CEACAM1, an inhibitory co-receptor in T lymphocytes

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Abstract

A number of reports examining the role of CEACAM1 (Carcinoembryonic Antigen-related Cell Adhesion Molecule 1) in T lymphocytes have defined for it both stimulatory or inhibitory co-receptor functions. Using our unique *Ceacam1*-/- mouse model, we previously observed that CEACAM1 is not involved in the development nor in the migration of T cells, but that it exerts a co-inhibitory effect on their activation and proliferation. Here, we report that its loss does not affect the ratios of naïve and memory helper or cytotoxic T cells. Moreover, cytotoxic T cells from OT-1:*Ceacam1*-/- mice, resulting from breeding OT-1 TCR transgenic mice with *Ceacam1*-/- mice, are hyperproliferative and secrete more interleukin-2 and interferon-γ than their OT-1 counterparts. Finally, studies involving CD2-CC1-L transgenic mice whose T cells overexpress CEACAM1 did not consistently yield the hypoproliferative behaviour expected. Together, these findings suggest that CEACAM1 is an inhibitory co-receptor in T cells.

Abrégé

Certains articles traitant du rôle de CEACAM1 (Carcinoembryonic Antigen-related Cell Adhesion Molecule 1) chez les lymphocytes T lui ont attribué une fonction de co-récepteur soit activateur ou inhibiteur. Utilisant notre modèle de souris *Ceacam1*-/-, nous avons observé que cette protéine n'affecte ni le développement ni la migration des cellules T. Par contre, elle co-inhibe leur activation et leur prolifération. Dans ce travail, nous révélons que l'ablation de CEACAM1 n'affecte pas l'abondance des cellules T auxiliaires et cytotoxiques naïves et mémoires. De plus, les cellules T cytotoxiques des souris OT-1:*Ceacam1*-/-, nées de croisements entre souris *Ceacam1*-/- et OT-1, dont ces cellules arborent un récepteur transgénique, sont hyperprolifératives et sécrètent davantage d'interleukine-2 et d'interféron-γ que leurs homologues OT-1. Finalement, l'étude des souris transgéniques CD2-CC1-L, dont les cellules T surexpriment CEACAM1, n'a pas donné de façon consistante le profil hypoprolifératif attendu. Nous confirmons donc certains des résultats précédents et concluons que CEACAM1 agit comme co-récepteur inhibiteur chez les cellules T.

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List of Abbreviations

aa: Amino acid

Ab: Antibody

Ag: Antigen

Ala: Alanine

AP-1; AP-2: Activating protein 1; Activating protein 2

APC: Antigen-presenting cell; Adenomatous polyposis coli gene

Arg: Arginine

AS: Ankylosing spondylitis

BCR: B cell receptor

BGP: Biliary glycoprotein

BHK: Baby hamster kidney cells

BI: Beef insulin hybridoma T cell line

CD: Cluster of differentiation

CD2-CC1-L: Tg mice overexpressing CEACAM1-L cDNA under the control of the hCD2 promoter

CD2-FF: Tg mice overexpressing the CEACAM1-L cDNA harbouring the Y488,515F mutation under the control of the hCD2 promoter

CD40L: CD40 ligand (also referred to as CD154)

CEA: Carcinoembryonic antigen

CEACAM: Carcinoembryonic antigen-related cell adhesion molecule

CFSE: Carboxyfluorescein diacetate-succinimidyl ester

CMV: Cytomegalovirus

CNS: Central nervous system

Con A: Concanavalin A

COPD: Chronic obstructive pulmonary disease

CRH: Corticotropin-releasing hormone

CTL: Cytotoxic T lymphocyte

CTLA-4: Cytotoxic T lymphocyte antigen 4

Cyt: Cytoplasmic

DAEC: Diffusely adhering *Escherichia coli*

DC: Dendritic cell

DC-SIGN: Dendritic cell-specific ICAM-3 grabbing nonintegrin

dNTP: Deoxyribonucleoside triphosphate

dpc: Day post-coitum

EC: Endothelial cell

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

Erk: Extracellular signal-regulated kinase

EVT: Extravillous trophoblast

FAK: Focal adhesion kinase

FAS: Fatty acid synthase

FBS: Fetal bovine serum

Fc; FcR: Immunoglobulin "fragment crystallisable"; Immunoglobulin Fc receptor

FFA: Free fatty acid

FITC: Fluorescein isothiocyanate

FMLP: n-formylmethionylleucylphenylalanine

G-actin: Globular actin

Gln: Glutamine

GPI: Glycosylphosphatidylinositol

Grb2: Growth factor receptor-binding protein 2

GST: Glutathione-S-transferase

GTD: Gestational trophoblastic disease

GTPase: Guanosine triphosphatase

HA4: Hepatocyte antigen 4

HB-EGF: Heparin-binding epidermal growth factor-like growth factor

hCD2: Human CD2

HNF-4: Hepatic nuclear factor 4

HSPG: Heparan sulphate proteoglycan

HUVEC: Human umbilical vein endothelial cell

IBD: Inflammatory bowel disease

ICAM-3: Intercellular adhesion molecule-3

IFNγ; Interferon-gamma

Ig: Immunoglobulin

IgC2-like: C2-type immunoglobulin constant-like domain

IgSF: Immunoglobulin superfamily

IgV-like: Immunoglobulin variable-like domain

iIEL: Intestinal intraepithelial lymphocyte

IL: Interleukin

IL-2R: IL-2 receptor

Ile: Isoleucine

IR: Insulin receptor

IRF-1: Interferon regulatory factor 1

ISRE: Interferon-stimulated response element

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibitory motif

Jak: Janus-associated protein tyrosine kinase

JNK: Jun-N-terminal kinase

LCR: Locus control region

Le^x; sLe^x: Lewis x glycan structure; Sialyl-Lewis x glycan structure

LFA-1: Lymphocyte function associated antigen 1

LN: Lymph nodes

LPL: Lamina propria lymphocyte

L-SACC1: Liver-specific Ser503Ala CEACAM1 transgenic mouse

M.W.: Molecular weight

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein 1

MDCK: Madin-Darby canine kidney cells

MHC: Major histocompatibility complex

MHV; MHV-A59: Mouse hepatitis virus; MHV strain A59

MHVR: Mouse hepatitis virus receptor

MIP-1α: Macrophage inflammatory protein 1α

MIP2: Macrophage inflammatory protein 2

MLR: Mixed lymphocyte response

mmCGM1/2: *Mus musculus* CEA gene family member 1 or 2

NCA-160: Nonspecific cross-reacting antigen of M.W.160 kDa

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor κB

NK cells: Natural killer cells

NKT cells: Natural killer T cells

NRS: Normal rabbit serum

NSCLC: Non small cell lung carcinoma

OMP: Outer membrane protein

Opa: Opacity-associated proteins

OPN: Osteopontin

OT-1: OVA-specific class I-restricted TCR transgenic mice

OT-2: OVA-specific class II-restricted TCR transgenic mice

$OVA_{257-264}$: Ovalbumin-derived peptide corresponding to amino acids 257-264

PAK: p21-activated protein kinase

PBS: Phosphate-buffered saline solution

PBS-2% FBS: Phosphate-buffered saline solution containing 2% fetal bovine serum

PCR: Polymerase chain reaction

PD-1: Programmed death 1

PE: Phycoerythrin

PECAM-1: Platelet-endothelial cell adhesion molecule 1

PHA: Phytohemagglutinin

Phe: Phenylalanine

PKC: Protein kinase C

PMA: Phorbol 12-myristate 13-acetate

PolyA: Polyadenylation site

pp: Phosphoprotein

PSG: Pregnancy-specific glycoprotein

RCC: Renal cell carcinoma

RT-PCR: Reverse transcription followed by polymerase chain reaction

S: Spike glycoprotein of the murine hepatitis virus

SDF-1: Stromal cell derived factor 1

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser: Serine

Shc: Src homology 2 (SH2) domain-containing α2 collagen-related protein

SHP-1/2: Src homology domain 2 (SH2)-containing phosphatase 1 or 2

STAT: Signal transducer and activator of transcription

SVEC4-10: SV40-immortalized endothelial cell line 4-10

TAP2: Transporter associated with antigen processing 2

TBS: Tris-buffered saline solution

TCR; TCR/CD3 complex: T cell receptor; TCR associated with the CD3 complex

Tg: Transgenic

Th1; Th2: Type1 helper T cells; Type 2 helper T cells

Thr: Threonine

TMB: Tetramethylbenzidine

TNBS: Trinitrobenzene sulfonic acid

TNF-α: Tumour necrosis factor α

Treg: Regulatory T cell

Tyr: Tyrosine

U: Unit

USF: Upstream stimulatory factor

UspA1: Ubiquitous surface protein A1

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

WT: Wild type

ZAP-70: ζ-chain associated protein 70

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CHAPTER 1: INTRODUCTION

1. CEACAM1, a unique member of the CEA family

CEACAM1, or Carcinoembryonic Antigen-related Cell Adhesion Molecule 1, was discovered in 1976 by Svenberg as a component of normal human gall-bladder and hepatic bile, and was thus originally named biliary glycoprotein (BGP) [1]. CEACAM1 belongs to the *CEA* gene family, a group of genes encoding glycoproteins related to carcinoembryonic antigen (CEA), a commonly used colorectal cancer marker [2]. CEA was the first member of this family to be identified in 1965 by Gold and Freedman as an antigen (Ag) present both in colon tumors and in the fetal digestive tract [3]. The CEA family, which is part of the immunoglobulin superfamily (IgSF) [4], is composed of two main branches: the CEACAMs (Fig.1-1, 1-2), expressed on the cell surface and usually attached to the plasma membrane via a transmembrane domain or a glycosyl phosphatidyl inositol (GPI) anchor, with the latter type being found only in human family members (Fig. 1-1); and the pregnancy-specific glycoproteins (PSGs), which are solely secreted. A third subgroup includes various pseudogenes [5]. Until recently, there were 29 genes ascribed to this family in humans, 22 in the mouse, and 7 in the rat, with nine new genes recently identified in the mouse genome, four of which possess orthologs in all three species [6].

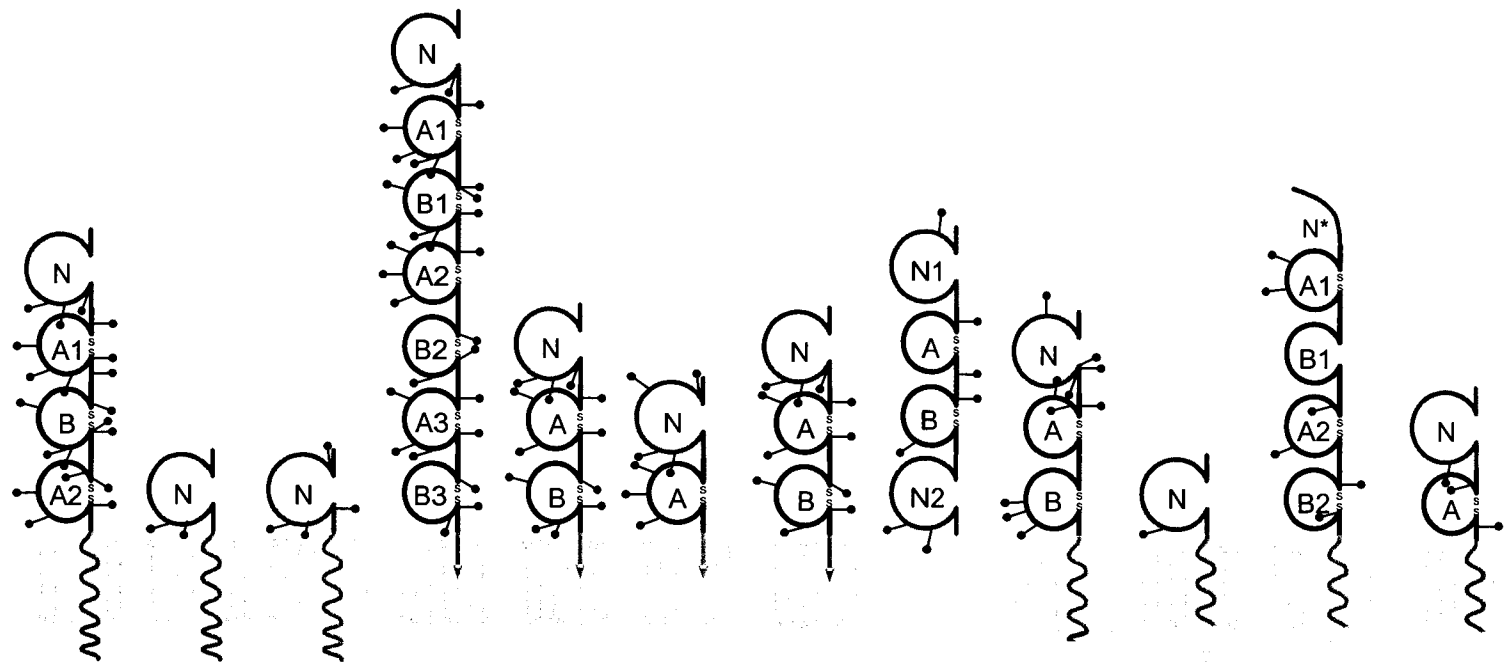
A number of features renders the glycoprotein formerly known as BGP, CD66a, NCA-160, Bgp, MHVR, mmCGM1/2, Cell-CAM 105, pp120, HA4, and ecto-ATPase unique within this family whose nomenclature was recently standardized, hence its rechristening to CEACAM1 (Table 1-1) [5]. First of all, it is considered the ancestor of the CEA family, for it is the only member which possesses "obvious orthologs" in the human, murine [7, 8], rat [9] genomes. It was also recently identified in the bovine [10] and canine [11] genomes. Furthermore, it is also the most conserved gene within this family, be it at the level of its structure, splice variants, patterns of expression, or functions [12]. CEACAM1 is also the most widely expressed member of this family [13], and the only one to harbour immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic (cyt) domain of some of its isoforms [14], a feature of many immune inhibitory receptors [15].

