. We therefore hypothesize that the IgC deleted molecules are targeted for early degradation during synthesis or have a higher turnover at the membrane. These results explain, at least in part, the MFI difference of IgC deleted molecules when compared to their wild type counterparts. CD80 and CD86 intracellular domains associate to the cytoskeleton (225, 226, 290). As expected, intracellular domain deletion of CD80 and CD86 molecules resulted in loss of their cytoskeletal association (figure 1, panel D). Importantly, CD80ΔC and CD86ΔC retained their cytoskeletal association even in the presence of the C-terminal fluorescent tag arguing again in favor of

conformational integrity conservation of these molecules. All isolated fractions were subjected to p38 probing and this molecule was either greatly enriched or only present in cytoplasmic fractions thereby confirming the specificity of our fractionation approach (data not shown). Altogether, despite IgC deleted proteins being expressed at lower levels, all generated clones were recognized by monoclonal antibodies of various specificities and the intracellular domains of both CD80 and CD86 retained their cytoskeletal association function indicating that the overall conformation of proteins under study was not dramatically altered.

FCET Detection of CD80 Dimers and CD86 Monomers in Live Cells

CD80 and CD86 molecular structure was assessed by flow cytometric energy transfer (FCET) between CFP and YFP tagged molecules. We adapted the donor fluorophore quenching method from He et al. (288) that allows FRET efficiency quantification during CFP to YFP FRET. Because detection of donor CFP quenching becomes more reliable when molar ratio between CFP and YFP equals to 1, we documented comparable expression levels for each tagged protein by flow cytometry and biochemistry. As shown in figure 2 panel A, cells expressing equal amounts of any given CFP and YFP tagged constructs also presented highly similar MFI. To confirm comparable expression of these fusion proteins in 293T cells, immunoblots using an antibody that reacts with the common protein portion of all proteins related to GFP (XFP) were performed. As shown in panel B of figure 2, loading-corrected densities measured from the immunoblots showed that there is roughly the same amount of protein between CFP and YFP tagged constructs for all transfectants. Being ensured that the molar ratio was roughly equal to one, we proceeded to FCET experiments in 293T. CFP intensities were measured in an YFP versus CFP plot from YFP-equalized FRET-negative and tested condition populations and FRET efficiency by FACS (f_e^f) was calculated using the formula presented in the material and methods section. Of note, FCET efficiencies are always greater than the microscopy-based FRET efficiencies. The difference possibly results from the fact that FRET detection in living cells by flow cytometry is achieved within microseconds, which minimizes laser

photobleaching effects. As shown in panel C of figure 2 and summarized in panel D, FCET was always detected in cells expressing CD80, independently of IgC or intracellular domain deletion. Removal of CD80 intracellular domain resulted in a higher FRET efficiency ($p \leq 0.0409$) while removal of its IgC domain resulted in somewhat of a decrease of FRET efficiency ($p \leq 0.0672$). For CD86, no FCET was measured in cells expressing wild type molecules. Removal of CD86 intracellular domain did not affect its molecular structure but notably, the deletion of CD86 IgC led to FCET detection. This is the first description of CD80 and CD86 monomers and multimers in live cells and these data suggests that the CD86 IgC domain restrains CD86 from forming dimers. Because of the inverse correlation that exists between FRET efficiency and molecular distances, the derived relative biological distance between CFP and YFP moieties was calculated from the FRET efficiencies according to equation mentioned in the materials and methods section and is presented in figure 2, panel D. Not only does our FCET data concur with previously published microscopy-based FRET data that had shown that CD80 could be detected as a dimer but not CD86 (257), our positive controls all showed good FCET signals. This was the case for the fusion protein coding for both CFP and YFP separated by a 15 amino acid linker where the two fluorophores are so close that FRET is always detected. Other positive controls were CD4-lck, a cytoplasmic-membrane interacting pair (291), and Fas, a molecule known to trimerize (292). Using this distinct FRET approach, we obtained the same results previously obtained by showing FCET detection for the CD4 molecule and lost of this FCET signal with the dimerization impaired CD4 K318E mutant (286, 293). Altogether, FCET experiments have led to the detection of a FRET singal for CD80 but not for CD86 thereby suggesting that CD80 exist as multimers and CD86 as monomers in live cells and deletion of CD86 IgC domain leads to the detection of a FRET signal therefore suggesting an inhibitory role for the IgC domain in multimer formation.

The IgC Domain Inhibits Multimer Formation in Both CD80 and CD86

FCET measurements allow the detection of energy transfer between two fluorophores-tagged molecules; however, it does not allow the differentiation between dimerization and higher orders of interaction nor the detection of monomers. To further describe the role of the IgC and intracellular domains in CD80 and CD86 molecular structure, non-denaturing gels experiments were performed with 293T transfected cells. Cells were lysed in a 2% CHAPS buffer. The lysates were ran either in non-denaturing loading buffer composed of bromophenol blue and glycerol or boiled in SDS and β-mercaptoethanol containing loading buffer. One of three representative experiments is shown in figure 3. As shown in panel A, CD80 was mainly detected in monomeric form, followed by dimers, trimers and multimers. Following boiling (labelled as +), these multimeric forms disappeared. Highly similar results were obtained with CD80ΔT. Of high interest, CD80ΔC expressing cells presented very low levels of CD80 monomers and high levels of multimeric forms composed of dimers, trimers, tetramers and multimers. Even after boiling, detection of CD80ΔC monomeric form was only slightly increased while the multimeric forms remained at the top of the gel although the dimeric, trimeric and tetrameric forms disappeared. This suggests that the multimers formed by CD80ΔC molecules somewhat resisted disruption by boiling. Results obtained for CD86 are shown in panel B, figure 3. Only monomers were observed for the wild type and CD86ΔT expressing cells. As anticipated from the FCET experiments, a dimeric form of CD86ΔC was detected. Following boiling in denaturing loading buffer, the dimeric form disappeared. The dimer and multimer ratio for each protein was calculated by dividing the loading-corrected density of the relevant form to the total density as presented in panel C of figure 3. CD80 and CD80ΔT showed similar dimer ratios but CD80ΔC showed a higher dimer ratio due to the higher amount of dimers detected. CD80 and CD80ΔT showed a somewhat different multimer ratio but more strikingly, CD80ΔC showed a much higher multimer ratio, reflecting its propensity to form higher order complexes. These findings revealed that, as seen for CD86ΔC in FCET and non-denaturing gels experiments,

the IgC domain of CD80 also inhibits multimer formation. Altogether, these results complement the FCET data by showing that the IgC domain of both CD80 and CD86 inhibit multimer formation.

CD80 and CD86 IgC Domains Impact CD28 and CTLA-4 Binding

To study the role of CD80 and CD86 domains in CD28 and CTLA-4 binding, we transfected 293T cells with CFP-tagged constructs and incubated the cells with various concentrations of soluble CD28 and CTLA-4 and then incubated with Fc specific secondary antibodies. Cytometry analysis was performed on cells presenting very comparable CFP MFI to control for expression levels. The results of one of three representative experiments are shown in figure 4. Results are shown either as % positive cells (figure 4 panel A) or as MFI values (figure 4 panel B). Even when approaching close to 100% positive cells, CD80ΔC and CD86ΔC MFI remained low when compared to their wild type and intracellular domain deleted counterparts. From these binding curves, it was possible to determine the EC50 value for all proteins under study (figure 4, panel C). The EC50 value gives the soluble receptor concentration that binds 50% of the cells. EC50 values were lower for CTLA-4 than for CD28 since both CD80 and CD86 bind CTLA-4 with higher affinity (184). The removal of the intracellular domain of both CD80 and CD86 appeared to confer a CD28 binding advantage since EC50 values were less than with the wild type molecules (0.83ng/ml versus 3.37ng/ml for CD86 and 0.08ng/ml versus 4.3ng/ml for CD80). Deletion of CD80 and CD86 IgC domain had a negative impact on both CD28 and CTLA-4 binding with CD86ΔC requiring 13.2 times more CD28Fc and 3.5 times more CTLA-4Fc and CD80ΔC requiring 183.3 times more CD28Fc and 73.7 times more CTLA-4Fc when compared to wild type molecules. These results clearly indicate a role for both IgC and intracellular domains in ligand binding.

Both IgC and Intracellular Domains of CD80 and CD86 Are Required for Effective CD28-dependent IL-2 Production

To study the functional relevance of CD80 and CD86 Ig domains in T cell activation, B2D cells, stably expressing HLA-DR0101, were electroporated with the various constructs. These cells were then cell sorted based on highly equivalent MFI and put in culture at a 1:1 ratio with 0, 3 or 10ug/ml of HA peptide and CD28 positive T cells expressing a HA-specific TCR. One of three representative experiments is shown in figure 5. Cells expressing mYFP did not induce any response showing the cosignaling dependence of this system. Cells expressing CD80 and CD86 wild type molecules led to a dose-dependent T cell IL-2 secretion. Deletion of the intracellular domain of both CD80 and CD86 almost completely abrogated IL-2 production. This result is in agreement with our previous findings and those of others (225, 226, 290) showing the critical role of CD80 and CD86 cytoskeletal association. Deletion of the IgC domain of both CD80 and CD86 also led to a defect in T cell activation as measured by IL-2 secretion. At all HA peptide concentrations, the IL-2 response of CD80ΔC and CD86ΔC expressing cells was lower than their wild type counterparts. CD80ΔC and CD86ΔC expressing cells show differences in their multimer ratio that impacts their binding to CD28. These results show that the receptor binding impact seen in the soluble CD28 binding assays is correlated with T cell activation. Altogether, this data shows a functional role for all CD80 and CD86 domains in co-stimulation of T cell activation.

The IgC domains Show Inhibitory Function in CD80/CD86 Chimeras

FCET and biochemical results using IgC deleted construct showed that both CD80 and CD86 IgC domains inhibit multimer formation. In order to deepen our understanding of the IgC impact on molecular structure, we measured FCET using chimeric CD80/CD86 molecules as shown in figure 6. V80C86T86, expressing the IgV domain of CD80 and the IgC and intracellular domain of CD86 showed a drastic reduction in its FRET efficiency when compared to the wild type CD80 molecule. In contrast, when the IgV domain of CD86 is

expressed with the IgC and intracellular domain of CD80, CD86 remains a monomer. These results show that although the IgV domain present the dimer interface, the IgC domain impacts the overall molecular structure since CD86 IgC domain introduction in CD80 leads to the loss of dimer detection in live cells.

Discussion

CD80 and CD86 are both type I membrane proteins members of the IgSF displaying a membrane distal IgV domain, a membrane proximal IgC domain and an intracellular domain. Crystal structures of CD80 and CD86 complexed to CTLA-4 clearly identified the crucial role of the IgV domain in mediating receptor binding (185, 186). Also, both CD80 and CD86 crystallized as dimers in complex with CTLA-4. The dimer interface is contributed by the back sheet of the IgV domain of both CD80 and CD86 (227). Although the dimer interfaces occupy the same positions in CD80 and CD86 primary sequences, their chemical properties greatly differ. Indeed, CD80 dimer interface is hydrophobic while CD86 dimer interface is hydrophilic (256). Moreover, although CD80 was shown to form parallel 2-fold rotationally symmetric homodimers, CD86 dimers deviated from ideal two-fold rotational symmetry. Moreover, free and complexed CD80 and CD86 exhibit high structural similarity suggesting that receptor binding does not promote or enhance dimerization. Because of these findings, it is believed that the observed CD86 dimers in the CD86: CTLA-4 crystals resulted from crystal packing. Importantly, Bhatia et al. (257) have shown by microscopy-based photobleaching FRET that CD80 predominantly exists as a dimer and CD86 as a monomer at the cell surface of fixed HELA cells. Moreover, even upon introducing a cysteine residue thought to favor dimer formation, CD86 gives a very low FRET efficiency suggesting that it may have a tendency to resist dimerization. Although no direct contact between the IgC domain of CD80 and CD86 and CTLA-4 were observed in the crystals, earlier mutational and structural studies have showed that amino acids within both the IgV and IgC domains of CD80 and CD86 can significantly affect receptor binding and the overall cosignaling function of these molecules (247, 250, 253, 285). These studies have shown that addition or deletion of certain amino acids in CD80 and CD86 Ig domains could significantly alter the function of these proteins and that neither the IgV domain nor the IgC domain can act independently to provide full cosignaling function.

In an attempt to better understand the role of CD80 and CD86 Ig domains in their functional properties and molecular structure, we generated deletion and chimeric constructs of CD80 and CD86 in frame with either the CFP or YFP protein. First, we established by antibody staining and biochemical fractionation the expression and functional integrity of these constructs. Then, using a donor-quenching flow-cytometry based FRET method we showed that CD80 is detected as a dimer at the surface of live cells while CD86 is only detected as a monomer. These results are in agreement with those of Bhatia et al. that used a different FRET method (257). Moreover, we showed that CD86 is detected as dimers upon removal of the IgC domain suggesting an inhibitory role of the IgC domain of CD86 in dimer formation. We extended these findings by studying the molecular structure of these proteins by non-denaturing SDS-PAGE. This led to the observation that upon removal of the IgC domain of both CD80 and CD86, higher orders of multimerization was observed as seen as an increase of the dimer and multimer ratio. It is therefore clear from our observations that the IgC domain of both CD80 and CD86 are important in regulating the molecular structure of these molecules by impacting their dimerization capacity. Crucially, the molecular state of CD80 and CD86 greatly impacts their function as measured by receptor binding affinities and cosignaling function as determined by IL-2 secretion.

In an earlier report, Bajorath et al. (224) identified CD80 and CD86 IgC sequences to resembled that of the β2-microglobulin. This suggested that like β2m, the CD80 and CD86 IgC region could be involved in protein-protein interactions. Because it is clear from the crystal that the IgC domains of CD80 and CD86 do not contact the CTLA-4 receptor, this plausible interaction might occur with the membrane distal IgV domain. Of note, numerous atomic contacts between the IgV and IgC domains of CD80 have been described and we believe that the interaction of the IgC with the IgV impacts the dimer interface present in the IgV domain.

Moreover, it was also proposed from the crystal structure of CD80/CTLA-4, that CD80 IgC glycosylation at Asn 173 might stabilize CD80 dimers by the interaction of sugars residues with the plasma membrane. Analysis of the amino acid sequence of CD86 and CD80 revealed interesting differences in the IgC domain of these molecules (257). Four extra amino acids either in the B-C loop (insertion 144–147 KKMS) or in the C-D loop (insertion 150–153 LRTK) in the CD86 molecule were observed. These insertions could lead to a conformation difference of the B-C or C-D loop of the CD86 molecule in comparison to CD80 that might explain the molecular structure difference between the two molecules. Interestingly, we have shown that introduction of the IgC domain in CD86 led to an almost complete abrogation of CD80 dimer detection by FCET. This result agrees with the fact that CD80 and CD86 IgC domains are different and impact the dimerization capacity of the molecules.

CD28 is a monovalent dimer and CTLA-4 is a bivalent dimer. Upon removal of the IgC domain of both CD80 and CD86, and therefore accumulation of higher order multimers, CD28 binding was greatly impacted as shown by the calculated EC50. CTLA-4 binding was also impacted by the presence of multimers. Although CTLA-4 prefers dimeric ligands such as CD80, CD86ΔC dimer binding to CTLA-4 was not favored. This might be explained by the observation of the CD86 dimer not being in appropriate conformation for receptor binding upon dimerization as observed in the crystal and its suggested tendency to resist dimerization. The CD28 binding data was correlated to functional read-out by measuring IL-2 secretion following peptide-specific interaction with CD28 positive T cells. At 10ug/ml HA peptide, the IL-2 response was lower with the CD86ΔC expressing cells when compared with wild type. The same phenomenon was observed with CD80 and CD80ΔC. These results are in accord with those of others (254, 255) that studied these molecules in other *in vitro* and *in vivo* contexts. Vasu et al. also showed that a chimeric CD80 molecule expressing CD86 IgC domain was better at initiating responses than CD80 wild type. *In vivo* studies (254) have also shown that immunization with a cosignaling molecule

expressing CD86 IgC domain but the rest of CD80 led to a better anti HIV response. In view of our findings, this can be explained by the fact that the monomeric form is favored in this chimeric molecule and is therefore a better ligand for CD28. In contrast to our results, in the Agadjayan study (254), CD80dC was shown to be a better cosignaling molecule. We have shown that upon removal of its IgC domain, CD80 now forms higher order multimers and therefore is not a CD28 appropriate ligand. This conflicting data can certainly be explained by the complex interplay between CD28 and CTLA-4 in this *in vivo* model. CD80ΔC is not a better co-activator but rather a less potent co-inhibitor due to its affinity loss for CTLA-4 resulting in an overall increase in cosignaling response.

In this clear from our data and that of others that the distinct molecular organization of CD80 and CD86 account for their overlapping yet distinct effects on T cell responses. Both CD86 and CD80 and their receptors, CD28 and CTLA-4, are concentrated at the IS (150, 155, 294). The different oligomeric states of CD80 and CD86 indicate that these molecules form cosignaling complexes with distinct cell surface organizations, which may represent an important mechanism modulating their functional properties. Interestingly, it was recently demonstrated that B7 binding ultimately determines the formation of dimer-dependent CTLA-4 lattices necessary for T cell inactivation (170). These results emphasize the crucial role of CD80 and CD86 molecular structure in their function. In this study, we have identified a role for the IgC domain of CD80 and CD86 proteins in dimer formation. IgC domains of both molecules regulate the molecular structure of CD80 and CD86 directly impacting on their ligand binding and cosignaling properties.

Conclusion

Despite having been discovered more than a decade ago (211-213), the structural-functional relationships of CD80 and CD86 Ig domains remained ill defined. Our current findings described a role for CD80 and CD86 IgC domains in multimerization. Although the receptor interaction and the dimer interface all localized to the IgV portion of the molecule, the IgC impacts the dimer interface thereby directly influencing receptor binding properties. Because CD28 favors the binding of monomeric ligands and CTLA-4 that of dimeric ligands, the ratio of available CD80 and CD86 multimeric forms impacts the ensuing immune response. CD80 and CD86 are key players in naïve T cell activation. By better delineating the structural functional relationships between the domains that composed these molecules, we have better chances in regulating immune responses for therapeutic purposes.

Acknowledgements

We thank S. Gimmig for his help in FCET experiments and the generation of supplementary data figure 1.

Figure Legends

Figure 1 All CD80 and CD86 Constructs Are Recognized at the Cell Surface by IgV Specific Antibodies

A) Schematic representation of the deletion constructs used in this study. CD80 and CD86 are wild type molecules. CD80ΔC and CD86ΔC are deleted of their extracellular IgC domain. CD80ΔT and CD86ΔT are deleted of their intracellular domain. B) CD80 antibody stainings of 293T cells expressing CD80, CD80ΔC or CD80ΔT. One of two representative experiments is shown. Bars represent MFI values obtained from the stainings while the % positive cells is shown as the line on top of the bars. C) CD86 antibody stainings of 293T cells expressing CD86, CD86ΔC or CD86ΔT. One of two representative experiments is shown. Bars represent MFI values obtained from the stainings while the % positive cells is shown as the line on top of the bars. D) Sub-cellular fractionation of 293T cells expressing various CD80 and CD86 forms. C: cytoplasmic fraction, M: membrane fraction, Sk: cytoskeletal fraction. The loading-corrected membrane expression density for each protein is shown.

Figure 2 FCET Detection of CD80 Dimers and CD86 Monomers in Live Cells

A) CFP and YFP MFI values for all constructs transfected in 293T cells. B) Biochemical analysis of the protein levels for all constructs. The loading-corrected expression of each protein is shown. One of two representative experiments shown. C) FCET results representative of five independent experiments. FRET efficiencies in % for each protein under study. D) FRET efficiencies and calculated molecular distances between CFP and YFP tags of given proteins.

Figure 3 The IgC Domain Inhibits Multimer Formation in CD80 and CD86

Samples were ran on SDS-PAGE gels in non-denaturing or denaturing (+) conditions. A) CD80 results. Monomeric and multimeric forms are identified. CD80ΔC presents low levels of monomers and higher levels of multimers. B)

CD86 results. Monomeric and dimeric forms are noted. C) Calculated dimer and multimer ratio from loading-corrected expression densities. Results pooled from three independent experiments.

Figure 4 CD80 and CD86 IgC Domain Deletion Impact CD28 and CTLA-4 Binding

293T cells were transfected with CFP relevant constructs. Cells were incubated with various concentrations of soluble CD28 or soluble CTLA-4. A secondary reagent was used to detect soluble receptors and cells were analyzed by flow cytometry. Legend shown on the right. A) Results from soluble receptor binding shown as % positive cells. B) Results from soluble receptor binding shown as MFI. C) Calculated EC50 values, representing the concentrations of soluble CD28 or CTLA-4 that resulted in 50% positive cells.

Figure 5 Both IgC and IgT Domains of CD80 and CD86 Are Required for Effective CD28-dependent IL-2 Production

B2D cells were electroporated and cell sorted based on YFP fluorescence. Sorted cells were put in overnight culture with CD28 positive T cells in the absence or presence of 3ug/ml or 10ug/ml HA peptide. IL-2 production was measured by ELISA. The obtained values are shown as IL-2 pg/ml levels while the legend on the right indicate the HA peptide concentration. 1 of 3 representative experiments shown.

Figure 6 IgC Domain Show Inhibitory Function in CD80/CD86 Chimera

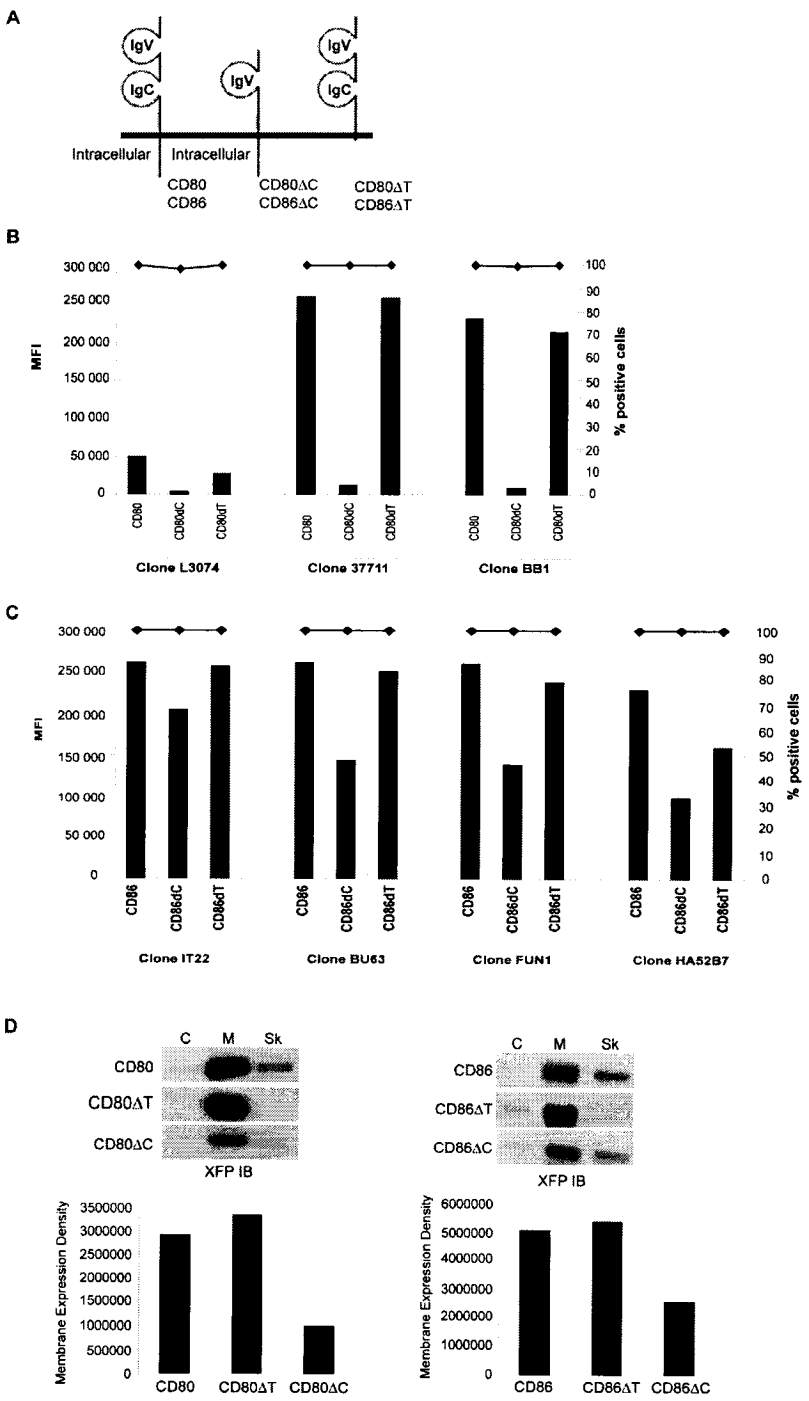
FCET experiments using chimeric CD80/CD86 constructs. FRET efficiency is greatly reduced upon presentation of the IgV domain of CD80 by CD86.

Legends for Data Not Shown

Figure 1 Validation of the Fractionation Technique by p38 Probing

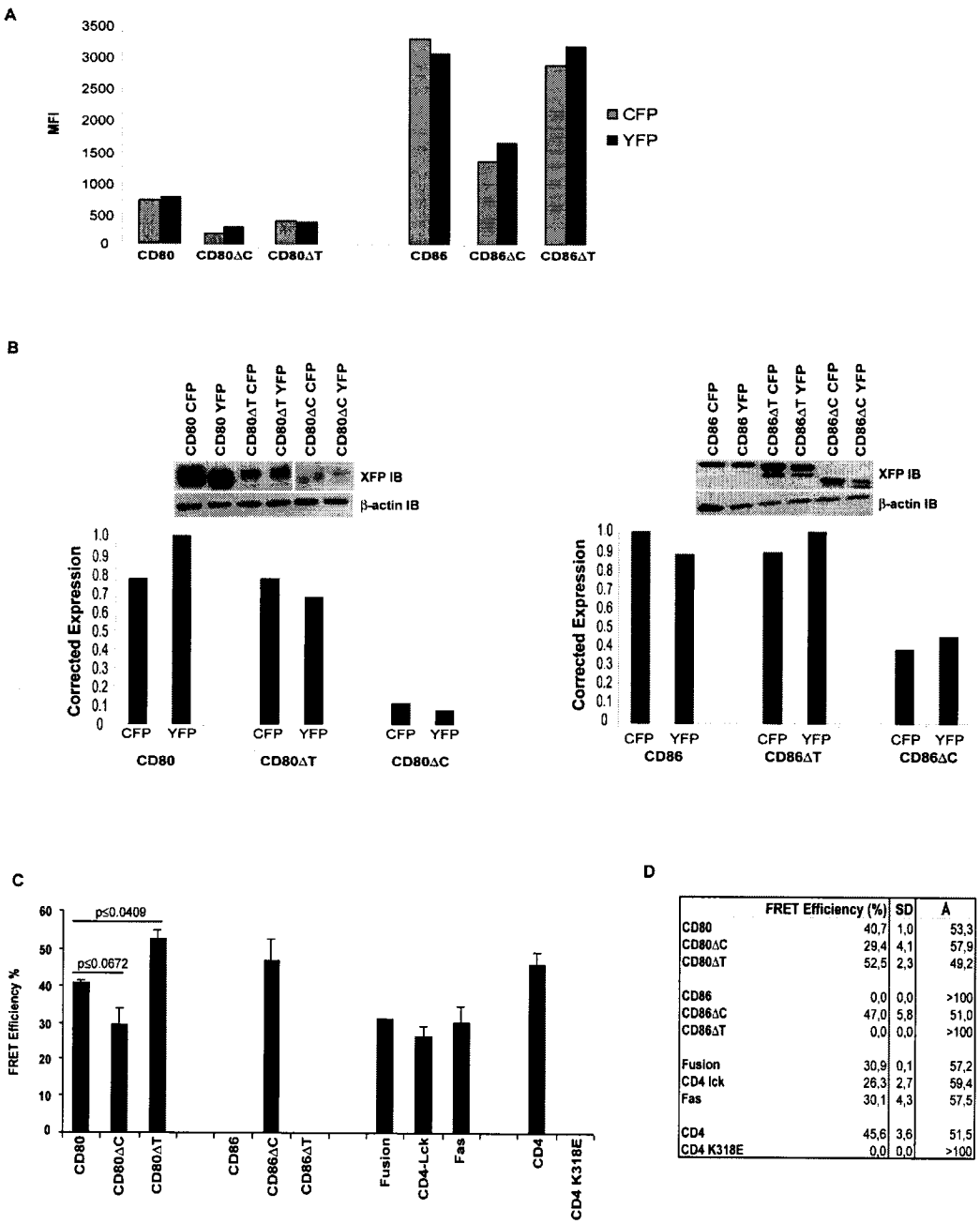
p38 immunoblot on subcellular fractions obtained from 293T cells. As expected, p38 is greatly enriched in the cytoplasmic fractions.