Figure 1-1: The CEACAM branch of the human CEA family.

Schematic representation of the protein structures of the human CEA family members belonging to the CEACAM branch. Red balloons represent the immunoglobulin (Ig) variable-like (IgV-like) N-terminal domains, whereas blue ones represent the C2-type Ig constant-like (IgC2-like) domains of the A and B subtypes, with their "intra-chain" disulfide bonds symbolized by S-S. Green wavy lines represent the transmembrane and cytoplasmic (cyt) domains, with downward green arrows indicating the presence of a glycosylphosphatidylinositol (GPI) anchor. The black "pinheads" represent presumed N-linked glycosylation sites.

Adapted with permission from the CEA homepage: http://cea.klinikum.uni-muenchen.de/

Figure 1-1


N
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B
A2
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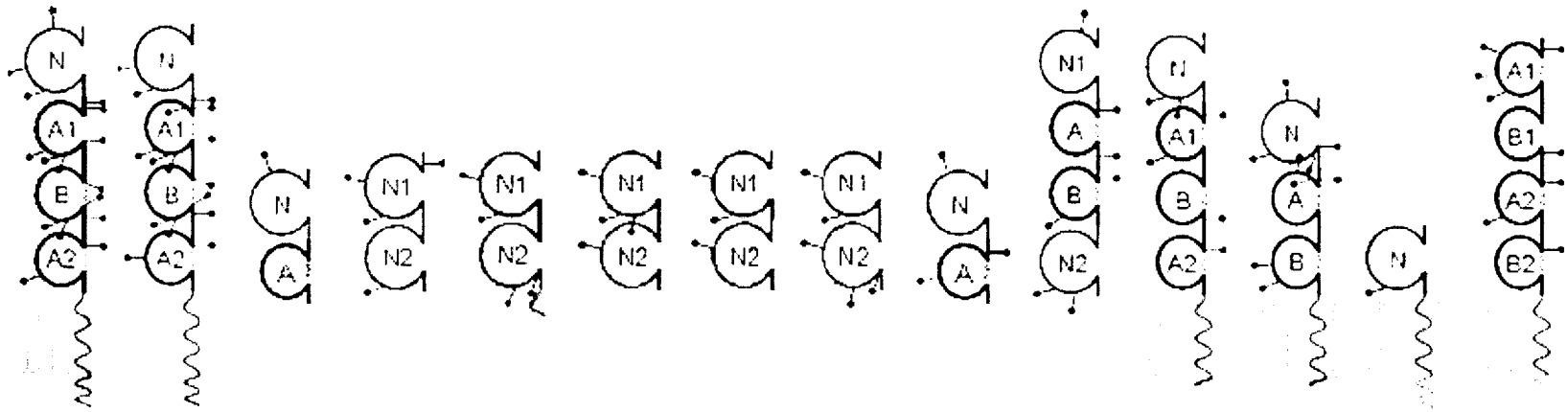
B1
A2
B2
N
A
CEACAM1
CEACAM4
CEACAM6
CEACAM8
CEACAM18
CEACAM20
CEACAM3
CEACAM5
CEACAM7
CEACAM16
CEACAM19
CEACAM21

Figure 1-2: The CEACAM branch of the mouse CEA family.

Schematic representation of the protein structures of the mouse CEA family members belonging to the CEACAM branch. Red balloons represent the IgV-like N-terminal domains, whereas blue ones represent the IgC2-like domains of the A and B subtypes, with their "intra-chain" disulfide bonds symbolized by S-S. Green wavy lines represent the transmembrane and/or cyt domains. The black "pinheads" represent presumed N-linked glycosylation sites.

Adapted with permission from the CEA homepage: http://cea.klinikum.uni-muenchen.de/

Figure 1-2



CEACAM1 **CEACAM9** **CEACAM11** **CEACAM13** **CEACAM15** **CEACAM17** **CEACAM19**

CEACAM2 **CEACAM10** **CEACAM12** **CEACAM14** **CEACAM16** **CEACAM18** **CEACAM20**

Table 1-1: New standardized nomenclature of *CEACAM1* genes and their corresponding protein splice variants in human, mouse and rat.

a, b, and a, b: allelic variants; N: IgV-like N-terminal domain; A1, A2: IgC2-like domains of the A subtype; B: IgC2-like domain of the B subtype; TM: Transmembrane domain; L: Long cytoplasmic domain; S: Short cytoplasmic domain; Cn: Different termini generated by altermative mRNA splicing; Alu: Alu sequence replacing an IgC2-like extracellular domain.

Adapted with permission of Dr N. Beauchemin from Beauchemin *et al.* Exp. Cell Res. **252**, 243 (1999)

Table 1-1

Species	Gene	New gene name	Old splice variant protein name	New splice variant protein name	Structure	Other names
Human	*BGP*	*CEACAM1*	BGPa	CEACAM1-4L	N, A1, B, A2, TM, L	BGP1, CD66a, TM-CEA
			BGPb	CEACAM1-3L	N, A1, B, TM, L	
			BGPc	CEACAM1-4S	N, A1, B, A2, TM, S	
			BGPd	CEACAM1-3S	N, A1, B, TM, S	
			BGPg	CEACAM1-4C1	N, A1, B, A2, C1	W211
			BGPh	CEACAM1-3	N, A1, B	W233
			BGPi	CEACAM1-3C2	N, A1, B, C2	W239
			BGPx	CEACAM1-1L	N, TM, L	
			BGPx'	CEACAM1-1S	N, TM, S	
			BGPy	CEACAM1-3AL	N, A1, B, Alu, TM, L	
			BGPz	CEACAM1-3AS	N, A1, B, Alu, TM, S	
Mouse	*Bgp1* [a]	*Ceacam1* [a]	BgpA	CEACAM1[a]-4S	N^a, $A1^a$, B^a, $A2^a$, TM, S	mmCGM1, mmCGM1a, MHVR1, mCEA1
			BgpC	CEACAM1[a]-2S	N^a, $A2^a$, TM, S	mmCGM2 B6
			BgpD	CEACAM1[a]-4L	N^a, $A1^a$. B^a, $A2^a$, TM, L	MHVR1(2d)$_s$, MHVR1(4d)L
			BgpG	CEACAM1[a]-2L	N^a, $A2^a$, TM, L	MHVR1(2d)L
	Bgp1 [b]	*Ceacam1* [b]	BgpB	CEACAM1[b]-2S	N^b, $A2^b$, TM, S	mmCGM2, MHVR2, mmCGM2(2d)
			BgpE	CEACAM1[b]-4S	N^b, $A1^b$, B^b, $A2^b$, TM, S	mmCGM1 SJL, mmCGM2(4d)
			BgpF	CEACAM1[b]-4L	N^b, $A1^b$. B^b, $A2^b$, TM, L	mmCGM2(4d)L, bb-1
			BgpH	CEACAM1[b]-2L	N^b, $A2^b$, TM, L	mmCGM2(2d)L
	Bgp2	*Ceacam2*	Bgp2C	CEACAM2-2S	N, A2, TM, S	
				CEACAM2-2L	N, A2, TM, L	
Rat	*C-CAM1* [a]	*Ceacam1* [a]	C-CAM1[a]	CEACAM1[a]-4L	N^a, $A1^a$. B^a, $A2^a$, TM, L	Cell-CAM105, pp120, HA4, gp110, ecto-ATPase, CBATP
			C-CAM2[a]	CEACAM1[a]-4S	N^a, $A1^a$, B^a, $A2^a$, TM, S	C-CAM2a
			C-CAM3	CEACAM1[a]-4C3	N^a, $A1^a$, B^a, $A2^a$, TM, C3	
	C-CAM1 [b]	*Ceacam1* [b]	C-CAM1[b]	CEACAM1[b]-4S	N^b, $A1^b$, B^b, $A2^b$, TM, S	C-CAM/n, cell-CAM105

1.1 CEACAM1 at the genomic level

The *CEACAM1* gene is found within the *CEA* family gene cluster, which regroups all members on human chromosome 19q13.2 [4]. This 1.8 Mb-*CEA* gene cluster is subdivided into two regions, separated by 700 kb: a 250 kb region where the majority of *CEACAM* genes are found, and a 850 kb region encoding the *CEACAM1*, and the *PSG* genes, and where the pseudogenes are scattered [13]. The murine *Ceacam1* gene is located within its family's 6.5 Mb-cluster (Fig. 1-3) [6] on mouse chromosome 7, in a region syntenic to that encoding *CEACAM1* on human chromosmome 19q [16], the 7A2-A3 region [17]. Moreover, another highly homologous gene, the *Ceacam2* gene, has been found in this region [18], and encodes the CEACAM2-2S, and -2L proteins [19]. This is unique to the murine *Cea* family, as there is only one *CEACAM1*-like gene in the human and rat genomes [20]. Also, two alleles of the *Ceacam1* gene have been identified in rodents: *Ceacam1a* and *Ceacam1b* [21-24]. These generate variations in a number of amino acids (aa) in the N-terminal domain of the encoded proteins (27 aa in the mouse [22], and 16 aa in the rat [23]), rendering mice carrying the *Ceacam1a* allele susceptible to mouse hepatitis virus (MHV) infection [25]. Interestingly, this allelic variation exists also in cattle [10], but not in humans [24] and has been suggested to result from a "pathogen-driven evolution [10]".

Furthermore, the structure of the *CEACAM1* gene, which is composed of nine exons and eight introns, is highly conserved amongst species [16, 26-28]. Figure 1-4 shows the structure of the murine *Ceacam1* gene. The first exon contains the 5' untranslated region (UTR), the start codon, and part of the sequence of the leader peptide, required to target the resulting protein to the membrane. The remainder of the leader peptide sequence is found within the second exon, which also encodes the extracellular immunoglobulin (Ig)-like N-terminal domain, termed IgV-like due to its resemblance to a variable Ig domain. Each of the three subsequent exons (3, 4, and 5) encode for a C2-type Ig constant-like (IgC2-like) domain (A1, B, and A2). The transmembrane domain sequence is encoded by exon 6, whereas that for the cytoplasmic (cyt) tail is spread over exons 6, 7, 8, and 9, with exons 8 and 9 containing stop codons used to generate the short- and long-tailed isoforms, respectively. This last exon also contains approximately 2 kb of 3' UTR sequence [28].

Figure 1-3: Organization of the murine *Cea* gene family cluster.

Distribution of the mouse *Cea* gene family members within its 6.5 Mb-gene cluster, located in the 7A2-A3 region of chromosome 7. *Ceacam* (Cc) genes are represented by white bars, with *Ceacam1*, *Ceacam2* and *Ceacam10* represented by black ones. A pseudogene, ps-1, is depicted as a dotted bar, and the pregnancy-specific glycoprotein (*Psg*) genes are shown in grey. The nine recently discovered genes and pseudogene have an asterisk beside their name, and the arrows underneath each gene indicate the orientation of transcription.

Figure 1-4: Structure of the mouse *Ceacam1* gene and patterns of alternative mRNA splicing resulting in its different protein isoforms.

Gene structure of the mouse *Ceacam1* gene, with its nine exons represented by boxes with the protein sequence they encode identified on them. Exon numbers are shown underneath. Dashed lines represent the mRNA splicing events taking place to generate the four mouse splice variants, which possess two or four extracellular domains, and either a short or long cyt tail. 5'UTR: 5' untranslated region; L: leader peptide sequence; N: N-terminal IgV-like domain; A1, A2: IgC2-like domains of subtype A; B: IgC2-like domain of subtype B; TM: transmembrane domain; C: Portions of the cyt domain; 3'UTR: 3' untranslated region; ATG: start codon; TGA(S): stop codon for the isoforms bearing a short cyt tail; TGA(L): stop codon for the isoforms bearing a long cyt tail.