Figure 1 All CD80 and CD86 Constructs Are Recognized at the Cell Surface by IgV-Specific Antibodies



Refer to Appendix 4:
'Antibody Stainings Gating Strategy and Exemplary Histograms'

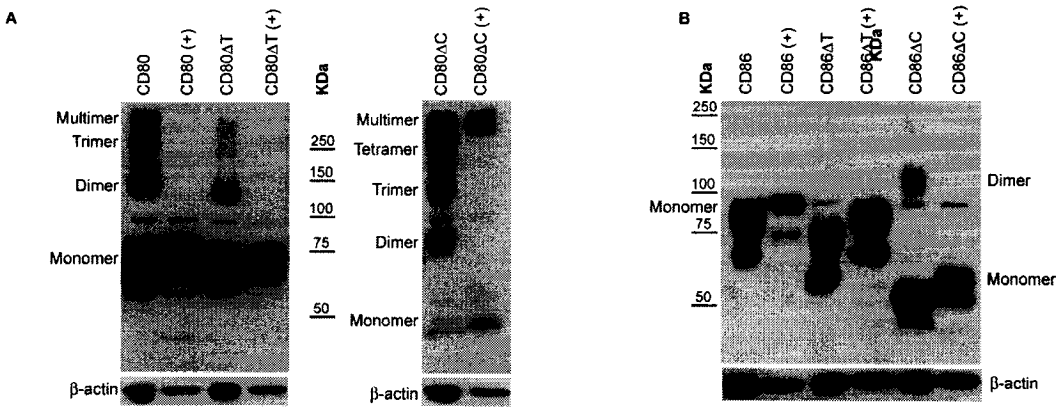
Figure 2 FCET Detection of CD80 Dimers and CD86 Monomers in Live Cells



	FRET Efficiency (%)	SD	A
CD80	40,7	1,0	53,3
CD80ΔC	29,4	4,1	57,9
CD80ΔT	52,5	2,3	49,2
CD86	0,0	0,0	>100
CD86ΔC	47,0	5,8	51,0
CD86ΔT	0,0	0,0	>100
Fusion	30,9	0,1	57,2
CD4 lck	26,3	2,7	59,4
Fas	30,1	4,3	57,5
CD4	45,6	3,6	51,5
CD4 K318E	0,0	0,0	>100

Refer to Appendix 5:
'Ligand Binding by FACS: Gating Strategy and Exemplary Histograms'

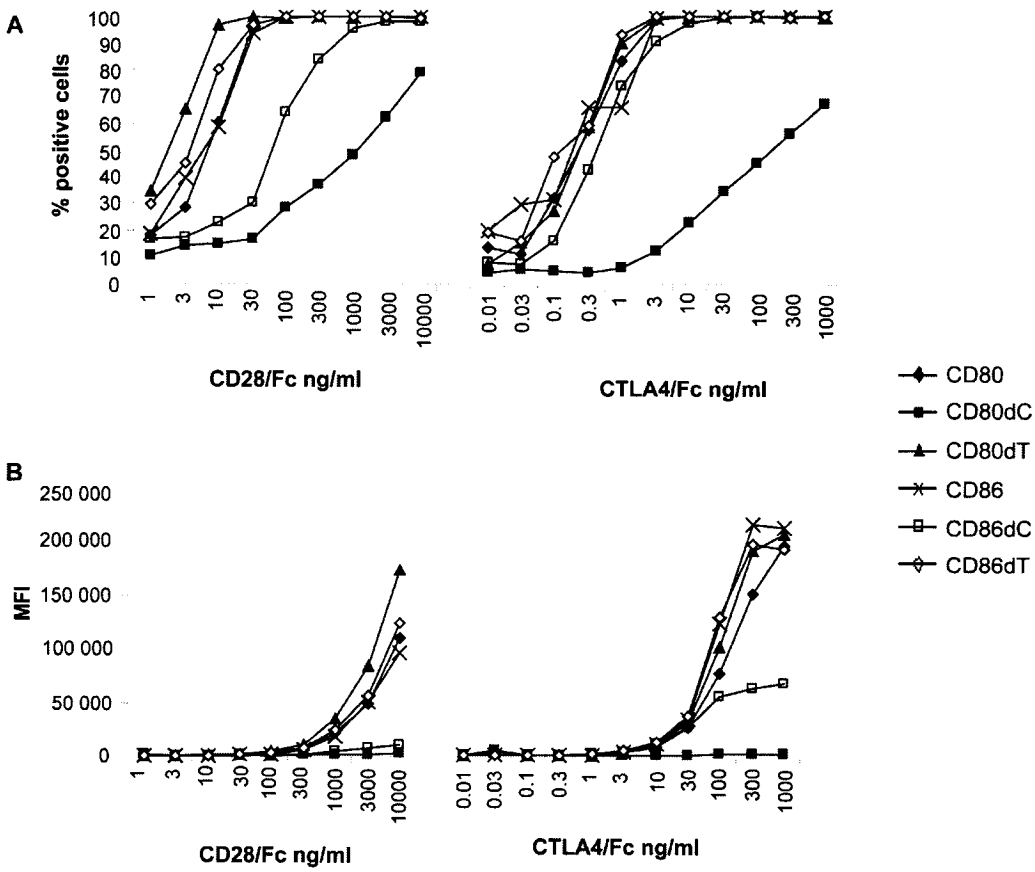
Figure 3 **The IgC Domains Inhibits Multimer Formation in CD80 and CD86**



C

	CD80 dimer ratio	SD	CD80 multimer ratio	SD	CD86 dimer ratio	SD
wt	0,28	0,04	0,27	0,04	na	na
ΔT	0,22	0,04	0,17	0,10	na	na
ΔC	0,40	0,06	0,61	0,10	0,25	0,08

Figure 4 CD80 and CD86 IgC Domain Deletions Impact CD28 and CTLA4 Binding



C EC50

	CD28Fc ng/ml	CTLA4Fc ng/ml
CD80	4.3	0.23
CD80dT	0.08	0.24
CD80dC	788.22	16.94
CD86	3.37	0.15
CD86dT	0.83	0.11
CD86dC	44.92	0.53

Figure 5 Both IgC and IgT Domains of CD80 and CD86 Are Required for Effective CD28-Dependent IL-2 Production

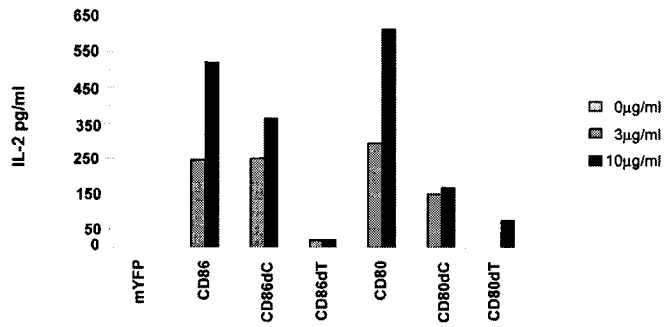
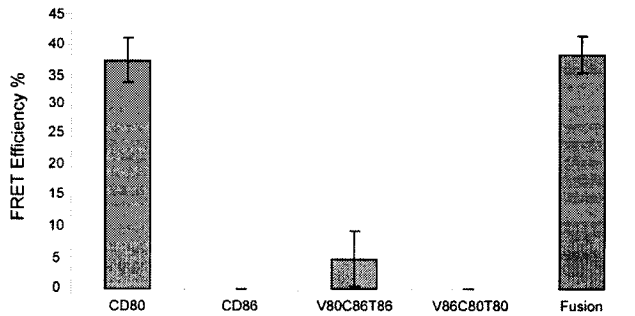
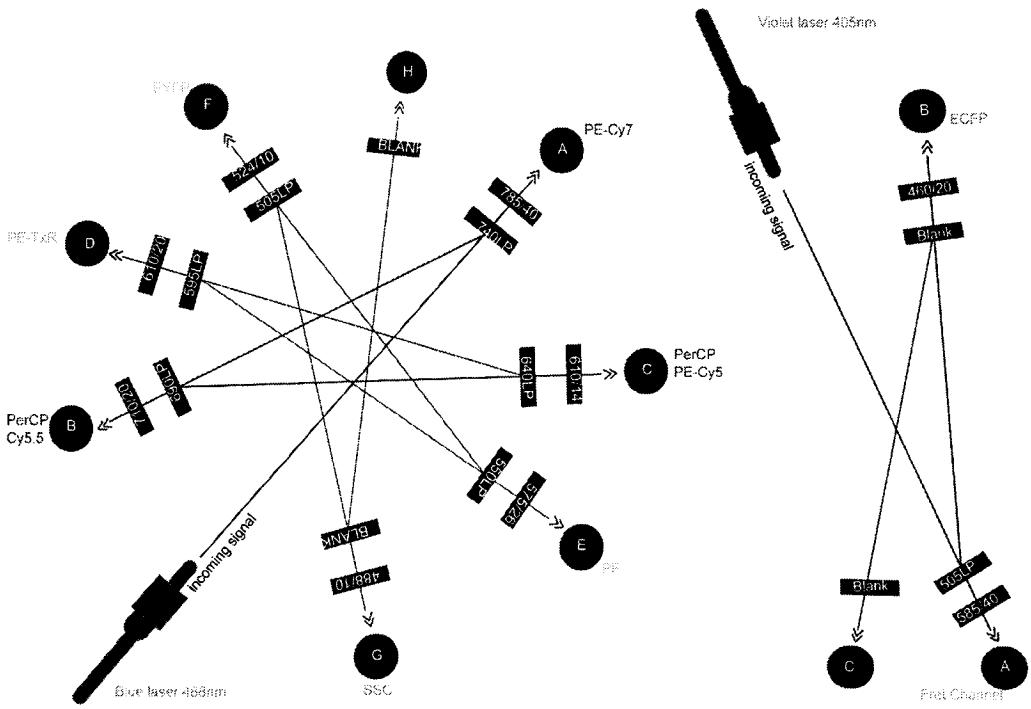


Figure 6 IgC domain Show Inhibitory Function in CD80/CD86 Chimeras

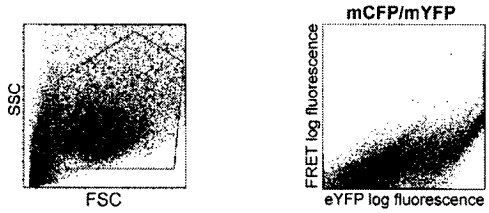


Supplementary Figure 1 **LSR II Configuration for FCET**

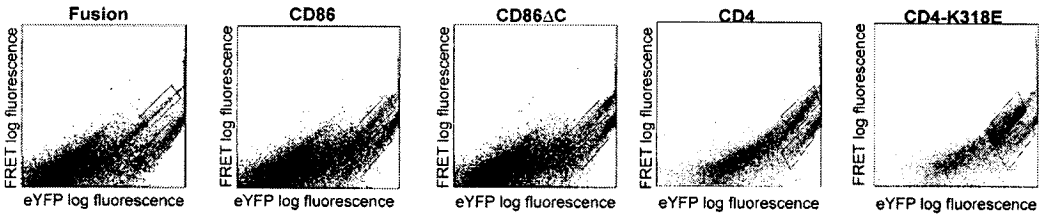


Supplementary Figure 2 FCET Gating Strategy

1. P1: Live cells population

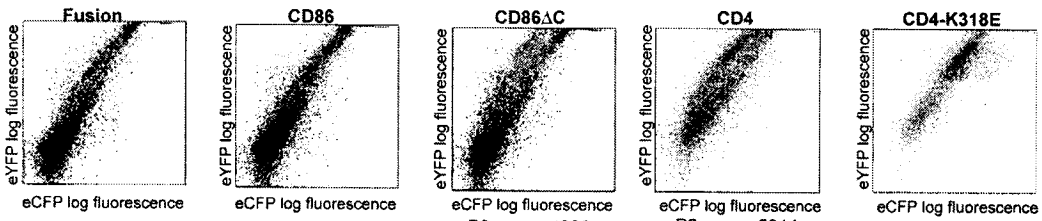


2. Selection of populations of highly similar MFI
P2: mCFP/mYFP population (bottom populations)
P3: tested condition (top populations)



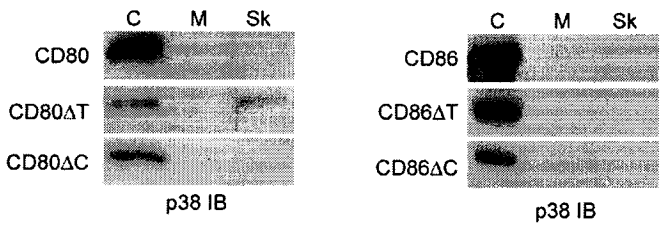
Fusion		CD86		CD86ΔC		CD4		CD4-K318E	
P2:	69312	P2:	68546	P2:	67220	P2:	90651	P2:	83562
P3:	69131	P3:	68198	P3:	67025	P3:	90329	P3:	83234

3. Comparison of CFP Mean Fluorescence of Both Populations



Fusion		CD86		CD86ΔC		CD4		CD4-K318E	
P2:	5253	P2:	5013	P2:	4800	P2:	5314	P2:	4940
P3:	4507	P3:	5356	P3:	2092	P3:	3399	P3:	5291

Data Not Shown (1) Validation of the Fractionation Technque by p38 Probing



Chapter 3

CD86 Cytoskeletal Association is Necessary for Immunological Synapse Localization and Effective Co-Stimulation

Work presented in chapter 3 investigates the role of CD86 intracellular domain in the cosignaling function of this molecule. The experimental questions are answered through the use of wild type, deleted or mutated CD86 constructs. Methods used to answer experimental questions are a peptide-specific cellular interaction system, conjugate formation assay by cytometry, soluble receptor assays, biochemical fractionation and confocal microscopy imaging.