Figure 1-3

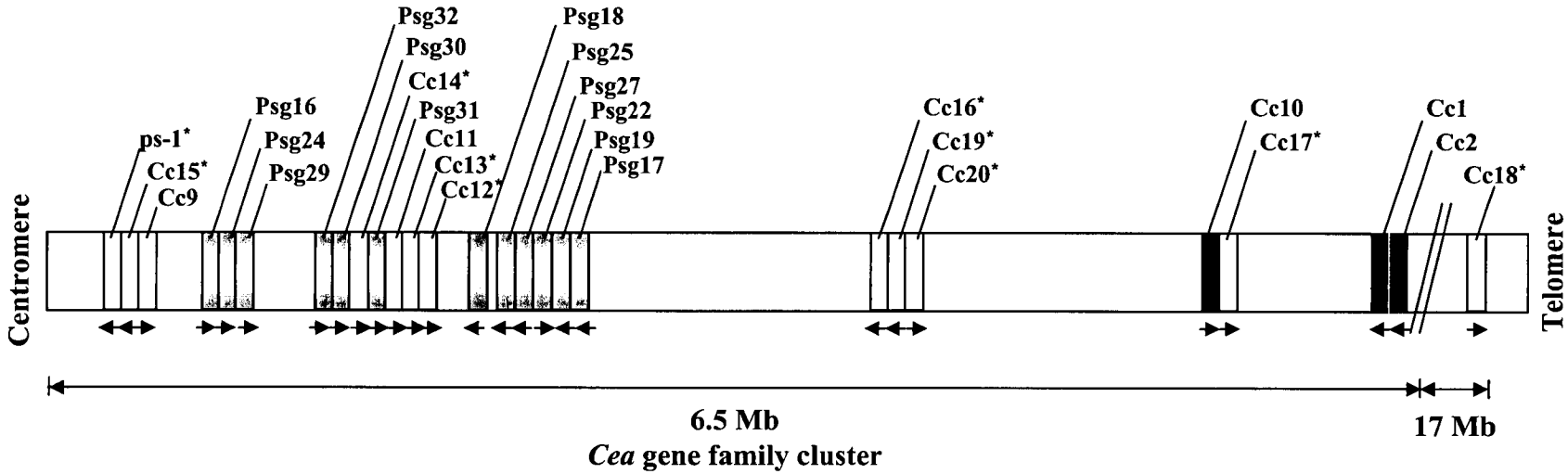
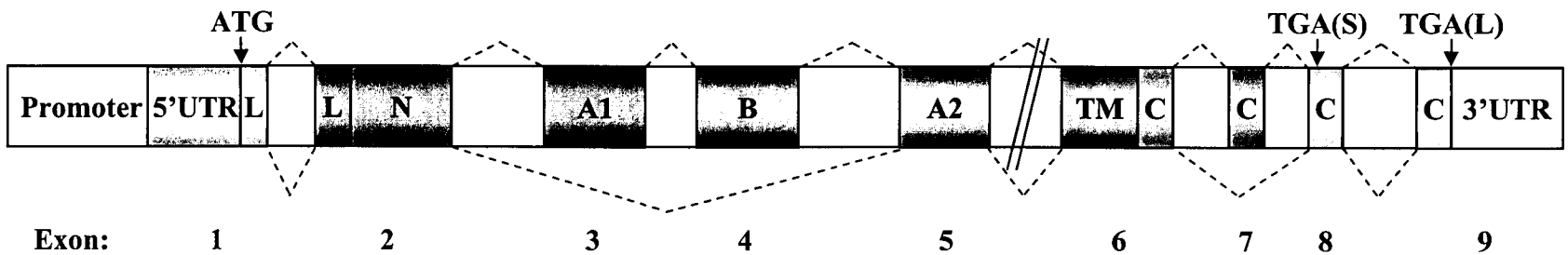


Figure 1-4



Thus, linkage of N to A2, or of all four exons (N, A1, B, and A2) generates murine splice variants with two or four extracellular Ig-like domains, respectively. Long-tailed isoforms result from the inclusion of exons 6-9, with the 53-bp exon 7 shifting the open reading frame, allowing translation to proceed until the stop codon in exon 9; omission of exon 7 stops translation at exon 8, generating short-tailed isoforms (Fig. 1-4) [21, 28]. The combination of these alternative mRNA splicing events leads to the different murine isoforms. Similar events occur in humans [26, 29], rat [27], and cattle [10], generating their specific isoforms, with the inclusion of a 53-bp exon 7 yielding long-tailed isoforms (a more detailed description of the mouse and human isoforms will be provided below).

1.2 Regulation of CEACAM1 expression at the transcriptional level

The promoter of the *CEACAM1* genes, like that of housekeeping genes, does not possess TATA or CAAT boxes, a characteristic it shares with the other members of the CEA family [13]. Instead, the basal level of transcription is regulated by transcription factors such as hepatic nuclear factor 4 (HNF-4), upstream stimulatory factor (USF), and the activating protein 2 (AP-2)-like factor [30, 31]. Binding sites for the latter two were also identified in the murine *Ceacam1* promoter [28], whereas additional sites for Sp1 and AP-1 were reported in its rat counterpart [32]. Moreover, other transcription factors can modulate CEACAM1 expression including nuclear factor κB (NF-κB) [33, 34] and Sp2 [35], which act as activators and repressors, respectively, in specific instances which will be described later. Furthermore, *CEACAM1* genes differ from housekeeping genes in that their expression can be influenced by various hormones, such as insulin [32], retinoids [36], corticotropin-releasing hormone (CRH) [37], androgen [38], progesterone and estrogen [39]; and cytokines, such as interleukins (IL)-2, -7, and -15 [40], and interferon gamma (IFNγ) [41]. The latter acts through interferon regulatory factor 1 (IRF-1), which binds an interferon-stimulated response element (ISRE) present in the *CEACAM1* promoter [41].

1.3 CEACAM1 at the protein level

The predominant CEACAM1 forms are type 1 transmembrane glycoproteins with variable molecular weights (M.W.) due to extensive glycosylation, and various isoforms

generated by alternative mRNA splicing. For instance, in human granulocytes, its bare polypeptide backbone has a M.W. of ~55-58 kDa, but the fully glycosylated protein is 140-170 kDa [42]. The different isoforms possess between one and four extracellular Ig-like domains. The IgV-like N-terminal domain is 108 aa in length, and a common characteristic of CEA family members is that its "intra-chain" disulfide bond is replaced by a salt bridge [13]. Moreover, this domain contains a CC' loop with a "unique stable convoluted conformation [43]", important for mediating homophilic interactions [44] (see Fig. 1 in [43] for a three-dimensional representation of CEACAM1 and of its CC' loop).

Within the CC' loop, key Arg43 and Gln44 residues in human CEACAM1 are involved in these interactions as well as in specific heterophilic interactions with CEA [45], whereas a projecting Ile41 residue participates in MHV heterophilic binding in mice [43]. The IgV-like N-terminal domain is followed by zero to three IgC2-like domains of subtypes A or B, which are 93 and 85 aa long, respectively, and which possess intra-chain disulfide bonds [13]. Finally, alternative mRNA splicing generates additional variation at the level of the cyt domains, which can be either a short tail of 10-12 aa (CEACAM1-S), or a long tail of 71-73 aa (CEACAM1-L) [20, 21, 23, 27]. This has a major impact on the functions exerted by these different isoforms, as it will become apparent later.

Four murine splice variants have been identified, which possess two or four extracellular domains, and either the short or the long cyt tail: CEACAM1-2S, CEACAM1-2L, CEACAM1-4S, and CEACAM1-4L (Fig. 1-5A) [5, 46]. In humans, eleven splice variants have been identified: six possess either one, three or four extracellular domains, with either a short or long tail; two possess an Alu sequence in their extracellular domain instead of the A2 IgC2-like domain [29] and either a short or long tail; the remaining three lack a transmembrane domain and are secreted (Fig. 1-5B) [5]. The prevalent human isoforms are CEACAM1-3S, -3L, -4S, and -4L [47]. Secreted splice variants are also found among the five different rat CEACAM1 isoforms [48].

In addition to the various isoforms generated by alternative mRNA splicing, more variation exists as a result of different glycosylation patterns. Indeed, CEACAM1 is heavily glycosylated, with its carbohydrate structures accounting for up to 50% of its molecular weight [2]. Studies of CEACAM1 from rat liver, which possesses fifteen potential N-glycosylation sites (Asn-X-Ser/Thr), have revealed the presence of high-man-

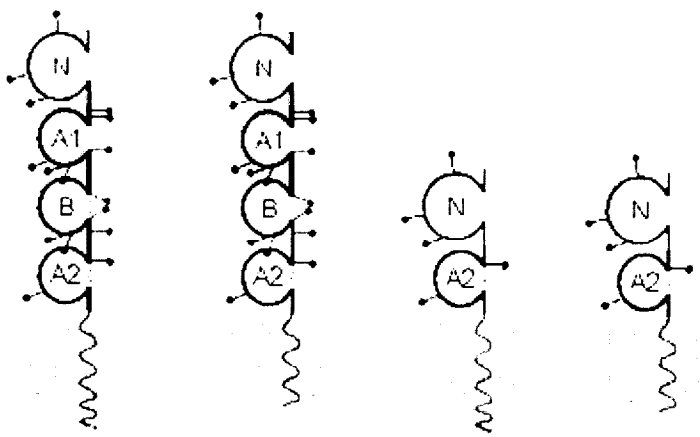
Figure 1-5: Structure of CEACAM1 isoforms expressed in mice and humans.

Schematic representations of the protein structures of the four mouse (A) and eleven human (B) isoforms of CEACAM1 generated by alternative splicing. Red balloons represent the IgV-like N-terminal domains, blue balloons represent the IgC2-like domains of the A and B subtypes, with their intra-chain disulfide bonds represented by S-S. Wavy lines represent the transmembrane and/or cyt domains, with downward green arrows indicating the presence of a glycosylphosphatidylinositol (GPI) anchor. Turquoise rectangles are indicative of the presence of an Alu sequence replacing an IgC2-like extracellular domain. The black "pinheads" represent presumed N-linked glycosylation sites. Secreted isoforms are depicted without an underlying plasma membrane. L or S: long or short cytoplasmic domain, respectively.

Adapted with permission from the CEA homepage: http://cea.klinikum.uni-muenchen.de/

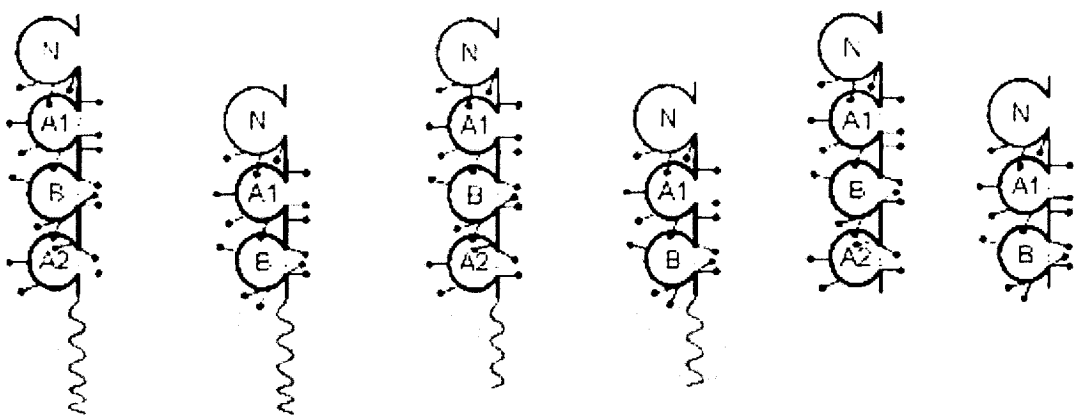
Figure 1-5

A

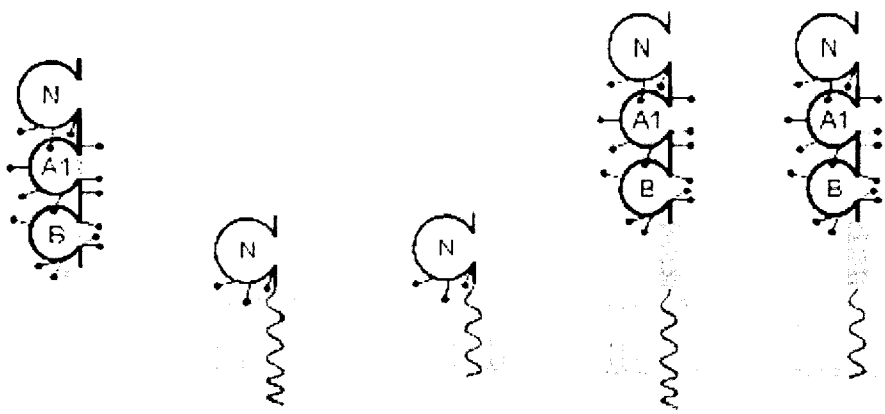


CEACAM1-4L CEACAM1-4S CEACAM1-2L CEACAM1-2S

B



CEACAM1-4L CEACAM1-3L CEACAM1-4S CEACAM1-3S CEACAM1-4C1 CEACAM1-3



CEACAM1-3C2 CEACAM1-1L CEACAM1-1S CEACAM1-3AL CEACAM1-3AS

nose, as well as highly sialylated complex N-glycans [42, 49]. Sialyl-Lewis x (sLe^x) [50] and Le^x structures have also been identified on CEACAM1 from human neutrophils [42], and play an important role in their adhesion [50, 51] and immune functions [52]. Furthermore, CEACAM1 present on human T cells presents a unique glycosylation pattern and does not harbour any sLe^x or Le^x moieties [40, 53].

2. Expression patterns of CEACAM1

2.1 Spatiotemporal patterns of expression in normal tissues

CEACAM1 expression begins early in embryonic development. For instance, CEACAM1 is first detected in the developing epidermis and gut of murine embryos by day 8.5 post-coitum (dpc), and 10.5 dpc, respectively. It is also expressed in the dermis, kidneys, lungs, meninges, and salivary glands of 15.5-dpc embryos, all areas of active epithelial-mesenchymal interactions [54]. Its expression was also reported in rat embryonic capillaries [55]. In contrast, in murine embryos, no expression of the highly homologous *Ceacam2* gene has been detected [56]. Furthermore, CEACAM1 expression has been linked to a number of developmental processes, such as odontogenesis [57], tooth eruption [57], myogenesis [54], spermatogenesis [58], as well as morphogenesis of the breast [59], in the sebaceous glands [60], and the vascularization of the central nervous system (CNS) [61].

Moreover, CEACAM1 is also expressed in trophoblasts in rodent and human placentae [54, 55, 62]. More precisely for the latter, it is highly expressed at the maternal-fetal interface, in the extravillous trophoblast (EVT) [62]. EVTs invade the endometrium, allowing the implantation of the embryo, and the creation of this interface. The latter, with specialized extracellular matrix (ECM) components, forms the decidua basalis. An important feature of the decidua is that 40% of its constituent cells are immune cells, mostly natural killer (NK) cells, and T cells [63]. As detailed later, the presence of CEACAM1 on EVTs might be important both for the proper implantation of the embryo [37], and for its protection against maternal immune responses, since elements from its paternal heritage constituting foreign/"non-self" Ags could lead to its rejection, and that collateral damage caused by too violent an immune response would harm it [63].

Furthermore, CEACAM1 expression is maintained in adult tissues, and, as mentioned earlier, is broadly distributed, being mainly expressed in epithelial, endothelial, and hematopoietic cells [20, 64]. These patterns of expression are similar in human, rat, and mouse, and in the latter species, the highly homologous *Ceacam2* gene is expressed in a more restricted manner, as it is found mostly in the spleen, testes, and kidneys [19, 56]. Moreover, in most tissues where CEACAM1 is found, both short- and long-tailed isoforms are coexpressed in ratios which vary among the different cell types, and which are also influenced by the activation and proliferative status of the cells [65, 66].

CEACAM1 is expressed on epithelial cells of the gastrointestinal tract, more precisely in the esophagus, stomach, small intestine; and in the caveolated, columnar and M cells [67] of the colon, where in the latter two it is part of the glycocalyx present at their apical surface, and is thought to participate in innate immunity [68, 69], and pathogen invasion [67], respectively. CEACAM1 is also found in the epithelial cells of tissues of the reproductive organs, such as the prostate, the endometrium, the ovaries; in the breast; in the pancreatic ducts; the liver bile canaliculi; the gall bladder; the proximal tubules of the kidney; the urinary bladder; as well as epithelia of the respiratory tract [13, 16, 70]. Moreover, CEACAM1 is found on endothelial cells of small blood vessels in humans and rodents, for example those found in the placenta and the endometrium [70], and only CEACAM1-L has been identified in endothelial cells [12]. In humans, larger, more mature blood vessels are devoid of CEACAM1 [71], whereas no such restriction exists in the mouse [72].

Furthermore, CEACAM1 is expressed on a variety of hematopoietic cells. These include platelets [73]; cells of the innate immune system, such as macrophages [74], neutrophils [73], monocytes [75], dendritic cells (DC) [76], and a subset of NK cells [77]; as well as cells of the adaptive immune system, namely B cells [65, 74], and T cells [77, 78]. Interestingly, a comparative study revealed that whereas only CEACAM1-L is present on the surface of neutrophils, T, and B lymphocytes in humans, their rodent counterparts express both CEACAM1-L and -S isoforms, giving rise to the hypothesis that GPI-linked family members (CEACAMs 6 and/or 8) took over the function of CEACAM1-S during evolution, hence its absence in these human immune cells [46].

More precisions regarding CEACAM1 expression in some of these cells will be added in a further section.

2.2 Altered expression during malignancy

Not only is CEACAM1 widely expressed in epithelia, but alterations in its expression occur in a number of human tumors and cancers of epithelial origin, some of which have also been observed in rodents [79-81]. For instance, in humans, CEACAM1 is downregulated in more than 85% of early colorectal adenomas and carcinomas [82-85], in about 30% of breast carcinomas [86, 87], as well as in hepatocellular [88], endometrial [89], prostatic [90, 91], and bladder [92] carcinomas when compared to normal tissues. Recently, complete downregulation of CEACAM1 in renal adenomas and renal cell carcinomas (RCC) was reported, alongside its reexpression due to the production of IFNγ by NK and T cells which had infiltrated these tumors in an attempt to eradicate them [93].

Furthermore, the downregulation of CEACAM1 occurs early in carcinogenesis, as it is observed in the adenoma stages of some of these malignancies, as described above. This occurs even earlier in the progression of colorectal carcinoma, as it is also downregulated in hyperplastic aberrant crypt foci and hyperplastic polyps [94], and microadenomas [95]. Systematic analyses from tissue samples corresponding to various stages of the evolution of prostate carcinoma led to similar conclusions, as decreased CEACAM1 expression was noted in prostatic hyperplasia [90]. In addition, this downregulation is often accompanied by a loss of cell polarity and adhesion, leading to profound changes in the architecture of the transformed tissues [12]. For instance, in endometrial and breast carcinomas, the apical localization of CEACAM1 changes to a non-specific cell-membrane one [87, 89], whereas another report revealed a conversion to a cytoplasmic localization for the latter [86].

The mechanisms leading to the downregulation of CEACAM1 in these malignancies are mostly unknown. Indeed, no mutations of the gene itself or its promoter have been reported; neither have deletions or chromosomal rearrangements in the region surrounding it [79], or modifications in the expression of the transcription factors regulating its basal level of transcription [30]. However, there are some indications that this loss involves transcriptional regulation. For instance, DNA methylation of its

promoter has been reported [79], and the Sp2 transcription factor, overexpressed in prostate carcinomas, represses CEACAM1 transcription through the recruitment of histone deacetylase to its promoter [35]. Finally, it was recently observed that activation of the protein kinase C (PKC) pathway with phorbol ester and calcium ionophore restored CEACAM1 expression in an endometrial carcinoma cell line through transcriptional regulation; hence, decreased PKC activity would lead to CEACAM1 downregulation [30].

Paradoxically, CEACAM1 is upregulated in gastric carcinomas [96]; primary non small cell lung carcinomas (NSCLCs) (e.g. adenocarcinomas and squamous cell carcinomas), as well as small cell lung carcinomas [97-99]; and malignant melanomas [100]. For all these latter malignancies, their healthy tissue counterparts did not express CEACAM1, or did so only at low levels. Moreover, for both NSCLCs and malignant melanoma, CEACAM1 expression indicates unfavourable outcomes for the patients. Indeed, for the former, it constitutes an independent prognostic factor associated with decreased overall disease-free survival [98, 99]. Similarly for the latter, the onset of metastases can be predicted more accurately from CEACAM1 expression in primary tumors than from their thickness [100]. Finally, high CEACAM1 expression has been identified in a number of benign and malignant lesions associated with abnormal trophoblast proliferation or invasion, termed gestational trophoblastic diseases (GTDs) [101, 102], and CEACAM1 positivity has been reported in acute lymphocytic leukemias [103, 104], as well as in 86% of multiple myelomas [105].

3. CEACAM1, a multifunctional protein

CEACAM1 is involved in several cellular processes, and has been ascribed six main functions so far: intercellular adhesion molecule, signal regulatory molecule, regulator of tumorigenesis, angiogenic factor, regulator of insulin and lipid metabolisms, and pathogen receptor. Moreover, based on recent developments, a seventh major function has emerged: that of a modulator of immune responses. As it will become apparent in the following sections, these functions are not mutually exclusive.

3.1 CEACAM1 serves as an intercellular adhesion molecule

This function, the first attributed to CEACAM1, was originally discovered in rat hepatocytes *in vitro* [106]. Since then, CEACAM1-mediated cell adhesion has been

identified in various cell types, as well as in humans [107] and mice [8, 108]. The mouse CEACAM2 protein is unable to mediate this function [19]. CEACAM1 interacts with other CEACAM1 molecules through homophilic interactions between their N-terminal CC' loops involving the Arg43 and Gln44 residues [43, 45]. Furthermore, various factors influence this function: the expression of the different isoforms, as CEACAM1-S is better at eliciting adhesion than CEACAM1-L [20]; caspase-3-mediated cleavage of the long tail, which enhances CEACAM1-L adhesiveness [109]; and the CEACAM1 *cis*-dimerization, as only monomeric CEACAM1 can mediate intercellular adhesion [110]. Indeed, CEACAM1-L and -S form mostly *cis*-homodimers (with the former more prone to dimerization) in equilibrium with their respective monomers on the cell surface [20, 111]. This is in turn regulated by the calcium-dependent association of calmodulin with both cyt domains [20, 111, 112], crosslinking by tissue transglutaminase of the tails of CEACAM1-L molecules [113], the expression levels and ratios of both isoforms [110], and possibly PKC-elicited phosphorylation of Ser/Thr residues in the calmodulin binding sites of their cyt domains [20].

Furthermore, CEACAM1 mediates this function at intercellular contacts; its occurrence and persistence at these sites involves targeting, *trans* homophilic interactions among CEACAM1 molecules, and their association with the cytoskeleton, which also implicates CEACAM1 in cellular migration [114, 115]. Indeed, interactions of CEACAM1-L and -S with the actin cytoskeleton and its associated proteins have been reported [114, 116-118]. Whereas the tyrosine (Tyr)-phosphorylated cyt tail of CEACAM1-L binds globular (G-) actin [117], paxillin [118] and tropomyosin [117], CEACAM1-S can bind directly the latter, G-actin, as well as fibrillar (F-) actin [117].

Hence, in CEACAM1-L-expressing Swiss 3T3 fibroblasts, CEACAM1-L targeted at cell-cell contacts becomes anchored to the cytoskeleton. This involves the activation of small RhoGTPases, with *trans* homophilic adhesion securing the interacting CEACAM1 molecules on the extracellular side [114]. A later study performed with epithelial MDCK cells revealed that signals from the small RhoGTPases Cdc42 and Rac1 in opposition to RhoA localized both isoforms to intercellular boundaries, ruffles and filopodia, using their transmembrane domain as a targeting cue [115]. Sundberg *et al.* reported that Tyr515 and Ser503 of the cyt tail of CEACAM1-L provided lateral targeting signals in

these cells, where it was localized in adherens junctions, and remotely disturbed the desmosomal organization [119]. Finally, the interaction of CEACAM1 with two other actin cytoskeleton-associated proteins, talin [120] and filamin A [121], revealed regulatory mechanisms of cell motility which involved small Rho and Ras GTPases, respectively.

Many developmental and biological processes rely on the intercellular adhesion properties of CEACAM1: the morphogenesis of the liver and intestinal epithelium during embryogenesis, odontogenesis, myogenesis, EVT invasion, CNS vascularization, angiogenesis, activation and extravasation of neutrophils, and the modulation of T and NK cell responses all involve homophilic interactions between CEACAM1 molecules [12]. In human, CEACAM1 is also capable of heterophilic interactions with CEA [122] (whose N-terminal domain also contains the Arg43 and Gln44 residues [45]). CEACAM1 also interacts with various cell surface components of viral and bacterial pathogens, as well as lectins, such as the dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) [52, 123], E-selectin [50], and galectin-3 [124], through their binding of its carbohydrate structures.

3.2 CEACAM1 serves as a signal regulatory molecule

Often, intercellular interactions involving opposing CEACAM1 molecules lead to signal transduction through their cyt domains. Indeed, key Tyr residues, critical for this function of CEACAM1, are solely present in isoforms bearing the long cyt domain. In mouse CEACAM1-L, there are two crucial phosphorylatable Tyr residues, Tyr488 and Tyr515; the counterparts of Tyr515 in rat and human isoforms are Tyr513, and Tyr516, respectively [20]. Hence, CEACAM1-L is a substrate of various protein Tyr kinases, notably the insulin receptor (IR) and epithelial growth factor receptor (EGFR) in hepatocytes [125], c-Src in epithelial cells [126], as well as c-Src, Lyn and Hck in activated neutrophils [127]. Tyr phosphorylation, especially of Tyr488, is crucial in enabling CEACAM1-L to interact with various proteins, in turn allowing it to mediate its diverse functions, as covered in greater depth in the next sections.