CD86 Cytoskeletal Association is Necessary for Immunological Synapse Localization and Effective Co-Stimulation

Running Title: Functional Association of CD86 to the APC Cytoskeleton

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Keywords : Dendritic Cells; Cell Surface Molecules; Costimulation; Cell Activation

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Abstract

T cell activation requires both an antigen specific and a co-stimulatory signal delivered by antigen presenting cells (APC) in the context of the immunological synapse (IS). Reorganization of the cytoskeleton is required for the formation and maintenance of the IS. Our results show that CD86 is constitutively associated to the cytoskeleton in primary human APC as well as in a murine APC model. A highly conserved sequence present in all CD86 intracellular domains of higher mammals, the K4 motif, is critical for this association and CD86 localization at the IS. Importantly, APC expressing this mutated CD86 are severely impaired in their capacity to trigger complete T cell activation upon peptide presentation probably due to their lack of physical association with the cytoskeletal ERM proteins. Altogether, our data reveals the critical importance of cytoskeleton-dependent CD86 polarization for effective co-stimulation.

Introduction

Interactions between naïve T cells and APC in the context of the immunological synapse (IS) provide the two key signals for T cell activation. The first signal requires TCR engagement by a foreign antigen presented by MHC molecules while the second signal involves the engagement of CD28 by CD86. Co-stimulation through B7 proteins such as CD86 (B7-2) and CD80 (B7-1) is essential for IS formation (153). CD80 is believed to be functionally linked to the cytoskeleton since removal of its intracellular domain impacts CD80 membrane redistribution following T cell interaction and inhibits full T cell activation (156, 225, 226). We identified a conserved region within human CD86 cytoplasmic domain at residues $Lys^{265-268}$. This motif, defined herein as the K4 motif, is conserved in all higher mammalian CD86 sequences examined. This region is reminiscent of CD80 RRNE region, previously shown to be important for CD80 mediated costimulation, leading to our hypothesis that CD86 is associated to the APC cytoskeleton and that this association is of functional relevance for its co-stimulatory function. We show here that CD86 is indeed associated to the APC cytoskeleton through its K4 motif and that CD86 physically interacts with ERM proteins. Importantly, CD86 cytoskeletal association is critical for T cell activation.

Material and Methods

Recombinant DNA Constructs

Cloning of CD86wt and CD86ΔT in the eukaryotic expression vector Srα neo was previously described (254). CD86-K4 was generated by overlap PCR using the following primers: K4fwd GAAATGGGCGGCGGCGGCGCGGCCTCGCAACTCTTATAAATG, K4rev CATTTATAAGAGTTGCGAGGCCGCGCCGCCGCCGCCCATTTCC, CD86fwd GGCTGACCCGGGTCTGAGCCACCATGGGACTGAGTAACATTCTC and CD86rev GTATCTTATCATGTCTGGATCC. CD86-K4 was cloned in the Srα neo vector as a BamHI and SmaI fragment.

Antibodies and Reagents

CD86 antibodies used in this study included clone IT2.2 (BD Pharmingen) for cytometry analysis and clone BU63 (ID labs, Canada) for immunoblotting. ERM and NF-κB antibodies were from Cell Signaling Technologies. Secondary antibodies and Prolong Antifade mounting media were from Molecular Probes. HRP-coupled cholera toxin was from Sigma-Aldrich. Antibodies against human MHC class I, CD83, TCR, CD19 and CD14 were from BD Pharmingen. Influenza hemagglutinin peptide ($HA_{306-318}$; PKYVKQNTLKLAT) was synthesized with >95% purity at the Sheldon Biotechnology Center (Montreal, Canada).

Cell Lines and Transfections

Murine B2D cells (287) were transfected with 30 μg DNA by electroporation at 260V and 950μF and selected with G418 (0.8μg/ml). Aseptic cell sorting (MoFlo, Cytomation) was used to generate cellular clones of the transfectants. CD28neg and CD28pos Jurkat T cells express a Vα1.2/Vβ3.1 TCR specific for $HA_{306-318}$ peptide restricted to HLA-DR0101 (195). The Daudi B cell line was obtained from ATCC.

Dendritic Cells Generation and Culture

Human PBMCs obtained from healthy donors were depleted of $CD3^+$ T cells by rosetteSep kit (StemCell) and $CD14^+$ cells were isolated by autoMACS. Monocytes were plated at a density of $1x10^6$ million cells per ml in RPMI1640 medium supplemented with 1% human serum (GemCell), 100U/ml penicillin, 100ug/ml streptomycin, 10mM HEPES, 2mM L-glutamine, 1% non-essential amino acids (GIBCO), 200U/ml IL-4 (Sigma) and 1000U/ml GM-CSF (Cangene). Cells were supplied with 1.5ml of fresh medium containing 200U/ml IL-4 (Sigma) and 1000U/ml GM-CSF (Cangene) on day 2, 4, and 6. mDCs were obtained by harvesting non-adherent cells on day 7 and stimulating them with TNF-α, 1 ηg/ml (Biosource), IL-1β 10ng/ml (Biosource), PGE2 1μg/ml (Sigma), and IL-6 1000U/ml (Biosource) for 48h. DC purity assessed by flow cytometry and was always higher than 95%.

Biochemical Subcellular Fractionation

The protocol is described elsewhere (289). Briefly, $10x10^6$ dendritic cells or $30x10^6$ B2D cell lines were resuspended in hypotonic solution (10 mM HEPES pH 6.9, 10 mM KCl, protease inhibitors) and incubated on ice for 20 minutes. Cells were disrupted by pipetting up and down 20 times. Nuclei were pelleted at 3200 rpm for 10 minutes at 4°C. Supernatant from pelleted nuclei was centrifuged further at 35000 rpm for 30 minutes at 4°C. The supernatant (cytosolic fraction) was separated and the pellet (cytoskeletal plus membrane fractions) was resuspended in NTENT buffer (500 mM NaCl, 10 mM Tris–HCl pH 7.2, 1 mM EDTA, protease inhibitors and 1% Triton X-100). This fraction was centrifuged at 14000 rpm for 30 minutes at 4°C. The resulting pellet, resuspended again in NTENT buffer, comprised the cytoskeletal fraction, while the supernatant comprised the membrane fraction.

Raft Isolation

Lipid rafts isolation was performed using sucrose gradient ultracentrifugation of cell lysates as described elsewhere (295). Brifely, 10^8 cells were washed in ice-cold PBS and lysed in 0.5 ml of cold buffer (1% Triton X-100, 20 mM of MES, and 150 mM of NaCl, pH 6.5, containing protease inhibitors (Roche). The lysates were then subjected to sucrose gradient fractionation using ultracentrifugation (100 000 x *g*, 4°C, 17 h). Eleven to 12 fractions of 1 ml were collected. A total of 10 µl of each fraction was subjected to dot-blot analysis using HRP-conjugated cholera toxin (Sigma-Aldrich) to detect GM1, a positive marker of rafts.

Immunoblotting

All electrophoreses were performed on 10% SDS-PAGE gels, transferred to PVDF membranes and blocked for 1h. Primary antibodies were incubated overnight. After washes, secondary antibodies coupled to HRP were incubated for 45 minutes. Blots were revealed using ECL (Amersham).

Co-immunoprecipitations

5 millions Daudi B cells were lyzed in a NP40 1% buffer containing proteases inhibitors, 10% glycerol and 50mM NaCl for 30 minutes on ice. The lysates were cleared by centrifugation and pre-cleared with protein-G sepharose for 1h (Amersham). CD86 specific or isotype-matched antibody was then added to the lysate and incubated overnight in a rotator at 4°C. Protein G beads were then added for 1h. Beads were washed four times in cold lysis buffer and proteins were detached by boiling in Laemli buffer for 5 minutes.

IL-2 ELISA

$1x10^5$ T cells and $1x10^5$ B2D cell lines were co-cultured overnight at 37°C in round-bottom 96-well plates with HA peptide. Supernatants were assayed for human IL-2 according to manufacturer's instructions (BD Pharmingen).

Conjugate Formation

APC were pre-pulsed with 10μg/ml of $HA_{306-318}$ peptide for 2 hours at 37°C. $1x10^6$ T cells and $1x10^6$ HA-loaded B2D cell lines were co-cultured in a 37°C water bath and then fixed in 2% paraformaldehyde for 20 minutes. Cells were stained with anti-human MHC class I and anti-human CD86 for 1h at 4°C. Cells were analyzed using a BD FACS Scan flow cytometer (Becton Dickinson).

Immunological Synapse Imaging

Images of conjugates were acquired on a Leica Confocal microscope, using a 63X oil-immersion objective. An average of 10 images per condition were taken for each experiment. Image analysis was performed with the Northern Eclipse software. A fluorescence ratio was obtained by dividing the Mean Fluorescence Intensity (MFI) at the interaction interface with the total cell MFI. A ratio of 1 indicates that the protein is homogenously distributed at the cell surface while a ratio greater than 1 indicates a specific accumulation of the protein of interest (296).

Results and Discussion

CD86 is Associated to the Cytoskeleton

The cytoplasmic tail of CD86 encompasses a highly conserved K4 motif reminiscent of the RRNE sequence of CD80 previously shown to be important for CD80 co-signaling function (226). We verified CD80 subcellular localization by biochemical fractionation using the Daudi B cell line. This fractionation technique allows the separation of cytoplasmic (C), membrane (M) and cytoskeletal (Sk) subcellular fractions (289). CD80 immunoblotting of the obtained fractions revealed that CD80 could be detected in both the membrane and the cytoskeletal fraction, confirming biochemically that CD80 is associated to the APC cytoskeleton (data not shown). The same procedure was performed to assess CD86 subcellular localization in primary human dendritic cells (DCs) (n=2). CD86 was detected in both the membrane and cytoskeletal fractions of both immature and mature human DCs (figure 1, panel B) clearly showing that CD86 is associated to the cytoskeleton of primary professional APC.

Constructs encoding either human CD86 wild type (CD86wt), human CD86 truncated of its intracellular domain (CD86ΔT) or human CD86 in which the four lysine residues of the K4 motif are mutated to alanines (CD86-K4) (figure 1, panel A) were used to generate stable cell lines in the murine mastocytoma B2D cell line (287). Following fractionation of these cell lines, CD86 immunoblotting showed that the CD86 molecule was detected in the cytoskeletal fraction of CD86wt cells but not in CD86-K4 cells (figure 1, panel B). As expected, CD86 from CD86ΔT cells was only detected in the membrane fraction. As a control, all fractions were subjected to NF-κB probing and this molecule was greatly enriched in cytoplasmic fractions thereby confirming the specificity of our fractionation approach (data not shown). These results clearly indicate that CD86 is associated to the cytoskeleton and that this association requires CD86 intracellular domain and most specifically the K4 motif.

Lipid rafts are membrane microdomains that are not readily solubilized in non-ionic detergents (297). Because the non-ionic detergent Triton X-100 was used in the subcellular fractionation protocol, raft isolation was undertaken to confirm that it was indeed cytoskeletal association rather than raft residence that conferred detergent resistance to CD86 molecules. Cholera toxin probing of dot blots showed that rafts were enriched in fractions 4 to 6 by (figure 1, panel C). CD86wt as well as its deleted and mutated forms were also found in comparable amounts in raft fractions obtained from the B2D cell lines (n=3) (figure 1, panel C). Therefore, the absence of the mutated (CD86-K4) and the truncated (CD86ΔT) forms of CD86 in the cytoskeletal fractions can only be explained by their lack of cytoskeletal association rather than lack of raft residence.

CD86 Cytoskeleton Association is Important for IS Localization but Does not Affect Conjugate Formation

Cytoskeletal association of cell surface proteins, such as CD80, has been shown to be crucial for their function in the IS (96, 156). We investigated the functional importance of CD86 cytoskeletal association on an early event of T cell activation, namely cell conjugation (n=3). CD28 positive or negative (CD28pos or CD28neg) Jurkat T cell lines expressing a HA specific TCR were co-cultured for 0 to 45 minutes with B2D APC presenting this peptide. Cells were then fixed and stained with PE-Cy5-conjugated anti-human MHC class I (T cell specific) and anti-human PE-conjugated CD86 (B2D specific). T cell-APC conjugates were assessed by measuring the number of PE/PE-Cy5 double positive events by flow cytometry (figure 2 and data not shown). A background level of conjugation, consistently below 3%, was observed when CD28neg Jurkat T cells were used. For CD28pos Jurkat T cells, kinetic analyses showed that a maximum frequency of conjugates (26.6%) was obtained at 45 minutes, representing a 12.5-fold difference as compared to CD28neg Jurkat T cells. No difference in the efficiency of conjugate formation was observed between CD86wt and CD86-K4 cells. These results confirm the initial adhesion role mediated by CD86/CD28 interactions in

the earliest events of T cell-APC contacts (298). However, CD86 association to the cytoskeleton is not required at this early stage.

To demonstrate the localization of CD86 at the IS, HA-pulsed primary mature DCs (figure 3, panel A) derived from a HLA-DR0101^{+} donor were co-cultured with CD28pos Jurkat T cells. The conjugates were then analyzed by confocal microscopy. An average of ten images per tested condition were taken in any given experiment. In mDCs, CD86 was reoriented towards the interaction interface with a fluorescence ratio of 1.85±0.33 (n=2) (figure 3, panel A and C) clearly demonstrating that CD86 is localized at the IS in primary professional APC. In B2D cell lines, CD86 was also strongly reoriented at the IS after a 30 minute interaction between CD86wt and CD28pos Jurkat T cells with a fluorescence ratio of 2.58±0.38 (n=3) (figure 2, panel B and C). Reorientation in CD86-K4 cells was much less significant ($p < 0.0001$) with a fluorescence ratio of 1.29±0.21 (n=3). These results clearly indicate that CD86 polarization to the IS is dependent on its interaction with the cytoskeleton through the K4 motif. Our results indicate that CD86 is actively recruited and/or retained at the IS in a cytoskeleton-dependent fashion. Mutation in the K4 motif prevents CD86 interaction with the cytoskeleton (figure 1, panel A) and its accumulation at the IS following T cell interaction. Our findings are in agreement with previous studies showing the importance of the APC cytoskeleton in the formation of peptide-dependent IS (34, 35, 299).

CD86 Cytoskeletal Association is Critical for T cell Costimulation and IL-2 Production

To assess the physiological relevance of CD86 association to the cytoskeleton in late events of T cell activation, IL-2 secretion of CD28pos Jurkat T cells in response to antigen specific TCR triggering was measured by ELISA following an overnight co-culture with APC expressing CD86wt or CD86-K4 (n=5). The system used herein is highly dependent on costimulation since absence of CD86 expression resulted in the lack of IL-2 secretion even in the presence of optimal

concentrations of HA peptide (figure 4). Two independently derived CD86-K4 clones expressing similar levels of CD86 when compared to CD86wt cells (data not shown) induced drastically lower IL-2 levels (3.6 to 13.1 fold lower) at all tested peptide concentrations. CD86 association to the cytoskeleton is thus of functional relevance for costimulation since IL-2 production by T cells is almost completely abrogated following stimulation by APC expressing CD86-K4. Impairment of T cell activation in this system could only result from the lack of cytoskeletal association of CD86 rather than inefficient ligand binding, since all B2D cell lines bound soluble CD28 with comparable affinity (data not shown). Further evidence for the importance of CD86 cytoskeletal association is provided through the use of Latrunculin B, a cytoskeleton inhibitor. Latrunculin B treated CD86wt cells showed a completely impaired co-stimulatory activity to the same extent of T cell treatment with the same inhibitor as measured by IL-2 secretion (data not shown).

CD28 has been proposed to be initially recruited to the synapse by TCR induced cytoskeletal remodeling, independently of CD86 binding; it is then stabilized through its interaction with CD86 on APC (155). CD86, located at the IS, is thus able to provide the necessary signal for proper co-stimulation of T cell activity. Our results and those of others (156, 225, 226) clearly indicate that the interaction of CD80 and CD86 with the cytoskeleton constitutes a general mechanism for the costimulatory function of these molecules by regulating their subcellular localization.

CD86 Physically Associates with ERM Proteins

CD86 K4 motif and CD80 RRNE motif are both positively charged amino acids clusters. These types of sequences are often present at the juxtamembrane region of integral proteins. However, some cell surface molecules such as CD43, CD44, L-selectin, ICAM-2 and ICAM-3 are linked to the cytoskeleton through these aforementioned sequences by interacting with a family of membrane-cytoskeleton linkers, the ezrin/moesin/radixin (ERM) proteins (127). ERM proteins link

transmembrane receptors to the cortical actin cytoskeleton. We thus hypothesized that the K4 region of CD86 was an ERM binding region. In preliminary experiments, we attempted to co-immunoprecipitate CD86 and ERM proteins in the B2D cell lines. The results were unconvincing most probably due to low levels of CD86 and/or ERM proteins in these cell lines. We therefore chose to use the Daudi B cell line that expresses high levels of CD86 and more than twice the levels of constitutive ERM proteins (data not shown) when compared to B2D cells. CD86 immunoprecipitation in the Daudi B cell line allowed us to detect the physical interaction between CD86 and ERM proteins as shown in figure 5 (n=3). To our knowledge, this is the first description of a binding partner for CD86.

The Two Signal Model in APC

A question remaining from both earlier studies and the current study is how are B7 proteins redistributed at the cell surface. We suggest a reciprocal two-signal model in APC that could involve a first signal triggered either through CD86 or MHC molecules (53, 300) inducing inactive cytoplasmic ERM proteins to adopt 'open' active conformations. Signaling within APC would require initial conjugate formation through cytoskeletal relaxation (121) and CD28-CD86 dependent adhesion. The second signal would involve recruitment or retention of CD86 to the IS by phosphorylated ERM proteins, allowing sustained T cell co-stimulation. It is only in the context of a mature IS that CD86 could act as a potent co-stimulator and induce full T cell activation (294). This reciprocal two-signal model highlights the bi-directional nature of signaling in the IS (230) that leads to cytoskeletal rearrangements thereby creating an optimal environment for CD28-B7 interaction and function. Altogether we provide evidence for the cytoskeletal regulation of CD86 cosignaling molecule function in APC.

Acknowledgements

We thank IG. Rodrigue-Gervais, A. Dumont, J. Thibodeau, W. Mourad and E. Haddad for helpful discussions and careful reviews of the manuscript. The authors have no conflicting financial interests.

Figure Legends

Figure 1 CD86 Sub-cellular Distribution in Antigen Presenting Cells

CD86 sub-cellular localization was assessed by fractionation in immature and mature primary dendritic cells (n=2) and in the B2D cell lines (n=3). A) Intracellular sequence of CD86 constructs used in this study B) Cytosolic (C), membrane (M) and cytoskeletal (Sk) fractions were isolated. CD86 immunoblotting reveals that CD86 is associated to the cytoskeleton in immature and mature dendritic cells and in the CD86wt cell line. CD86 is not associated to the cytoskeleton in the CD86ΔT and CD86-K4 cell lines. C) CD86 from all B2D cell lines was present in rafts enriched in fractions 4 to 6 as shown by cholera toxin and CD86 dot blot analyses.

Figure 2 CD86 Association to the Cytoskeleton Does not Affect Conjugate Formation

Average results of 3 independent conjugate formation experiments. HA peptide pre-pulsed B2D cell lines were co-cultured with CD28pos and CD28negJurkat T cells for 0 to 45 minutes and then stained with PE-conjugated anti-human CD86 and PE-Cy5-conjugated anti-human MHC Class I. Double positive (PE^+/PE-$Cy5^+$) events represent conjugates and is shown here as % conjugates.

Figure 3 CD86 Localization to the IS Requires the K4 Motif

Representative images of two (A) and three (B) independent experiments. An average of 10 images were taken per condition in each experiment. A) Human mature dendritic cells derived from a HLA-$DR0101^+$ donor were pre-pulsed with HA peptide and co-cultured with CD28pos Jurkat T cells for 15 minutes at 37°C. Cells were then spun onto a slide, fixed and stained for CD86 (shown in green). All images were acquired on a Leica Confocal microscope using a 63X oil-immersion objective. Confocal microscopy analysis reveals that CD86 relocates to the T cell:mDC interface after 15 minutes. B) CD86wt cells also present relocalisation of CD86 at the interface after 30 minutes of interaction with CD28pos Jurkat T cells whereas CD86-K4 cells do not show such relocalisation.

C) The calculated fluorescence ratio was obtained by dividing the MFI at the interaction interface with the MFI of the entire cell.

Figure 4 CD86 Co-stimulatory Activity is Dependent on its K4 Motif

CD86wt and two different CD86-K4 cellular clones expressing comparable levels of CD86 were co-cultured overnight with CD28pos Jurkat T cells at a 1:1 ratio with various HA peptide concentrations. IL-2 production was measured by ELISA in five independent experiments.

Figure 5 CD86 and ERM Proteins Physically Interact

CD86 was immunoprecipitated from Daudi B cell lysates (n=3). CD86 co-immunoprecipitated a doublet representative of the 78Kda forms of radixin and moesin and the 80Kda ezrin protein. Ig = Isotype control, IP = immunoprecipitation.

Figure 1 CD86 Sub-Cellular Distribution in APC

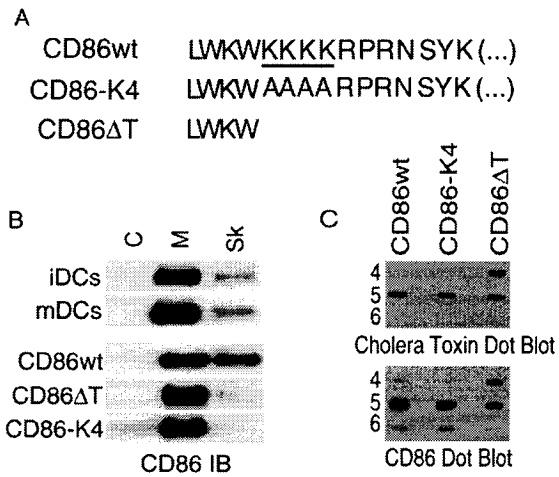


Figure 2 CD86 Association to the Cytoskeleton Does Not Affect Conjugate Formation

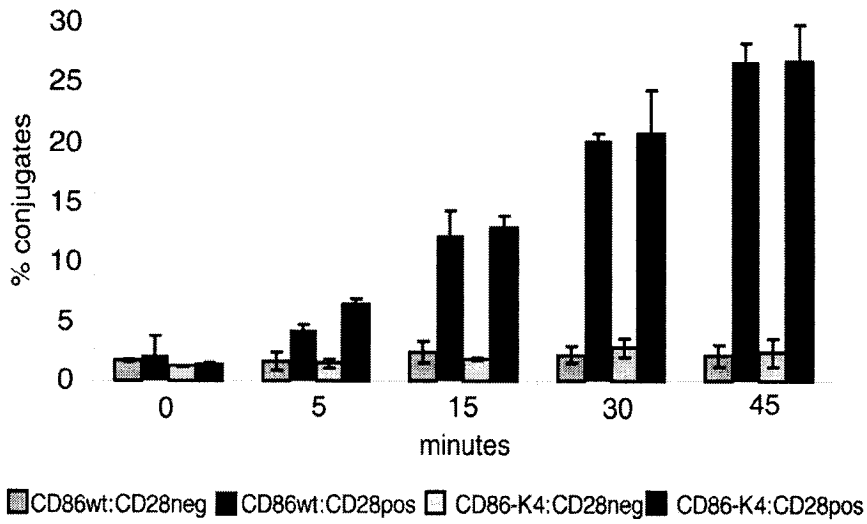
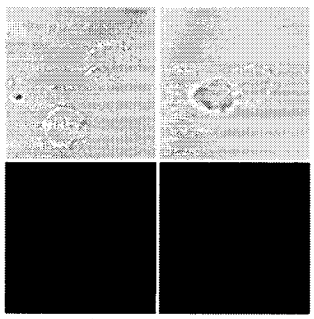


Figure 3 CD86 Localization to the IS requires the K4 Motif

A



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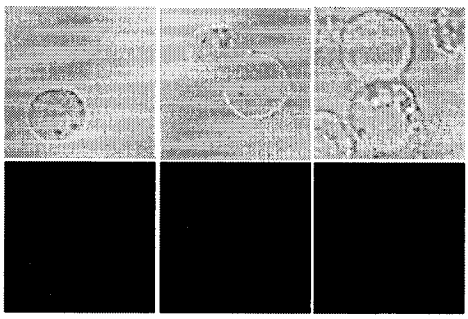
B



t=0' CD86wt t=30' CD86-K4 t=30'

C

	Fluorescence Ratio	SD
t=0'	1.02	0.04
DC t=15'	1.85	0.33
CD86wt t=30'	2.58	0.38
CD86-K4 t=30'	1.29	0.21

Figure 4 CD86 Co-stimulatory Activity is Dependent on its K4 Motif

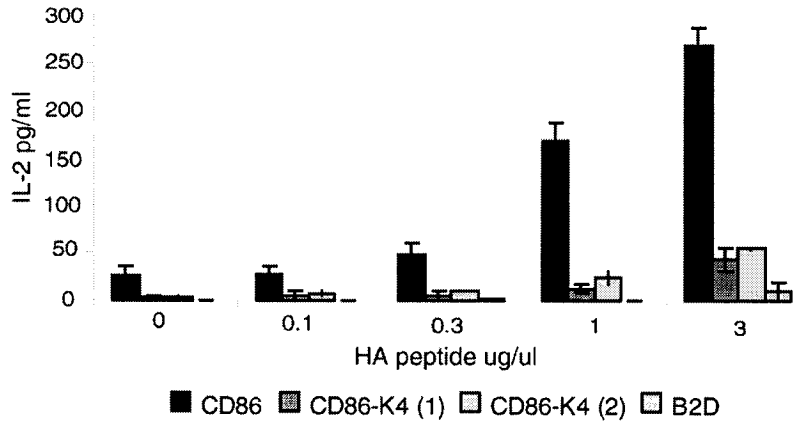
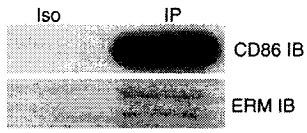


Figure 5 CD86 and ERM Proteins Physically Associate



Legends for Data Not Shown

Figure 6 CD80 Sub-Cellular Distribution in Daudi B Cells

CD80 sub-cellular localization was assessed by sub-cellular fractionation in the Daudi B cell line. Cytosolic (C), membrane (M) and cytoskeletal (Sk) fractions were isolated. CD80 immunoblotting reveals that CD80 is associated to the cytoskeleton of these cells.