Moreover, each of these Tyr residues is part of an ITIM, a consensus V/I/L/SxYxxL/V/I sequence shared by many inhibitory receptors (x is any aa). Phosphorylation of Tyr residues within an ITIM leads to the recruitment of Src homology

2 (SH2)-domain containing phosphatases, which act as inhibitory downstream signalling molecules [15]. Thus, Tyr phosphorylation of CEACAM1 allows the docking of the SH2-domain containing phosphatases 1 and 2 (SHP-1 and -2), and intact Tyr488 and Tyr515 are needed for this [128, 129]. SHP-1 then conveys its inhibitory signals during tumorigenesis [128, 129], insulin metabolism [130], and immune responses [14, 78]. Another unique phosphorylation site of CEACAM1-L is Ser503, which is essential for its role in tumor suppression [131, 132], and in insulin metabolism [133]. These processes and signalling mechanisms will be revisited in greater depth in subsequent sections.

On the other hand, the signal regulatory functions of CEACAM1-S, which lacks these particular Tyr and Ser residues, have started to emerge. For instance, transfection of Jurkat T cells with CEACAM1-3S resulted in co-stimulation of these cells [64]. Although the mechanism for this remains to be uncovered, it might involve its phosphorylation, as CEACAM1-4S can nevertheless undergo Ser phosphorylation by PKC on Ser449 [64, 134]. Also, CEACAM1-4S associates with the β_1 integrins [59], and with annexin II [135] during breast morphogenesis.

3.2.1 CEACAM1 and contact inhibition of cell cycle progression

In vitro analyses demonstrated that the inhibitory effects of CEACAM1 on the proliferation of epithelial NBT-II bladder carcinoma cells involves its effect on $p27^{Kip1}$ levels, an important inhibitor of cyclin-dependent kinases controlling both the G1/S and G2/M checkpoints. Disruption of the intercellular interactions at cell-cell contacts by anti-CEACAM1 antibodies (Abs) induces the proliferation of "quiescent, confluent" cells by downregulating the expression of $p27^{Kip1}$, resulting in increased DNA synthesis, and thereby revealing the implication of contact inhibition in this phenomenon. Accordingly, "subconfluent, proliferating" cells exhibit reduced $p27^{Kip1}$ levels and enhanced DNA synthesis [66]. Further studies revealed that these modulations of $p27^{Kip1}$ levels are due to the activation or inhibition of the Erk1/2 MAPKs by CEACAM1, depending on both the state (as dictated by exposure to growth factors) and density of the cells [136]. Finally, studies performed with *Ceacam1*-/- mice showed that the absence of CEACAM1 enhances cell proliferation in their colonic crypts compared to those of wild type (WT)

mice due to a reduction in the levels of both $p27^{Kip1}$ and $p21^{Cip1}$, another cyclin-dependent kinase inhibitor [137].

3.2.2 Implication of CEACAM1 in apoptosis

Another facet of the signal regulatory role of this protein is its involvement in apoptosis. For instance, Nittka *et al.* reported that CEACAM1 appears to mediate the maintenance of "tissue homeostasis and maturation" in the colonic epithelium through the pro-apoptotic signals resulting from its crosslinking on the cell surface *in vitro*, although they did not define which isoforms were involved in this process [94]. These findings were recently corroborated in the *Ceacam1* knockout mouse model, whose colonic villi display reduced apoptosis compared to those of WT mice [137]. Similarly, CEACAM1-4S is implicated in mammary morphogenesis [138]. Its expression is predominant in the breast [86], where it promotes lumen formation by the MC7 breast carcinoma cell line *in vitro* through apoptosis of the central cells, thereby abolishing their cancerous phenotype. Its recently discovered interaction with and modulation of the cell surface levels of integrin β_1 has also been proposed to participate in this process [59]. Finally, CEACAM1-L can transduce anti-apoptotic signals, as *trans* homophilic interactions retard spontaneous and Fas ligand-induced apoptosis in rat granulocytes. Tyr phosphorylation of CEACAM1-L and its consequent recruitment of SHP-1 are involved, and lead to reduced caspase-3 activity through the activation of the mitogen-activated protein kinases (MAPK) Erk1/2 [139].

3.3 CEACAM1 serves as a regulator of tumorigenesis

3.3.1 Tumor suppressor function of CEACAM1

As described earlier, CEACAM1 expression is downregulated in a number of human malignancies, implying it may act as a tumor suppressor. Experimental systems, namely cell lines derived from these malignancies which restored CEACAM1 expression, and murine xenograft models [12], confirmed this function of human [140], rat [80, 81, 141] and mouse [79, 142, 143] CEACAM1. Furthermore, downregulation of CEACAM1 is more predominant in early colorectal tumors than mutations in the adenomatous polyposis coli (APC) tumor suppressor gene (whose sole inactivation can initiate the

chain of genetic modifications responsible for 80% of colorectal cancers). This suggests that CEACAM1 downregulation would occur prior to APC mutations, hence further supporting its tumor suppressor role [94]. Finally, recent *in vivo* confirmation was obtained through studies involving *Ceacam1*-/- mice, which did not spontaneously form colon tumors, but formed form larger and more numerous tumors than their WT counterparts upon treatment with the DNA-methylating carcinogen azoxymethane [137].

The importance of the long cyt domain of CEACAM1-L in mediating this function has emerged from these studies [141, 142, 144]. For instance, mouse colorectal carcinoma cells whose CEACAM1-L expression has been re-established form 80% less tumors after injection in syngeneic mice, a phenomenon which does not occur with cells transfected with CEACAM1-S [142]. Also, a physiological ratio of both CEACAM1-L and -S is required for this function in epithelial cells, with the inhibitory effect of the former predominating, although it can nevertheless be influenced by the latter [144]. This requirement for the long cyt domain was subsequently attributed to the Tyr488 residue located within the membrane-proximal ITIM [143], and Ser503 [131, 132], as their respective conversion to non-phosphorylatable Phe or Ala residues abolished this function [131, 143], probably through impaired recruitment of SHP-1, SHP-2 or a serine phosphatase [12]. Also, the extracellular domain of CEACAM1-L is not necessary for its tumor suppressor function, as mutants lacking the entire extracellular domain or only the N-terminal domain can still inhibit tumor cell growth *in vitro* and *in vivo* [143, 145].

Furthermore, apoptosis and contact inhibition of cell cycle progression constitute two other means through which CEACAM1 acts as a tumor suppressor. Indeed, for the former, diminished apoptosis was reported in human hyperplastic colorectal tumors whose CEACAM1 expression has been downregulated or lost [94]. For the latter, it has been suggested that since CEACAM1-L regulates cell proliferation by influencing the cell cycle, damaged epithelia displaying reduced or lost CEACAM1-L expression, hence deprived of the regulation mediated by contact inhibition, would then continue to proliferate even after repair, thereby setting the stage for tumorigenesis [136]. Finally, studies involving *Ceacam1*-/- mice revealed the significance of this *in vivo*, as enhanced proliferation accompanied by reduced levels of $p27^{Kip1}$ and $p21^{Cip1}$ were observed in colonic crypts of the CEACAM1-deficient mice compared to those of their WT

counterparts; this, as well as the aforementioned diminution of apoptosis observed in the colons of *Ceacam1*-/- mice, confirms the importance of CEACAM1 in suppressing the progression of colorectal tumorigenesis [137].

3.3.2 Implication of CEACAM1 in invasion

On the other hand, CEACAM1 is upregulated in some cancers: for malignant melanoma, this is most pronounced at the invasive front of primary tumors, where CEACAM1-L colocalizes and associates in *cis* with integrin β_3 [146]. The greater invasive and migratory capacities conferred by CEACAM1-L to melanoma cells through this association have been highlighted *in vitro*, as well as the essential phosphorylation of Tyr488 that enables binding of β_3 [147]. Similarly, CEACAM1-L colocalizes with β_3 in the invasive EVT at the maternal-fetal interface during normal implantation and placentation [146], and might be involved in the regulation of EVT invasiveness together with osteopontin (OPN), an ECM glycoprotein also highly expressed in the EVT and interacting with β_3 [148]. The potential importance of CEACAM1 for the proper unfolding of these processes is reflected by the fact that corticotropin-releasing hormone (CRH), secreted by both the trophoblasts and the decidua to favour embryonic implantation, can regulate EVT invasion by modulating CEACAM1 expression [37]. Finally, a complex comprising of CEACAM1, OPN, and β_3 might participate in the progression of malignant gestational trophoblastic diseases and of endometrial carcinoma, as CEACAM1 and OPN, colocalizing at the normal EVT and endometrium, display similar altered patterns of during the progression of these malignancies [102, 149].

3.4 CEACAM1 serves as an angiogenic factor

The implication of CEACAM1 in angiogenesis, "the sprouting of new blood vessels from pre-existing ones" [150], was first inferred from its presence in microvessels of the nascent rat CNS [61], as well as its restricted expression to small blood vessels of the endometrium, and placenta, both "regenerating and proliferative" tissues [70, 71]. Subsequently, CEACAM1 expression was found to be inducible by the vascular endothelial growth factor (VEGF), a crucial pro-angiogenic factor promoting endothelial cell (EC) proliferation, migration, and tube formation, three effects also exerted by CEACAM1 both *in vitro* and *in vivo*. Not only is CEACAM1 an angiogenic factor in its

own right, but it also potentiates the effects of VEGF, and represents an important effector for the latter in early angiogenesis [71, 150].

Recently, some insight into the mechanistic aspects of this process revealed that rat brain EC adhesion and motility mediated by CEACAM1-L involves the focal adhesion kinase (FAK), downstream opposing RhoA and Rac1 signals, as well as the interaction of CEACAM1-L with talin, which modulates integrin signalling; the interplay between these molecules as well as their effects on cell morphology and motility varies with the ECM context [120]. Also, experiments using the EC line SVEC4-10 have revealed that phosphorylation of Tyr488 and Ser503 of CEACAM1-L are important for EC motility and invasion, respectively [72]. Furthermore, studies involving *Ceacam1*-/- mice and the CEACAM1^{endo+} transgenic (Tg) mice specifically overexpressing CEACAM1-L in their ECs as a result of the insertion of its cDNA downstream of the Tie-2 receptor promoter (a crucial player in angiogenesis), confirmed the implication of CEACAM1-L in angiogenesis *in vivo*. These analyses revealed that the ablation of CEACAM1 prevented the invasion and formation of new blood vessels in aortic ring explant assays and following ligation of the femoral artery of *Ceacam1*-/- mice compared to WT counterparts. In contrast, this resulted in enhanced invasion and blood vessel generation in CEACAM1^{endo+} Tg mice compared to WT mice. The implication of *trans* CEACAM1 homophilic interactions in this process was also revealed by the ability of anti-CEACAM1 Abs to destabilize and prevent the formation of blood vessels by aortic ring explants originating from WT and CEACAM1^{endo+} Tg mice [72].

Finally, CEACAM1 is expressed in small blood vessels of human urinary bladder and prostatic carcinomas, RCC, and Leidig cell tumors [150], suggesting a role in tumor angiogenesis. For instance, the parallel downregulation in epithelial cells, and upregulation of CEACAM1 in neighbouring angiogenic blood vessels recently revealed its implication in a "switch" converting "non-invasive and nonvascularized" urinary bladder tumors to "invasive and vascularized" ones through the induction of VEGF-C and VEGF-D [92]. A similar effect has just been reported for prostate cancer [151]. Thus, the involvement of CEACAM1 in angiogenesis, which plays a critical role in tumorigenesis [152], represents another means through which it can mediate the latter.

3.5 CEACAM1 serves as a regulator of insulin and lipid metabolism

Another important function of CEACAM1 which involves its signal regulatory properties is the regulation of insulin sensitivity through insulin clearance in the liver, the main organ where this occurs [133]. Briefly, following binding of insulin to its receptor, the latter undergoes auto-phosphorylation on Tyr960 and Tyr1316 in its β-subunit. The IR then Tyr phosphorylates various substrates, among them CEACAM1-L [153]. *In vitro* studies revealed that CEACAM1-L undergoes phosphorylation on Tyr488 in rat hepatocytes [125], a process requiring prior phosphorylation of Ser503 by Ser/Thr kinases (irrespective of the presence of insulin) [133] and of Tyr1316 of the IR [154]. Once phosphorylated, CEACAM1-L partakes in the complex promoting the endocytosis of the insulin-bound IR in clathrin-coated vesicles, leading to insulin degradation in endosomes while the IR is subsequently recycled to the cell surface [153, 155]. Also, IR phosphorylation on Tyr960 is necessary for CEACAM1-L to promote its internalization [156], and the importance of SHP-1 in conveying CEACAM1 signalling in hepatic insulin clearance was recently reported [130].