Figure 7 NF-kB is Greatly Enriched in the Cytoplasmic Fractions

NF-kB immunoblots on subcellular fractions obtained from DCs or the B2D cell lines. As expected, NF-kB is either only present or greatly enriched in the cytoplasmic fractions.

Figure 8 CD86wt and CD86-K4 Clones Express Comparable CD86 Protein Levels

Flow cytometer histograms showing CD86 staining of CD86wt cells and two independently derived CD86-K4 clones. MFI values of these different cellular clones show comparable CD86 expression. These cells were used in the co-culture IL-2 production experiments.

Figure 9 Conjugate Formation Density Plots

HA peptide pre-pulsed B2D cell lines were co-cultured with CD28pos and CD28negJurkat T cells for 0 to 45 minutes and then stained with PE-conjugated anti-human CD86 and PE-Cy5-conjugated anti-human MHC Class I. Double positive (PE^+/$PE\text{-}Cy5^+$) events represent conjugates.

Figure 10 B2D APC Cell Lines Bind CD28 in a Similar Fashion

B2D cell lines were incubated with soluble CD28 at the various concentrations indicated. After washing, cells were incubated with a secondary antibody coupled to Alexa 488. After washing, cells were analyzed on BD LSR II flow cytometer. Results indicate that all cell lines bind CD28 with a comparable avidity (n=3).

Figure 11 LatB Treatment Negatively Impacts CD86 Costimulation

CD86wt or CD28pos Jurkat T cells were pre-treated with Latrunculin B for 30 minutes at 37°C. Concentrations of Latrunculin B used are shown in parentheses following the treated cell type. After extensive washing, cells were co-cultured overnight with untreated APC or T cells and various concentrations of HA peptide as indicated. Following the overnight co-culture, viability of cells was monitored by flow cytometry using FSC/SSC parameters and IL-2 production was measured by ELISA. One of three representative experiments is shown.

Figure 12 Surface Expression of CD86 is Not Altered by Latrunculin B Treatment

CD86wt cells were treated with Latrunculin B for 30 minutes and then washed and put in culture for 16 hours. CD86 expression was monitored by flow cytometry before treatment (red histogram) and after the overnight culture (blue histogram). CD86 surface expression albeit lower following treatment (MFI X versus Y) was not considerably altered following latrunculin B treatment.

Figure 13 ERM Proteins Expression Levels of Daudi B Cell and B2D Cell Line

Total ERM protein levels were measured by intracellular staining in B2D and Daudi B cells.

Data Not Shown

Figure 6 CD80 Sub-cellular Localisation in the Daudi B cell line

Figure 7 NF-kB is Greatly Enriched in the Cytoplasmic Fractions

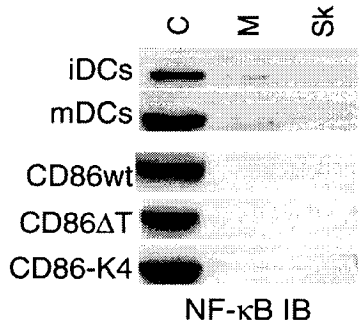


Figure 8 CD86 Expression Levels on CD86-K4 Cells Versus CD86wt Cells

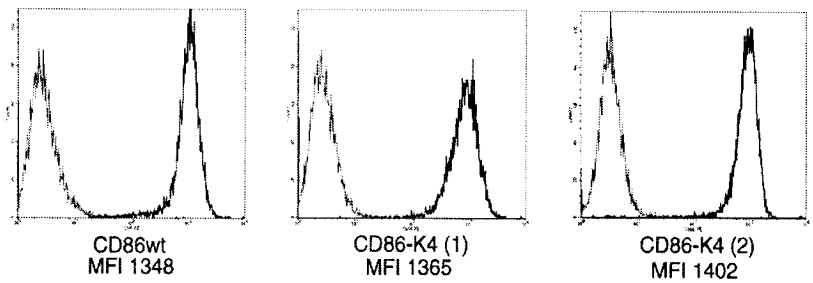


Figure 9 Conjugate Formation Density Plots

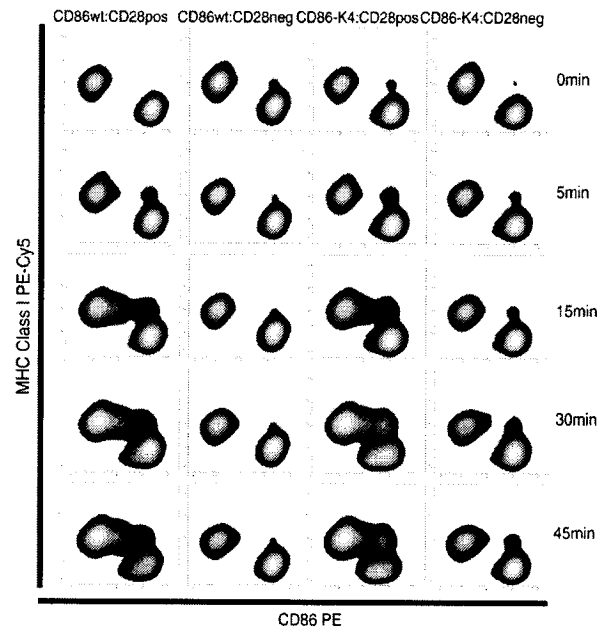


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Figure 10 B2D Cell Lines Bind CD28

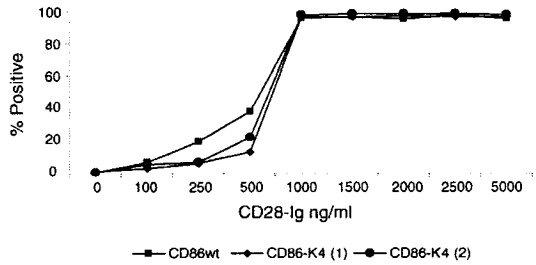


Figure 11 LatB Treatment Negatively Impacts CD86 Costimulation

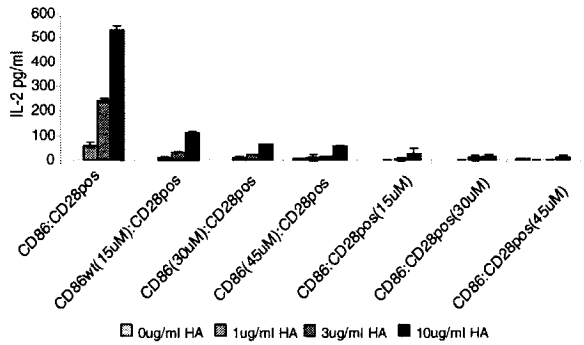


Figure 12 CD86 Surface Expression is Not Altered by Latrunculin B Treatment

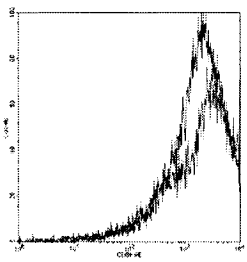
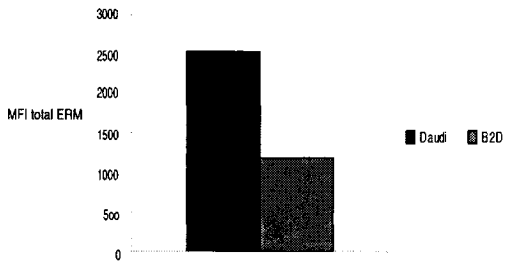


Figure 13 Comparison of Total ERM Proteins Between the Daudi B Cell and B2D Cell Lines



Chapter 4

General Discussion

4.1 Molecular structure of CD80 and CD86

4.1.1. Only wild type CD80 can form homodimers

The crystal structure of CD80 and CD86 complexed with CTLA-4 were both reported in 2001 (185, 186). The crystals highlighted the crucial importance of the IgV domain of CD80 and CD86 in the interaction with CTLA-4. However, earlier evidence from mutagenesis and biophysical studies suggested that both IgV and IgC domains were implicated in CD80 and CD86 receptor binding. When complexed with CTLA-4, both CD80 and CD86 crystallized as homodimers. In contrast to the CD80 dimer, the CD86 dimers deviate from 2-fold symmetry and presents a hydrophilic rather than hydrophobic dimer interface. These observations, in addition to previous studies that showed CD86 to be monomeric, lead to believe that the reported CD86 dimers resulted from crystal packing. Therefore, the exact molecular structure of CD80 and CD86 remained to be confirmed. Moreover, the role of CD80 and CD86 functional domains in molecular arrangement needed to be established.

In 2005, *Bhatia et al.* showed that CD80 existed as a mixed monomeric-dimeric population and CD86 as a monomer at the surface of fixed cells using microscopy-based FRET (257). The results presented in chapter 2 confirm and extend these findings in live cells. Cytometry-based FRET experiments show that CD80 is detected as a homodimer and CD86 as a monomer at the surface of live cells (figure 2, chapter 2). Interestingly, upon deletion of CD86 IgC domain, CD86 is detected as homodimers in live cells. Non-denaturing polyacrylamide gel electrophoresis (PAGE) experiments (chapter 2, figure 3) corroborate these findings and also reveals that CD80 IgC domain deletion leads to higher orders of CD80. Clearly, the IgC domain affects the multimeric structure of both CD80 and CD86. Additional FCET experiments confirmed the inhibitory effect of the IgC domain as shown by the loss of CD80 homodimer formation when the IgC domain of CD86 replaced the IgC domain of CD80 while keeping CD80 IgV domain (figure 6, chapter 2). A summary of the obtained results regarding the molecular structure of CD80 and CD86 is shown in figure 1.

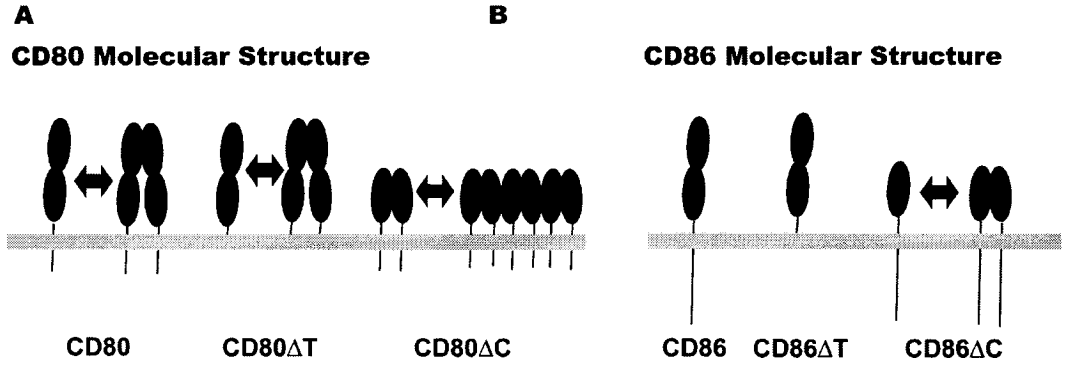


Figure 1 Molecular structure of CD80 and CD86
The molecular structure of CD80 (panel A) and CD86 (panel B) as observed from FCET and biochemical experiments using wild type and deletion forms of CD80 and CD86. CD80 wild type molecules exist as a mixed monomeric and dimeric population. Upon removal of CD80 IgC domain, higher orders of multimers are observed. CD86 exists as a monomer. Upon deletion of CD86 IgC domain, some homodimers are detected. Removal of intracellular domains in both CD80 and CD86 does not greatly impact their molecular structure.

4.1.2 The inhibitory function of CD80 and CD86 IgC domain

CD86 presents two insertions of four amino acids within its IgC domain when compared to CD80. This divergence might explain the difference between CD80 and CD86 molecular structure. Clearly, CD86 IgC domain differs from that of CD80 since V80C86T86 molecules did not homodimerize even if CD80's dimer interface is favourable for such formation. Conversely, CD80 IgC domain coupled to CD86 IgV domain in the V86C80T80 molecule did not allow homodimers formation. This clearly shows the dual requirement for a dimer interface with appropriate properties and the IgC domain presentation of the dimer interface for multimer formation. In the crystals, numerous atomic contacts between the IgV and IgC domains of CD80 were uncovered; these interactions might stabilize the conformation of the IgV domain (185, 227). The IgC domain could support the IgV mediated CTLA-4/CD28 binding by, for example, shielding the hydrophobic receptor binding domain. Glycosylation of the IgC domain might have a profound effect on the stabilization of CD80 dimers perhaps through the interaction of the sugar moieties with the membrane (185).

4.1.3 CD80 and CD86 molecular structure impacts receptor binding and cosignaling function

Collins et al. (184) published pioneer data in 2002 that showed that CD86 is the preferential ligand of CD28 and CD80 the preferential ligand of CTLA-4. That same study showed that CTLA-4 is a bivalent homodimer while CD28 is a monovalent homodimer. This valency difference between CD28 and CTLA-4 was finally explained with the recent crystallization of CD28 in complex with an antibody Fab fragment (167). Although both CD28 monomers are available for binding, simultaneous docking of two B7 monomers is prevented by the physical clash of their IgC domains. Interestingly, *Dennehy et al.* showed that CD28 monovalency is essential for the cosignaling phenomenon since ligation of an engineered bivalent CD28 induced responses in the absence of TCR engagement (246).

Results presented in figure 4 of chapter 2 show that a change in the molecular structure of CD80 and CD86 directly impacts their receptor binding properties. Deletion of the IgC domains leads to higher order of CD80 and CD86 multimerization, greatly affecting binding to monovalent CD28 as seen with the increased EC50 values. Binding defects were also seen when using soluble CTLA-4. Although CTLA-4 is bivalent and can form higher order arrays with CD80, multimeric CD80ΔC was not a better binder to CTLA-4. Assumingly, multimeric CD80 could not allow appropriate CTLA-4 binding perhaps through structural and physical constraints. Moreover, although present at the surface in a mixed monomeric-dimeric population, CD86ΔC binding to CTLA-4 was not favored. This could be explained by the fact that as observed in the crystal lattice, CD86 does not form symmetrical dimers leading to defective CTLA-4 binding. Altogether, these results clearly highlight the critical importance of molecular structure in the interactions of the B7/CD28 system.

In this thesis, CD80 and CD86 cosignaling function was studied in a peptide-specific cellular interaction model. As an APC, the murine B2D cell line that stably expresses HLA-DR0101 was transfected with the CD80 or CD86 construct of interest. The responding T cell line was either a CD28 negative or CD28 positive T cell expressing a Vα1.2/Vβ3.1 TCR restricted to HLA-DR0101 and specific for the $HA_{306-318}$ peptide. Upon HA peptide presentation, responding T cells secreted IL-2 in a dose-dependent and cosignaling-dependent manner. The impact of the altered CD28 binding properties of CD80 and CD86 IgC deleted molecules was corroborated using this cellular interaction system. IgC deletion in both CD80 and CD86 lead to reduced IL-2 secretion as expected from their CD28-binding defect (figure 5, chapter 2). These results are in accord with results from *Agadjanyan et al.* (254) and *Vasu et al.* (255) who showed in 2003 that the chimeric molecule expressing the IgV domain of CD80 and the remaining portion of CD86 (V80C86T86) enhanced T cell activation *in vivo* and *in vitro*. In view of our results, this observation can be explained by CD80 IgV being mostly present as a monomer at the cell surface and therefore being more suited for CD28 binding and cosignaling. Altogether, our findings and those of others show that both the IgV and IgC domains are crucial for CD80 and CD86 function. A summary of the interaction properties and ensuing responses is presented in figure 2.

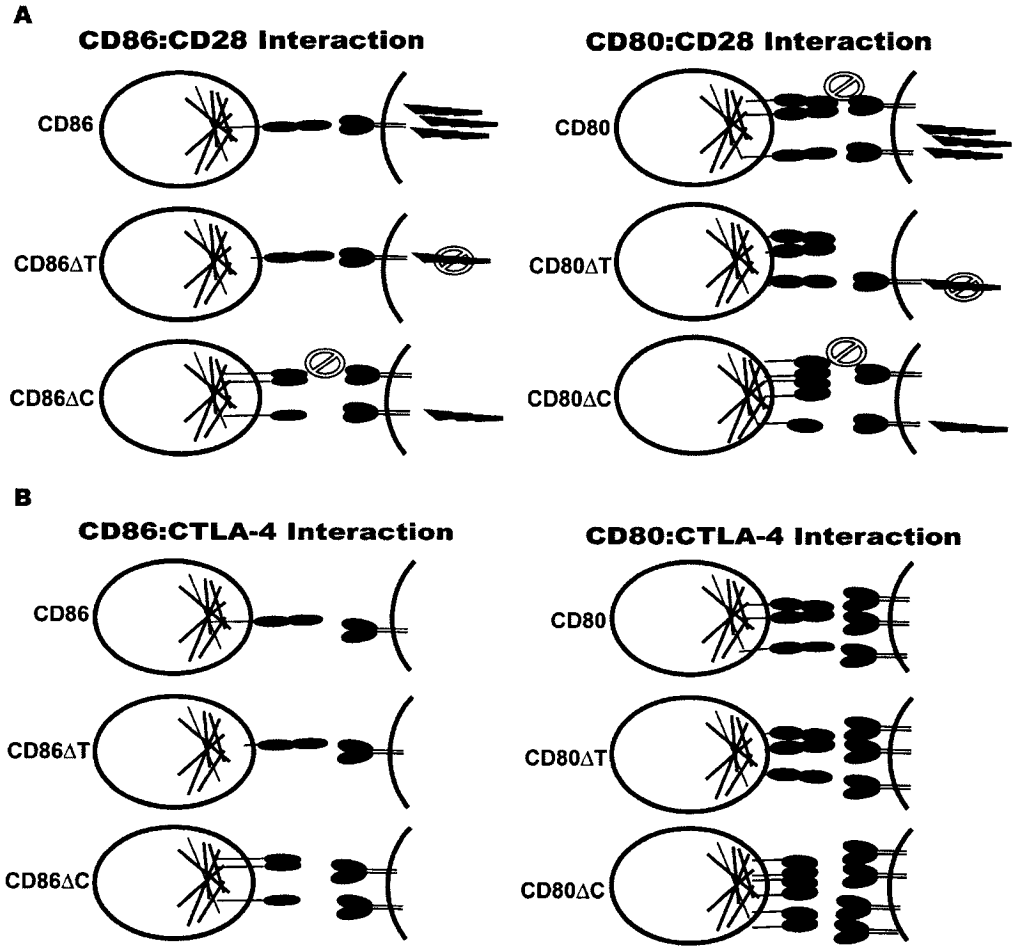


Figure 2 Molecular interactions of wild type and deleted forms of CD80/CD86
A) Physiological Impact of CD28 Interactions. For discussion purposes, both CD80 and CD86 are presented but CD28:CD86 interactions are more likely than CD28:CD80 in a physiological setting. CD28 favors binding to monomeric counter receptors such as CD86 and monomeric CD80. Intracellular domain deletion does not negatively impact CD28 binding but impacts the overall cosignaling function due to the lack of association to the cytoskeleton and IS localization of these molecules. IgC deletion leads to dimeric and multimeric CD80 and CD86 negatively regulating CD28-dependent cosignaling. B) **Interactions with CTLA-4.** For discussion purposes, both CD80 and CD86 are presented but CTLA-4:CD80 interactions are more likely than CTLA-4:CD86 in a physiological setting. CTLA-4 is a bivalent homodimer that can bind to two counter receptors. Although CD86 deleted of its IgC domain is present as a dimer, binding is negatively impacted perhaps because CD86 forms asymmetric dimers. CD80 deleted of its IgC domain forms high order multimers that are not ideally suited for CTLA-4 binding. Intracellular domain deletion in both CD80 and CD86 does not impact CTLA-4 binding. The physiological impact of such interactions remains to be elucidated.

4.2 CD80 and CD86 are functionally linked to the APC cytoskeleton

Interaction between a T cell and an APC during the initiation of adaptive immune responses leads to the formation of an immunological synapse. At the time where the work presented in this thesis was initiated, the importance of the APC and the role of APC surface molecules in the context of the IS were ill-defined. The prevailing view was that the APC cytoskeleton played a passive role in formation of such a structure and that APC membrane receptor accumulation at the IS was the result of passive diffusion or trapping of these molecules following T cell interaction (147, 301-303). The bias of these original studies perhaps stemmed from the fact that experiments made use of B cells or artificial lipid bilayers as APC and not DC, the only APC capable of activating naïve T cells. Therefore, the significance of such a conclusion remained to be established.

4.2.1 The APC cytoskeleton

In a 2001 report, *Al-Alwan et al.*(35) provided evidence for the active role played by the DC cytoskeleton in the establishment of the IS. They demonstrated that during T cell interaction, the DC actin cytoskeleton is polarized and that DC treatment with actin polymerization inhibitors leads to reduced T cell activation. They extended their findings in 2003 by showing that DC rearrange their actin cytoskeleton only when encountering CD4 T cells presenting a peptide of the appropriate specificity and showed that MHC class II signaling plays a central role in this process (34). These results highlighted a difference between the APC and the T cell cytoskeleton since T cells polarize their cytoskeletal proteins even in the absence of specific antigen recognition. The DC cytoskeleton has also been shown to be important in polarizing MHC class II endosomes towards the site of T cell contact (32). We showed that the integrity of the cytoskeleton was also crucial for effective T cell activation in our interaction model since treatment of APC and/or T cells with the cytoskeleton inhibitor latrunculin B led to an almost completely abrogated IL-2 response as shown in figure 11 of chapter 3.

4.2.2 CD80 and CD86 intracellular domains interact with the cytoskeleton

In earlier studies, *Doty and Clark* (225, 226) suggested that CD80 was associated to the cytoskeleton since a tailless mutant of CD80 failed to induce T cell activation. Two key regions in CD80 intracellular domain (RRNE 275-278 and S284) important for CD80 cosignaling function were identified. We confirmed CD80 association to the cytoskeleton in Daudi B cells using a biochemical sub-cellular fractionation technique (figure 6 of chapter 3). Furthermore, CD80 cytoskeletal association was loss upon deletion of its intracellular domain as shown in figure 1 of chapter 2. The functional relevance of such an association was also demonstrated in our peptide-specific system by showing that tailless CD80 failed to induce significant IL-2 secretion from T cells (figure 5, chapter 2). Significantly, subcellular fractionation also revealed that CD86 is associated to the cytoskeleton in human DC, B2D cells and 293T cells as shown in figure 1, chapter 2 and figure 1, chapter 3. We also identified the highly conserved K4 (275KKKK278) region in CD86 intracellular domain as being crucial for this physical linkage. Upon mutation of the four lysines residues to alanines, CD86 lost its cytoskeletal association, as did a tailless CD86 (figure 1, chapter 3).

4.2.3 CD80 and CD86 are localized at the IS

In 2001, *Bromley et al.* (294) established that CD80 is present at the center of the IS. In 2002 *Wetzel et al.* (153) demonstrated that blockade of CD80:CD28 interaction alters synapse morphology and leads to reduced T cell proliferation (153). *Pentcheva-Hoang et al.* further defined the importance of CD80 and CD86 IS localization in a 2004 report (155) by describing a differential activity of CD80 and CD86 in receptor recruitment at the T cell surface. In agreement with *Collins et al.* (184), CD86, the preferential ligand of CD28, was shown to specifically stabilize CD28 at the synapse, while CD80 preferentially recruits and stabilizes CTLA-4 at the synapse (155). Finally, in a recent report, *Tseng et al.* showed that the recruitment of CD28 and CTLA-4 by CD80 specifically required its cytoplasmic domain (156).

As shown in figure 3 of chapter 3, CD86 is present at the IS in both primary human DC and in our model APC. These findings were further refined by showing that CD86 IS localization is dependent on its cytoskeletal association since CD86 mutated in the K4 region failed to redistribute at the interaction interface and has a drastically reduced cosignaling function as measured by T cell IL-2 secretion. CD86 was also shown to be important for conjugate formation, as shown in figure 2 of chapter 3.

4.2.4 CD86 physically associates with ERM proteins

The first description of a binding partner for CD86 is demonstrated by co-immunoprecipitation experiments in the Daudi B cell line where CD86 physically associates with ERM proteins (figure 5, chapter 3). ERM proteins act as adapters between transmembrane receptors and the cortical actin cytoskeleton. Ezrin has been shown to accumulate at the IS (304). Moreover, *Tomas et al.* showed that ezrin localization at the IS is triggered by both TCR and CD28 signaling (117). In that same report, ERM proteins were also shown to be associated to lipid rafts. ERM proteins may play a role in the formation or stabilization of signalosomes at the IS. Interestingly, a 2004 report by *Faure et al.* (121) showed that ERM proteins function at the IS is tightly regulated by selective phosphorylation. Transient Thr dephosphorylation of ERM proteins in T cells at initial stages of the IS leads to cytoskeletal relaxation and more efficient APC:T cell conjugate formation. ERM proteins are then rapidly rephosphorylated to act as adapters between transmembrane receptors and the cortical actin cytoskeleton.

4.2.5 The two signal model in APC

From our results and those of others, a question that remains is the mechanism of CD80 and CD86 recruitment at the IS. We propose a reciprocal two-signal model in APC that takes into account all previous research. This model is presented in figure 3. We have shown that wild type CD86 proteins physically associate with ERM proteins. Because CD86 mutated in the K4 region looses its cytoskeletal association, we hypothesize that this region is an ERM binding region.

Interestingly, CD80 RRNE region characterized by *Doty and Clark* (226) also fits the definition of an ERM binding region since it is a juxtamembrane positively charged cluster.

The initial signal leading to B7 recruitment at the IS would occur just following the formation of peptide-specific conjugates. This signal could be brought about either through direct CD80/CD86 signaling or signaling through other receptors such as MHC class II molecules. This first signal would involve the phosphorylation of inactive cytoplasmic ERM proteins to adopt open conformations. *Al-Alwan et al.* findings support this first step since it is only in the context of specific peptide: MHC clustering that DC rearrange their cytoskeleton. The second signal would involve recruitment and/or retention of CD86 to the IS by phosphorylated ERM proteins allowing sustained T cell co-stimulation. CD80 and CTLA-4 upregulation and interaction would stop cosignaling activity. This reciprocal two signal model highlights the bi-directional nature of signaling in the IS that has been described in recent years.

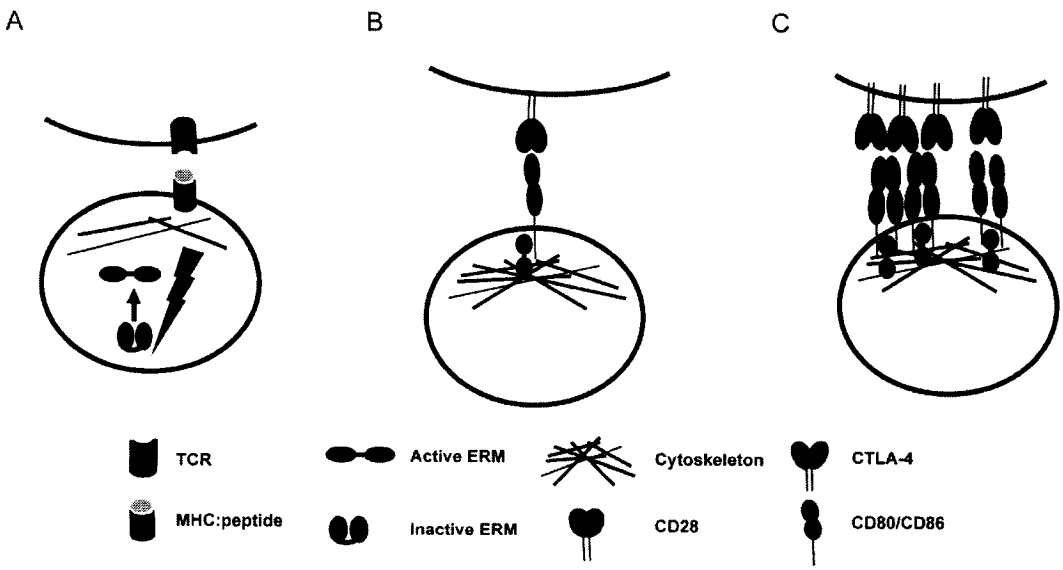


Figure 3 The Two Signal Model in APC

A) **Signal 1.** MHC: peptide cross-link or B7 cross-link (not shown) induces signaling events leading to the phosphorylation of ERM proteins. B) **Signal 2.** ERM-bound CD86 localize at the immunological synapse. C) Termination of the response by the CD80:CTLA-4 interaction.