The physiological importance of CEACAM1 in this process was uncovered through liver-specific Ser503Ala CEACAM1 (L-SACC1) Tg mice, essentially overexpressing a dominant-negative CEACAM1-L unable to sustain phosphorylation on Ser503. These mice exhibit impaired insulin clearance, leading to hyperinsulinemia, insulin resistance, and random hyperglycemia, as well as visceral adiposity accompanied by increased levels of circulating free fatty acids (FFAs) and triglycerides, with the latter also elevated in the liver [133]. Furthermore, recent studies revealed the interrelatedness between the regulation of insulin and lipid metabolisms by CEACAM1 in the liver [157, 158]. Indeed, the increased release of FFAs in the circulation associated with visceral obesity participates in insulin resistance, as normalization of plasma FFA levels without directly affecting receptor-mediated insulin endocytosis alleviated the hyperinsulinemic and hyperglycaemic phenotypes of L-SACC1 mice [157]. Moreover, CEACAM1-L also associates with and inhibits the activity of a major liver enzyme in the synthesis of FAs, fatty acid synthase (FAS), upon insulin-induced phosphorylation by the IR, as highlighted with *Ceacam1*-/- mice; these mice, displaying very similar metabolic anomalies, lose this insulin-based regulation of hepatic FAS [158].

These intertwined biological processes could be regarded as another mechanism through which CEACAM1 regulates tumorigenesis [159]. For instance, CEACAM1 downregulates both insulin- [160] and EGFR-mediated cell proliferation, with the latter being more frequently involved in advanced epithelial cancers [159]. Following Tyr488 phosphorylation by the IR or EGFR, CEACAM1-L can associate with and sequester their substrate Shc, thereby uncoupling the mitogenic Ras/MAPK pathway from these receptors by preventing Shc interactions with Grb2 [159, 160]. Finally, in L-SACC1 mice, not only does the abolition of CEACAM1-L phosphorylation lead to cell proliferation via this pathway by preventing Shc sequestration from the EGFR, but also by activating the EGFR by bypassing its need for EGF. The latter outcome is brought about by their hyperinsulinemic state, which causes visceral adiposity accompanied with enhanced release of FFAs and heparin-binding EGF-like growth factor (HB-EGF) from adipose tissues. HB-EGF can then induce EGFR signalling, leading to hepatocyte proliferation [159]. Thus, CEACAM1-L appears to be a link between obesity, insulin resistance and tumorigenesis through its effect on the EGFR [159].

3.6 CEACAM1 serves as a pathogen receptor

CEACAM1 serves as a receptor for various human-specific pathogenic [161] and commensal [162] bacterial species, as well as for MHV, with as recurring theme the exploitation of its cellular adhesion function to gain entry into the host's cells. For instance, a recombinant peptide corresponding to the CEACAM1-binding domain of an outer membrane protein (OMP) of *Moraxella catarrhalis* not only prevented adhesion and infection by this pathogen, but also that of *Neisseria gonorrhoeae*, *Neisseria meningitidis,* and *Haemophilus influenzae*, which use distinct, structurally unrelated proteins to bind common sites within its N-terminal domain [161]. Through their interaction with CEACAM1 these pathogens were also reported to promote the adhesion of infected cells *in vitro*, hence possibly preventing their exfoliation [163], an innate defence mechanism which limits the spread of the infection [164]. Finally, some of these pathogens use the signal transducing capacity of CEACAM1 to alter the host's immune responses.

3.6.1 Exploitation by Neisseria gonorrhoeae *and* Neisseria meningitidis

N. gonorrhoeae and the closely related *N. meningitidis* are two pathogenic bacteria which can solely colonize human genitourinary and nasopharyngeal mucosae, respectively, leading to gonorrhoea, a sexually transmitted disease for the former, and to meningitis in susceptible individuals for the latter. Often asymptomatic, these infections can lead to sterility in the case of *N. gonorrhoeae*, whereas *N. meningitidis* infection can become rapidly lethal if untreated [165, 166]. After an initial interaction involving neisserial type IV pili, the outer membrane colony opacity-associated (Opa) proteins allow tight adhesion of the bacterium and its subsequent uptake by host cells [167]. These adhesins are subdivided into two major classes. The first class is specific for heparan sulphate proteoglycan (HSPG)-harbouring receptors, such as Opa_{50} of gonococcal strain MS11. Opa proteins belonging to the second class (which comprises the majority of them) recognize CEACAMs, such as Opa_{52} of the same gonococcal strain [165].

CEACAM-binding Opa proteins display single or multiple specificity for the N-terminal domains of CEACAMs 1, 3, 5 (CEA), and/or 6, reportedly resulting in different mechanisms of internalization and host cell responses during *N. gonorrhoeae* infection, as the combinations of these receptors vary among different cell types [168]. The density of CEACAMs on the host's cells also comes into play during *N. meningitidis* infection [166]. Furthermore, CEACAM1 appears to be the receptor of choice, as 95% of Opa-expressing gonococci and meningococci recovered from samples from infected patient recognize its N-terminal domain [169], and that gonococci can enhance cell surface expression of CEACAM1 on epithelial and endothelial cells through the activation of transcription by NF-κB [33, 34].

Furthermore, *Neisseriae* expressing CEACAM-specific Opa proteins can corrupt the host's immune system through their interaction with CEACAM1. For instance, binding of Opa_{52}^{+} gonococci to CEACAM1-L on neutrophils leads to its Tyr phosphorylation, resulting in the activation of the Src-family kinases Hck and Fgr, thereby activating Rac1, its substrate p21-activated protein kinase (PAK), and culminating with Jun-N-terminal Kinase (JNK) stimulation, which promotes gonococcal internalization through cytoskeletal reorganization [170]. This is accompanied by

increased phosphorylation of SHP-1, hindering its inhibitory functions in this process [171]. Hence, *N. gonorrhoeae* seemingly uses the phagocytic machinery of neutrophils to colonize these cells and survive within them for subsequent transmission [170]. Also, the specific interaction of Opa_{52} with CEACAM1-L on the surface of helper T cells delivers inhibitory signals through its ITIMs, impeding their activation and proliferation [14], as described further in the next section. An inhibition of B cell functions has also been documented recently, as $OpaI^{+}$ gonococci specifically target human peripheral B cells expressing CEACAM1 and induce pro-apoptotic signals through the latter without involving its ITIMs, thereby preventing Ab secretion [172].

3.6.2 Exploitation by Haemophilus influenzae

H. influenzae, like *N. meningitidis* and *M. catarrhalis*, is a common bacterial commensal of the human nasopharyngeal mucosa, capable of provoking severe infections in susceptible hosts [161]. Indeed, capsulate/typeable *H. influenzae* strains harbouring a polysaccharide capsule can cause bacteraemia and meningitis in children [173], whereas the more common acapsulate/non-typable strains have been associated with about one third of childhood otitis media, recurring respiratory infections in patients suffering from cystic fibrosis and chronic obstructive pulmonary disease (COPD), purulent conjunctivis, and Brazilian purpuric fever [173, 174]. Both typeable and non-typeable strains bind the CC' loop of CEACAM1 [173] through the P5 variable OMP, but also through other yet-to-be defined ligands [175].

3.6.3 Exploitation by Moraxella catarrhalis

M. catarrhalis can be at the source of the same illnesses as non-typeable *H. influenzae*, and it is not uncommon to find both species at sites of infection [176]. Third prevailing cause of otitis media in children, it also causes recurring infections in patients with COPD, and sinusitis in children and adults [177]. This pathogen also interacts with the N-terminal domain of CEACAM1 through an OMP, the ubiquitous surface protein A1 (UspA1) [176], belonging to the Oca family of oligomeric coiled-coil adhesins [161]. The significance of this in host colonization is manifested by the specificity for this receptor displayed by all studied *M. catarrhalis* strains [176].

3.6.4 Exploitation by Escherichia coli *and* Salmonella typhimurium

In contrast, *E. coli*, and *S. typhimurium*, the latter causing intestinal infections ranging from gastroenteritis to typhoid fever if gone systemic [178], bind the high-mannose structures of CEACAM1 expressed on human neutrophils and intestinal epithelial cells via type 1 pili (fimbriae), which possess mannose-specific lectins [179, 180]. Similarly, they bind CEA and CEACAM6, with the difference that CEA is not expressed on neutrophils [67, 180]. Hence, it has been suggested that CEACAM1 and CEA, present on the apical glycocalyx of the intestinal epithelia and M cells would participate in the interactions with both the commensal flora and pathogens. Moreover, M cells are targeted and facilitate infection by the latter, as their function involves the phagocytosis of whole Ag (i.e. bacteria and food particles) to offer them to underlying lymphoid tissues, leading to tolerogenic or immune responses in the gut as deemed appropriate [2, 67]. Furthermore, virulent "uropathogenic and diarrhoeagenic" *E. coli* strains, more precisely the diffusely adhering *E. coli* (DAEC) strains expressing specific Afa/Dr adhesins, bind to CEACAMs 1, 6, and CEA, and induce their recruitment into lipid rafts around them, a process postulated to mediate signalling in the infected cells. Finally, whereas CEA and CEACAM6 can be found within lipid rafts of uninfected cells, CEACAM1 is only present in these structures following infection by this subset of Afa/Dr DAEC [181].

3.6.5 Exploitation by the mouse hepatitis virus

The mouse hepatitis virus (MHV) is a murine coronavirus causing enteric and respiratory infections in mice, as well as hepatitis, splenolysis, acute encephalitis, chronic demyelinating disease, and immune dysfunction [182]. Susceptibility to MHV infections, which range from being asymptomatic to severe and lethal [16], is conferred by the expression of *Ceacam1a* allelic variant [25]. Indeed, the majority of inbred laboratory mouse strains, such as the C57BL/6 strain, are homozygous for this allele and therefore susceptible, whereas mice of the SJL/J strain, homozygous for the *Ceacam1b* allele, are resistant [16, 22]. Outbred CD1 and wild mice, heterozygous for these alleles, can still be infected by this virus, and all MHV strains investigated use CEACAM1a as receptor [16].

This susceptibility is due to the high affinity of CEACAM1a for the 180 kDa spike (S) glycoprotein of MHV [183], which is responsible for its binding and fusing with host cell membranes [184]. More precisely, S binds to the projecting Ile41 residue of the CC' loop of the N-terminal domain of all four mouse isoforms of CEACAM1a [22, 43], and the Tyr162 residue of S from MHV strain A59 (MHV-A59) has recently been identified as crucial for hydrophobic interactions with CEACAM1a in this region [185]. The CEACAM1b variant differs from CEACAM1a by 27 aa in its N-terminal domain that encompass the CC' loop [22, 43], thereby decreasing its affinity for this pathogen, hence its protective effect [186]. CEACAM2 also displays significantly reduced MHV binding compared to CEACAM1a, and can serve as a receptor when overexpressed in MHV-resistant BHK cells [18]. However, CEACAM1a constitutes the sole receptor for this pathogen *in vivo*, as recently confirmed by the total resistance of *Ceacam1*-/- mice to infection by massive doses of MHV-A59 [187]. Finally, MHV can also use CEACAM1a to corrupt its host's immune response, as S can prevent the production of IFNγ by both differentiating and differentiated type 1 helper T (Th1) cells [188], which are involved in cell-mediated immune responses against virally infected cells [189].

3.6.6 Potential exploitation by the human cytomegalovirus

The majority of adults are believed to be carriers of the human cytomegalovirus (CMV), which has perfected several mechanisms to escape immune recognition, especially by NK and cytotoxic T cells, to live undetected in healthy hosts. Also, CMV is the main cause of "congenital viral infections" in the Western world, as it can infect foetuses via the maternal-fetal interface, and 1-2% of babies born each year in the United States are estimated to suffer from birth defects caused by infection during pregnancy [63, 190]. Based on the facts CMV-infected cells express a ligand specific for CEACAM1, and that decidual T and NK cells from infected pregnant women display enhanced expression of the latter, it has been suggested that CMV would use the inhibitory function of CEACAM1 in these cells (see below) as another means to avoid elimination [63].

3.7 CEACAM1 serves as a modulator of immune responses

3.7.1 CEACAM1 and innate immunity: Inhibition of natural killer cell responses

NK cells are innate immune cells whose main function consists in killing cells no longer expressing MHC class I molecules, such as virus-infected cells and tumor cells [191]. Following activation by IL-2, CEACAM1 is expressed on a subset of human NK cells expressing the CD56 marker common to all of them, but not CD16, one of the stimulatory receptors for their "natural cytotoxicity" [63, 77, 192, 193]. Moreover, these $CD16^{-}CD56^{+}$ NK cells predominate in the decidua basalis, where they represent 70-80% of its lymphocyte population, whereas their almost exclusively $CD16^{+}CD56^{+}$ counterparts in circulation account for 10% of lymphocytes found in peripheral blood [63, 77]. Studies regarding the expression and role of CEACAM1 in murine NK cells are currently lacking.