4.3 Future research

The work presented in this thesis deepened our understanding of CD80 and CD86 function; it also opened new research avenues.

4.3.1 Molecular structure of CD80 and CD86

The role of CD80 and CD86 IgC in regulating dimerization has been revealed from the work presented in this thesis. The exact mechanism by which the IgC domain exerts this function remains to be established. Two main hypotheses, not mutually exclusive, have been suggested to account for this function of the IgC domain. The first hypothesis involves the sugar residues within the IgC domain. These sugar residues might impact CD80 and CD86 dimerization capacity by interacting with the plasma membrane or by creating a shield around these molecules. The second hypothesis involves a possible stabilizing interaction between the IgC and the IgV domain that could directly impact the dimer interface present within the IgV domain. Mutational studies targeting CD80 and CD86 glycosylation sites along with the residues believe to be at the interface between the IgC and IgV domains could be instigated. FCET, biochemical and physiological readouts of the functions of these mutated molecules could deepen our understanding of the IgC domain mechanism of action. IgC deleted CD80 and CD86 molecules do not bind CD28 as well as their wild type counterparts. This can be explained by their existence as higher order multimers. However, the observed defect in IL-2 secretion upon T cell activation could also be due to the decreased size of the IgC deleted molecules. It would therefore be interesting to study the impact of CD80 and CD86 molecular structure in the context of the immunological synapse.

The data presented in this thesis along with the data of others have clearly established that CD80 exists as a mixed monomeric and dimeric population. Bhatia et al. (257) have suggested that CD80 predominantly exists as a dimer but the non-denaturing PAGE experiments performed in the course of this thesis do not support such a finding. Of note, both studies were performed in non-lymphoid

cells. It would be judicious to clearly establish the monomer:dimer ratio of CD80 molecules at the surface of cells of lymphoid origin, preferably in CD80/CD86 deficient APC. Moreover, it shall prove interesting to study the mechanisms by which CD80 is expressed as a monomer or as a dimer at the cell surface. Although overexpression of CD80 molecules does not change the FRET efficiencies, it may affect the proportions between CD80 monomers and dimers. This experimental question could be answered by non-denaturing PAGE experiments.

4.3.2. CD80 and CD86 binding partners and signaling

CD86 and ERM proteins have been shown to physically associate in the Daudi B cell line during the course of this thesis. Moreover, data has been gathered in recent years on the signaling cascades induced within APC following CD80 and CD86 ligation (94, 230, 231, 233, 235). It will be interesting to better delineate the region responsible for ERM binding in CD86 and to determine whether CD80 is also associated to ERM proteins. It shall also prove interesting to establish whether ERM proteins can act as signal conveyers through their interaction with CD80 and CD86 molecules. A careful analysis of CD80 and CD86 induced signaling will certainly lead to a better understanding of the function of these molecules. ERM proteins function is regulated by selective phosphorylation in T cells. Analysis of the Tyr and Thr phosphorylation of ERM proteins in APC, prior, during and following T cell interaction in the context of the IS could lead to a better understanding of cytoskeletal dynamics in APC.

4.3.3. Physiological function of CTLA-4 interactions

During the course of this thesis, a CD28-dependent cosignaling model was used to study the function of CD80 and CD86. To deepen our comprehension of CD80 and CD86 function, the relevance of CD80 and CD86 cytoskeletal association and molecular structure should be assessed in the context of CTLA-4 co-inhibitory signaling.

In past years, cutting edge microscopy techniques have been developed. To better understand the overall function and interplay between CD28/CTLA-4/CD86/CD80 molecules, the development of models that could allow the study of these interactions in real-time peptide-specific live cell interactions or even by visualizing these interactions *in vivo* is of utmost interest.

4.4 CD80 and CD86 are different

CD28 and CTLA-4 were discovered 20 years ago and CD80 and CD86 have been described over a decade ago. Although considerable progress has been made in studying B7-mediated cosignaling, important functions of these key molecules are still being described. Analysis of the properties of the classical members of the now extending cosignaling family could certainly help in the study of the newly identified molecules. In upcoming years, it will be important to investigate these newly described molecules to better understand how their various structures, expression levels, binding stochiometry, interaction affinities and signal integration regulate their function. Also, it remains a daunting task to integrate data of the multiple signaling pathways from both the IgSF and TNF/TNFR cosignaling superfamilies leading to proper T cell activation and function. It will be important to integrate all generated data to better understand T cell deregulation in autoimmune and allergic diseases, to better modulate transplant and tumor immunity and for to design new immunotherapeutics.

In the literature, CD80 and CD86 have been interchangeably used as the CD28 and CTLA-4 counter receptors. However, our data, combined with that of others, clearly show that these molecules are not equivalent. CD86 is a monomeric co-receptor that is constitutively or rapidly induced at the APC surface and will bind to monovalent CD28 inducing a co-activating signal. CD80 is a receptor that can be monomeric or dimeric, that is upregulated following initial T cell activation and that will bind to bivalent CTLA-4. The structural change that lead to a switch

between CD28 monovalent and CTLA-4 bivalent binding could certainly have initiated the functional diversification of this signaling system (184).

The work presented in chapter 2 and chapter 3 leads us to a better understanding of the structure and function of these molecules and their constituent domains as summarized in figure 4.

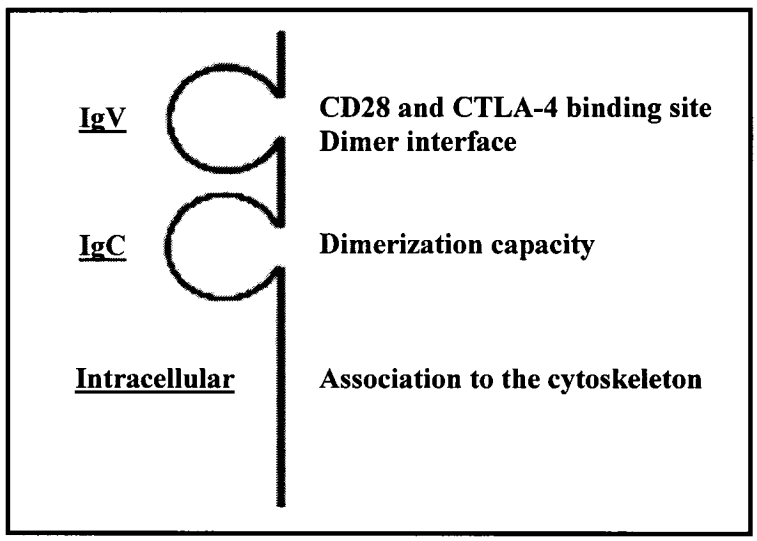


Figure 4 Role of CD80 and CD86 Functional Domains
The work accomplished during this thesis as enabled the characterization of CD80 and CD86 domains function. The IgV domain contains the receptor binding site and dimer interface. The IgC domains negatively regulate dimer formation for both molecules. Moreover, the intracellular domains of both molecules are functionally linked to the cytoskeleton. Altogether, all 3 domains of CD80 and CD86 are important for their full cosignaling function.

The results highlight the distinct yet coupled roles of CD80 and CD86 domains. The binding region resides in the IgV portions of CD80 and CD86 along with the dimer interface. This dimer interface is stabilized through the IgC domain interaction with the plasma membrane and/or with the IgV domain directly impacting the receptor binding properties and overall function. Finally, CD80 and CD86 intracellular domains functionally link these molecules to the cytoskeleton. Altogether, the results acquired during the course of this thesis have established the functional role of CD80 and CD86 domains in their molecular structure and overall cosignaling function. These results may facilitate the design of structure-based immunotherapeutics.

Chapter 5

References

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Appendix 1

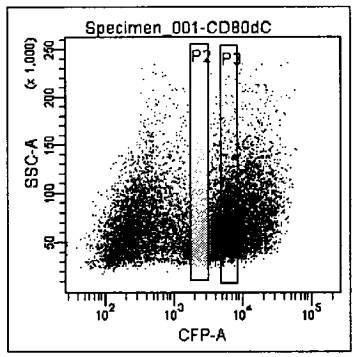
Appendix 2

Appendix 3

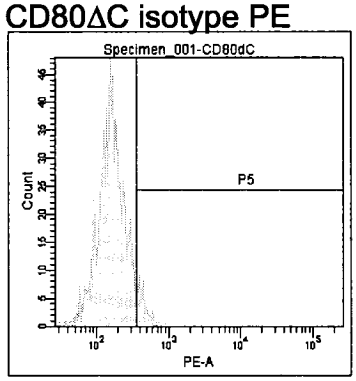
Appendix 4

Antibody Stainings Gating Strategy and Exemplary Histograms

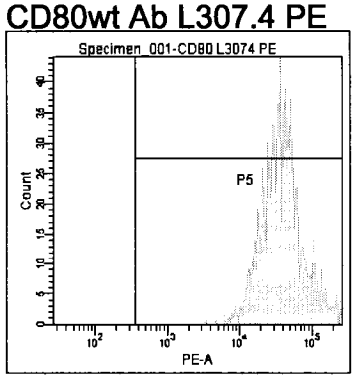
1. Gating on live cells (P1)
2. Gating on equal MFI populations (P2 or P3)
3. PE signal histogram on selected populations (P2)



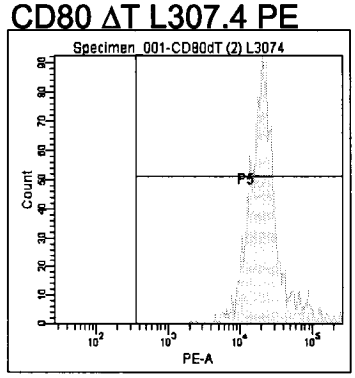
CD80ΔC isotype PE



CD80wt Ab L307.4 PE



CD80 ΔT L307.4 PE



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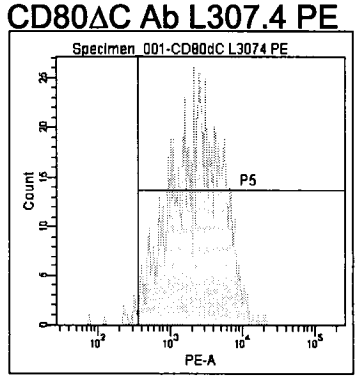


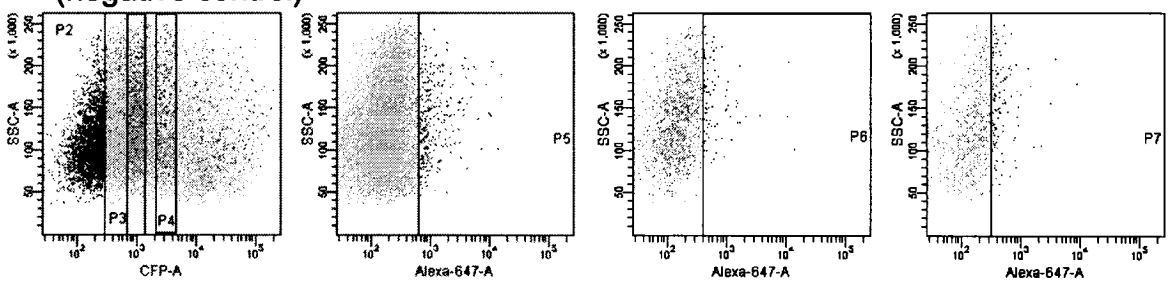
Appendix 5

Ligand Binding by FACS: Gating Strategy and Exemplary Histograms

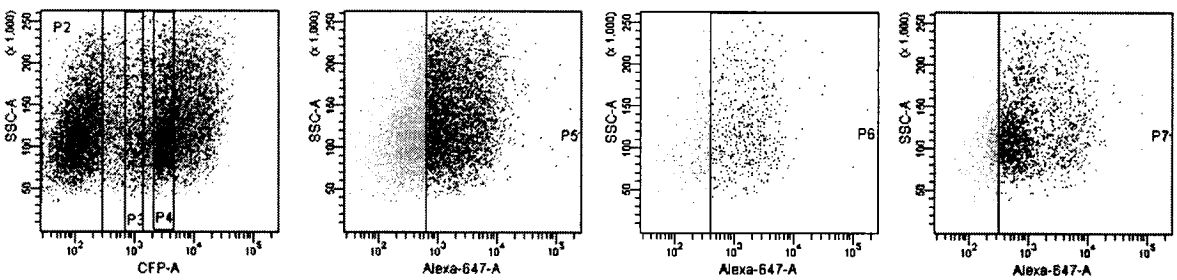
Gating Strategy

1. Live cells gating (P1)
2. Equal MFI Gating (P2, P3 and P4)
3. Alexa 647 Signal Histogram on Selected Populations (P5/P6/P7 parent of P2/P3/P4)

CD80ΔC Cells 1.25μg human Fc + Alexa 647 coupled secondary antibody (negative control)



CD80ΔC Cells 1.25μg CTLA-4Fc + Alexa 647 coupled secondary antibody



CD80wt Cells 1.25μg CTLA-4Fc + Alexa 647 coupled secondary antibody