A co-inhibitory role has been ascribed to CEACAM1 in the functions of NK cells, as *in vitro* studies involving a MHC class I-deficient melanoma cell line revealed that *trans* homophilic interactions between CEACAM1 molecules on their surface and that of NK cells prevented their elimination in a mechanism of inhibition of cytotoxicity that does not involve MHC class I molecules, but rather the levels of CEACAM1 on both cell types [192]. Heterophilic interactions between CEA on melanoma cells and CEACAM1 on NK cells also inhibited their cytolytic action [194], and in both instances, the ITIM motifs contained within the cyt domain of CEACAM1-L were crucial for this [192, 194]. Additionally, these findings implicate CEACAM1 and CEA as hallmarks for tumor cells to evade immunosurveillance [45, 192, 194], a notion further supported by the overexpression of CEACAM1 on NK cells of patients afflicted with malignant melanoma compared to those of healthy individuals [192]. Moreover, the observation that CEACAM1 transfection of target cells suppressed their elimination by activated decidual NK cells in lysis assays, compared to untransfected cells, led to the proposal that homophilic interactions between decidual NK cells and the EVT would inhibit their cytotoxicity *in vivo*, thereby protecting the foetus from maternal rejection, as well as from the collateral damage caused by local immune responses during pregnancy [63].

Moreover, the negative regulation of NK cell responses by CEACAM1 is also involved in some immune diseases. Indeed, individuals lacking the transporter associated

with Ag processing 2 (TAP2) protein, responsible for loading peptides onto MHC class I molecules for Ag presentation, lose the cell-surface expression of latter, rendered unstable without bound peptides [195]. In late childhood, this genetic defect leads to respiratory insufficiency due to repeated respiratory tract infections and collateral damage to the lungs [195], but unexpectedly, NK cell-mediated autoimmunity rarely occurs [196, 197]. This is mostly attributed to the inhibitory role of CEACAM1, whose expression is enhanced on their NK cells compared to healthy subjects [196], as well as reduced serum levels of soluble CEACAM1 isoforms that normally block the required homophilic interactions for this inhibition in a dose-dependent fashion [197]. Finally, a recent study of patients with ankylosing spondylitis (AS), a debilitating inflammatory disease of the joints, revealed that their more numerous NK cells also expressed more CEACAM1 as a result of elevated levels of circulating IL-8 and stromal cell derived factor 1 (SDF-1). Here, the inhibited NK cells are hypothesized to participate in this disease through their inability to destroy autoimmune cells or pathogenic Ags believed to be at the source of this disease [193].

3.7.2 CEACAM1 and innate immunity: Stimulation of neutrophilic responses

Neutrophils, phagocytic cells of the innate immune system constituting the first line of defence against pathogens [52, 191], express a variety of CEA-family members in humans, namely CEACAMs 1, 3, 6, and 8, whose cell surface expression is enhanced following activation [46, 198, 199]. By constrast, only CEACAM1 is expressed in their rodent counterparts [46]. CEACAM1 is involved at various stages of the immune responses of these cells. For instance, it mediates heterophilic interactions with E-selectin on activated ECs during the initial rolling of circulating neutrophils [50, 51]. *In vitro* studies using Abs directed against CEACAM1 revealed that it promotes the subsequent tight adhesion of these cells to activated ECs through the integrin β2 also known as Mac-1 and CD11b/CD18 [52, 198]. CEACAM1 also interacts with ECM components which would be encountered after neutrophil extravasation to the site of pathogen invasion, such as fibronectin and laminin [51], in processes involving integrins β1, β2 and/or galectin-3 [51, 124]. Finally, the combination of anti-CEACAM1 Abs and prior stimulation with

formylated peptide (FMLP) induces neutrophils to produce more reactive oxygen species [200, 201], compounds they use to destroy phagocytosed invaders [51].

Furthermore, CEACAM1 intercellular homophilic adhesion are important in these responses, as synthetic peptides corresponding to its N-terminal domain concurrently increased the expression of Mac-1 and decreased that of L-selectin (CD62L) on the surface of neutrophils, thus increasing their adhesion to human umbilical vein ECs (HUVECs) [202]. CEACAM1-L signal regulatory functions are also involved in neutrophilic responses, since following neutrophil activation, CEACAM1-L undergoes Tyr phorphorylation, enabling it to interact with the protein Tyr kinases c-Src, Lyn, and Hck [126, 127], the latter being activated alongside Fgr during infection by *N. gonorrhoeae* [170]. Finally, ligation of the GPI-linked CEACAM8, believed to have replaced CEACAM1-S in human neutrophils during evolution, activates the Erk1/2 MAPKs in these cells through its association in *cis* with CEACAM1-L, much like *cis* dimers formed by rat CEACAM1-L and –S [46].

3.7.3 Bridging innate and acquired immunity: CEACAM1 and dendritic cell responses

One study has shed some light on the function of CEACAM1 in DCs, phagocytic innate immune cells which are the most powerful APCs inducing T cell responses [189, 191]. Indeed, both mature and immature mouse DCs express CEACAM1-L and –S *in vitro* and *in vivo* [76]. CEACAM1 engagement by means of anti-CEACAM1 Abs led to their maturation and activation, as revealed by their cell-surface upregulation of MHC class II molecules [76] required for Ag presentation to helper T cells [191], and that of CD40, CD54, CD80, and CD86 [76], which are T cell co-stimulatory molecules [191]. CEACAM1 ligation on DCs also promoted their secretion of the chemokines macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 2 (MIP2), and monocyte chemoattractant protein 1 (MCP-1), which consequently attracted T cells, monocytes, neutrophils, and immature DCs [76]; and that of the cytokines IL-6 and IL-12 [76], respectively co-stimulating T cells [189], and inducing Th1 differentiation [203]. These findings thus suggest that CEACAM1 engagement favours the "efficient polarization" of T cell responses to this type [76].

Furthermore, neutrophils can also provoke the maturation of DCs by the production of tumour necrosis factor α (TNF-α), and by binding DC-SIGN expressed on the surface of DCs, resulting in T cell activation and Th1 differentiation by DCs [52]. These interactions involve the Le^x glycans of Mac-1 and of CEACAM1, both among the rare proteins on the surface of neutrophils to harbour these structures [42, 52]. Also, both are upregulated following activation of neutrophils, allowing them to interact more tightly with DCs than when they are in their resting state [52]. Another group reported that CEACAM1 is the main ligand of DC-SIGN, and that prior addition of fucose sugars by fucosyltransferase IX to finalize the synthesis of Le^x moieties on CEACAM1 in human neutrophils is key for these interactions [123]. Thus, not only does CEACAM1 directly modulate the responses of DCs, which are pivotal in both innate and acquired immune responses [204], but it also serves as a link between DCs and neutrophils, thereby allowing the latter to influence T cell responses by affecting DC responses [52].

3.7.4 CEACAM1 and acquired immunity: Modulation of B cell responses

CEACAM1 is constitutively expressed on the surface of human and rodent B cells [46, 65, 74], with resting mouse B cells equally expressing CEACAM1-L and –S, a ratio increasing threefold towards CEACAM1-L upon activation with various stimuli [65]. Nevertheless, the role of CEACAM1 in these cells is not clear. Analyses involving mouse B cells showed that concurrent B cell receptor (BCR) and CEACAM1 engagement by means of Abs resulted in their proliferation, enhanced intercellular adhesion involving the lymphocyte function associated Ag 1 (LFA-1), as well as Ig production without switching to other Ig classes (or isotypes). This clearly co-stimulatory role presumably involved *trans* homophilic interactions between CEACAM1 molecules, and the activation of JNK [65]. However, in the DT40 B cell line, parallel engagement of the BCR and of a fusion protein comprised of the extracellular and transmembrane portions of the inhibitory receptor FcγRIIB and of the long tail of human CEACAM1-L revealed a co-inhibitory role for the latter. Indeed, the release of intracellular calcium ions mirroring the stimulation of protein Tyr kinases conveying downstream BCR activating signals was prevented in this system, and involved Tyr488 as well as SHP-1 and SHP-2 [205]. Further analyses will be needed to clarify the role of CEACAM1-L in B lymphocytes.

3.7.5 CEACAM1 and acquired immunity: Modulation of T cell responses

Before describing the impact of CEACAM1 on the responses of T cells, let us briefly go over the salient features of their anatomy and physiology. T lymphocytes (or simply T cells) are thus christened because they arise from bone marrow progenitor cells, and migrate to the thymus to complete their maturation. Most T cell receptors (TCR) are composed of an α and a β chain, each possessing a constant and a variable region; these αβ T cells recognize Ags in the form of derived peptides complexed with either MHC class I molecules, expressed on the surface of all nucleated cells, or MHC class II molecules, present only on the surface of professional APCs (i.e. macrophages, B cells, and DCs). A minority of T cells (1-10%) harbour a γδ TCR, and dispose of other means of Ag recognition. In mice, a good proportion of γδ T cells constitute intraepithelial lymphocytes of the skin, and of the intestinal and genitourinary tracts [206]. Finally, because the cyt tails of its constituent chains are very short, downstream TCR signals are conveyed during activation by the associated CD3 complex, comprised of CD3 molecules (γ, δ, and ε) and ζ chains, which harbour immunoreceptor Tyr-based activation motifs (ITAMs).

However, for T cells to be activated, and mount an immune response *in vivo,* additional co-stimulatory signals (intercellular interactions or cytokines) must be provided. For instance, the CD28 co-stimulatory receptor must interact with CD80 or CD86 molecules (also respectively called B7.1 and B7.2) present on the surface of APCs. Also, these interactions can be mimicked *in vitro* through the use of anti-CD3 and anti-CD28 Abs [207]: the former crosslink the TCR/CD3 complex, thereby providing a first activation signal, which can be potentiated by the latter as they engage CD28 co-stimulatory signalling, resulting in T cell activation, and subsequent proliferation. Other co-stimulatory receptors present on the surface of T cells include CD154 (also called CD40 ligand (CD40L)) and CD2, which interact with CD40 and CD58, respectively. Co-inhibitory receptors can also be engaged, such as the cytotoxic T lymphocyte Ag 4 (CTLA-4), which also interacts with CD80 and CD86.

Moreover, T cells can be subdivided into two subsets based on their function and harbouring of co-receptors, whose contribution is crucial for the TCR/CD3 complex. Cytotoxic T lymphocytes (CTLs), whose main function is to kill virus-infected cells,

express the CD8 co-receptor ($CD8^+$), and thus recognize peptide:MHC class I complexes. In contrast, helper T cells, helping with the progression of the immune response mainly by secreting cytokines, display the CD4 co-receptor ($CD4^+$), and recognize peptide:MHC class II complexes. They can be further subdivided into type 1 (Th1) and type 2 (Th2) based on cytokine production. Th1 cells secrete IL-2, which induces T cell proliferation following activation in autocrine and paracrine fashions, and IFNγ,. which activates macrophages, enhances MHC class I expression, and prevents viral spread by conferring resistance to neighbouring cells. This favours the progression of immune responses termed cell-mediated, as both CTLs (also producers of IL-2 and IFNγ) and macrophages are involved. Th2 cells secrete IL-4, -5, -6, and -10 (as well as IL-2), thereby helping B cells to secrete Abs and thus favouring humoral immune responses [189, 191, 203, 204, 208]. (For depictions of these key features and interactions, see Fig.9 and 10 in [189], and Fig. 3 in [208]).

As mentioned earlier, CEACAM1 is widely accepted as the only CEA family member expressed on both human and murine T cells following their activation, with resting cells displaying little or no expression of this molecule [53, 77, 78]. More precisely in human T cells, CEACAM1-3L, and -4L isoform (with the latter predominating) [46, 53] expression can be induced on $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, small intestinal intraepithelial lymphocytes (iIELs), and most decidual T cells by various stimuli *in vitro*, namely IL-2; IL-7 and IL-15, both highly secreted in the intestine; the mitogenic lectin [209] phytohemagglutinin (PHA); anti-CD3 Abs with or without additional co-stimulation with anti-CD28 Abs; and the phorbol ester PMA, which stimulates T cells through the activation of PKC [14, 40, 47, 53, 63, 210]. Moreover, the expression of CEACAM1-L on human T cells *in vivo* has also been reported on lamina propria lymphocytes (LPLs), as well as on activated small iIELs from patients suffering from inflammatory bowel disease (IBD), and active celiac disease, respectively [40, 210]. Finally, the kinetics of CEACAM1 expression are reciprocal to that of the IL-2 receptor (IL-2R). Whereas both molecules are hardly expressed on resting T cells, maximal IL-2R levels are achieved after 3 days of stimulation, and are reduced fourfold by the time CEACAM1 reaches its peak levels, i.e. after 7 to 9 days [47].

Early on, a controversy arose pertaining to the role of CEACAM1 in these cells, as two initial reports involving human T cells ascribed a co-stimulatory role to it. For instance, in *in vitro* assays in which an anti-CEACAM1 Ab was present during anti-CD3-mediated TCR/CD3 complex stimulation, T cell proliferation was significantly increased compared to that occurring with the latter alone, albeit to a lesser extent than that induced by co-stimulatory anti-CD28 Abs [53]. Another group reported that similar stimulation of T cell clones augmented their production of TNF-α and IFNγ, and further corroborated these observations by transfecting cells of the Jurkat T cell line with either human CEACAM1-4L or a truncated version deprived of most of its cyt tail and similar to CEACAM1-4S. Stimulation of the former with anti-CD3 Abs enhanced their IL-2 production compared to the latter, mirroring their increased activation. This effect was even more pronounced when both anti-CD3 and anti-CEACAM1 Abs were used, and seemingly arose from increased translocation of NF-κB to the nucleus following its binding to activating protein 1 (AP-1). Thus, a co-stimulatory function for CEACAM1-4L in T cells, for which its cyt tail is crucial, emerged [40].

At about the same time, other groups reported a co-inhibitory role for CEACAM1 in human T cells. The first evidence came from studies involving small iIELs, characterized by a $CD8\alpha\beta^{+}TCR\alpha\beta^{+}CD28^{-}$ phenotype. CEACAM1, initially identified as an activation Ag of these cells, could inhibit their cytotoxicity upon engagement with anti-CEACAM1 Abs in "redirected lysis" assays where these cells were activated with anti-CD3 Abs prior to coculture with P815 target cells. CEACAM1 also prevented their cytotoxic functions in this setting without anti-CD3 stimulation, thereby revealing its inhibitory function in the lymphokine-killer activity characteristic of these cells [210].

Subsequent supporting evidence was provided by a key report identifying CEACAM1 as a target receptor on helper T cells whose sole engagement allowed *N. gonorrhoeae* expressing Opa_{52} proteins, but not the HSPG-binding Opa_{50}, to inhibit their activation and proliferation. Indeed, ligation by Opa_{52}^{+} gonococci or anti-CEACAM1 Abs similarly reduced the expression of CD69, an early activation T cell marker; as well as the proliferation, in a dose-dependent manner, of peripheral blood $CD4^{+}$ T cells activated *in vitro* with combinations of anti-CD3, anti-CD28, and/or IL-2. Also, ligation by means of anti-CEACAM1 Abs inhibited the stimulation of helper T cells induced by

Opa_{50}^{+} gonococci. Finally, this reduced proliferation was not the product of apoptotic induction by *N. gonorrhoeae* or this Ab, but instead that of inhibitory signalling through the ITIMs of CEACAM1-L, as infection of anti-CD3-stimulated helper T cells by Opa_{52}^{+} gonococci resulted in pronounced SHP-1 and SHP-2 recruitment, nonexistent during similar infection with Opa_{50}^{+} gonococci [14].

Yet more evidence arose from studies involving human T cells of the decidua, which constitute only 10% of its immune cell population. Indeed, CEACAM1 homophilic interactions reduced the proliferation of $CD4^{+}$ decidual T cells stimulated with staphylococcal enterotoxin B (a superantigen acting by binding to MHC class II molecules, and TCRs harbouring specific β chain variable regions): coculture *in vitro* with 721.221 cells transfected with CEACAM1-L significantly decreased their proliferation compared to that with untransfected cells. This suggested that similar interactions between CEACAM1 on the EVT and activated T cells *in vivo* would inhibit fetal rejection by CTLs, as well as inhibit immune responses initiated by CMV infection during pregnancy. In this situation, CEACAM1 upregulation on T and NK cells is presumed to occur as an attempt from the maternal immune system to spare the foetus from their collateral damage [63]. Finally, anti-CEACAM1 Abs also greatly reduced the secretion but not the de novo synthesis of IFNγ by IL-2 activated decidual NKT cells, a subset of T cells displaying characteristics of both NK cells and $CD4^{+}$ T cells [189], and also upregulating CEACAM1 upon IL-2 activation [63].

In addition, two joint studies have recently refined these observations, and provided deeper insight regarding the mechanism underlying this inhibitory function. For instance, anti-CEACAM1 Abs maximally suppressed the proliferation and IL-2 secretion of human peripheral blood T cells when stimulated with a both anti-CD3 and anti-CD28 Abs. Engaging CEACAM1-L also caused its Tyr phosphorylation with consequent recruitment of SHP-1 to its ITIMs during their activation; increased association of the ζ-chain associated protein 70 (ZAP-70), an essential Tyr kinase conveying downstream activation signals from the TCR/CD3 complex and a substrate of SHP-1; as well as enhanced calmodulin binding, which prevents CEACAM1 association with the actin cytoskeleton, whose rearrangement is an integral part of T cell activation. Furthermore, this study hinted at the recruitment or occurrence of CEACAM1 within the TCR/CD3

complex, as it cocapped in lipid rafts with CD3, whose presence in these structures is a "hallmark of TCR engagement and T cell activation" [47].

The same study revealed the interweaving between the signal transduction pathways of CEACAM1 and of the IL-2R, first hinted at by their reciprocal kinetics of induction. Indeed, analyses of transfected Jurkat T cells, secreting but not requiring IL-2 to proliferate, showed that Ab-mediated CEACAM1-4L ligation significantly decreased the IL-2 production by cells transfected with this isoform upon anti-CD3 and anti-CD28 stimulation. However, CEACAM1-4S engagement did not alter IL-2 production, but inhibited more markedly their proliferative response than -4L. In contrast, transfection of Kit-225 T cells, producing and requiring IL-2 to proliferate, revealed that both CEACAM1-4L and -4S reduced the expression of the IL-2R β and γ subunits, with -4L associating with them and apparently colocalizing with them in lipid rafts, and decreasing Kit-225 T cell proliferation by six-fold, whereas -4S only slightly did so. Finally, the inhibitory effect of CEACAM1-4L on IL-2R signalling was revealed by reduced Tyr phosphorylation of the first effector in its signalling cascade, Janus-associated protein Tyr kinase 3 (Jak3), also subject to the action of SHP-1, which is postulated to be recruited to the TCR/CD3 complex by CEACAM1-4L [47].

Another study also examining the function of the human CEACAM1-3L and -3S isoforms ascribed co-inhibitory, and co-stimulatory roles to -3L, and -3S, respectively, with that of -3L prevailing, using Jurkat T cells co-transfected with either or both isoforms, as well as the nuclear factor of activated T cells (NFAT)-AP-1 elements of the IL-2 promoter linked to a luciferase reporter gene. Indeed, CEACAM1-3S was able to enhance the "spontaneous" luciferase activity occurring without anti-CD3- and anti-CD28-mediated activation in this model, with equimolar amounts of -3L reducing both this spontaneous activity and co-stimulation by -3S. CEACAM1-3L also inhibited luciferase activity during stimulation with both Abs, with or without -3S being present, and again decreased its co-stimulatory effect when both isoforms were found at equimolar amounts. CEACAM1-3S also potentiated the co-stimulatory effect of anti-CD28 Abs until reaching optimal levels during TCR/CD3 complex activation with anti-CD3 [64].

Delving into the mechanistic aspects of the co-inhibitory function of CEACAM1-3L through mutational analyses revealed that both ITIMs are required for inhibition, as

converting both of their Tyr residues to Ala abolished its inhibitory effect not only on spontaneous and anti-CD3- and anti-CD28-induced luciferase reporter activity, but also on associated IL-2 production. In addition, activation of Jurkat T cells transfected with CEACAM1-3L using the aforementioned Abs resulted in a reduction of both Th1 (IFNγ), and Th2 (IL-4) cytokines. This arose from the inhibition of MAPK pathways by CEACAM1-3L resulting in decreased phosphorylation of JNK, and Erk. When phosphorylated, these kinases are involved, respectively, in Th1, and Th2 differentiation, and accompanying cytokine production. Moreover, -3L requires both of its ITIMs, and the presence of SHP-1 to exert these inhibitory effects (again predominant over the opposite effects of exerted by -3S), as SHP-1 silencing via small interfering RNA molecules alleviated them. Finally, the impact of CEACAM1 homophilic interactions with a chimeric protein fusing the extracellular part of -3L to an Ig Fc fragment was illustrated by an inhibition of peripheral blood T cell proliferation almost as strong as that elicited by a similar CTLA-4 fusion protein [64].

Futhermore, CEACAM1 displays a similar expression pattern in mouse T lymphocytes, which express all four murine isoforms as revealed by RT-PCR and Western blot analyses [46, 78]. More precisely, flow cytometric analyses revealed that both murine splenic $CD4^+$ and $CD8^+$ T cells express CEACAM1 on their surface as early as 30 min following activation with either the mitogenic lectin [209] concanavalin A (Con A) or anti-CD3 Abs and that it persists for 72h, with maximal expression being reached after 12 to 24h [78]. Also, its kinetics of expression are almost identical to that of CD69. Its rapid upregulation on their surface upon activation arises from its export from intracellular reserves, where presynthesized molecules would be stored in resting T cells, as actinomycin D, cycloheximide, and brefeldin A, respectively inhibitors of mRNA synthesis, protein synthesis, and protein trafficking through the ER and Golgi apparatus, could not prevent its cell surface expression in this setting. Such a behaviour is reminiscent of that of CTLA-4, also brought to the T cell surface upon activation from intracellular stores, but after 72h [78].

Subsequent *in vitro* assays revealed an inhibitory role of CEACAM1 in mouse T cell functions. Indeed, ligation of CEACAM1 via Abs directed against it on splenic T cells during their stimulation with a combination of anti-CD3 and anti-CD28 Abs

decreased their proliferation compared to those exposed to an isotype-matched control under the same conditions. This anti-CEACAM1 Ab also had an inhibitory effect on an allogeneic mixed lymphocyte response (MLR) in which splenic cells from C57BL/6 mice were cocultured with and stimulated by inactivated splenic BALB/c mouse cells as the latter express different, "non-self" MHC molecules. In both instances, the extent of inhibition by this Ab occurred in a dose-dependent manner. Additionally, the potential implication of the long cyt domain of CEACAM1-L was revealed through glutathione-S-transferase (GST) pull-down assays involving fusion proteins composed of GST and of its long tail: in resting splenic T cells, its phosphorylation enabled SHP-1 binding, whereas its lack thereof allowed adaptor protein 1 to bind its recognition motif, hence presumably confining CEACAM1 inside these cells in a similar fashion than it does for CTLA-4, as adaptor protein 1 is part of clathrin-mediated endocytosis [78].

Subsequently, the function of CEACAM1 in mouse T cell functions *in vivo* was assessed through two models of inflammation, akin to human IBD, in which colitis was induced either with the "haptenating agents" trinitrobenzene sulfonic acid (TNBS) or with oxazolone (not immunogenic themselves but rendering self-proteins so by adding chemical groups to them [211]). This is a process governed by Th1, or by both Th1 and Th2 cytokines, respectively, secreted by intestinal LPLs, which also express CEACAM1 *in vivo*. In both models, treating these mice with anti-CEACAM1 Ab both before the initial skin application of either compound (the "sensitization phase") and before a second exposure to them through injection in the colon (the "effector phase"), or only before the latter, dramatically reduced the pathological consequences of this inflammation, i.e. weight loss, shorter and thicker colons whose walls were also infiltrated by immune cells, and ulcers [188]. Similar protection by this Ab had also previously been reported in a model of T-cell mediated delayed type hypersensitivity of the skin involving oxazolone [78].

Moreover, this specific inhibition of inflammatory damages by the anti-CEACAM1 Ab occurred through the specific inhibition of Th1 cytokine secretion, more precisely that of IFNγ, but not Th2 cytokine secretion. This was further confirmed by the addition of anti-IL-4 Abs alongside that of anti-CEACAM1 Abs, which enhanced the protective effect of the latter, as then the adverse effects of both Th2 and Th1 cytokines in

oxazolone-induced colitis were prevented. Furthermore, the potential importance of *trans* homophilic CEACAM1 interactions in this process *in vivo* was pinpointed when mice underwent prior treatment with a chimeric protein made of the extracellular domain of CEACAM1-4L fused to an Ig Fc fragment instead of the anti-CEACAM1 Ab, resulting in the same protective effect by reducing IFNγ production by LPLs [188].

Finally, a closer look into the mechanism leading to this reduced IFNγ production in these models revealed that it resulted from CEACAM1 decreasing the levels of the T-bet transcription factor involved in its production by Th1 cells, and did not result from an effect on Th1 differentiation (and ultimately their secretion of IFNγ) by affecting IL-12 signalling, as the levels of signal transducer and activator of Ag 4 (STAT-4), the transcription factor via which this DC-produced cytokine acts, remained untouched (Fig. 1-6) [188, 204, 212].

Therefore, the interplay between the intercellular adhesion and signal transducing properties of CEACAM1 appears to be important to mediate its function in both human and murine T cells, with the most recent evidence indicative of an inhibitory role.

4. Previous work performed in our laboratory

Although the above studies have provided considerable insight regarding the function of CEACAM1 in T cells of both human and murine origin, they nevertheless present some limitations. For instance, *in vitro* studies involving anti-CEACAM1 Abs cannot discriminate between a blockade of co-stimulatory signals or an actual engagement of co-inhibitory signals [78], and *in vivo* modulation of these responses can only be inferred through models involving pathological conditions, as revealed by the murine colitis models. Hence, we initially decided to use our *Ceacam1* knockout mouse model to bypass some of these limitations, as well as attempt to offer some insight which would resolve the controversy regarding its function in these cells. Additionnally, the use of this unique model would also allow us to gain some knowledge regarding the implication and/or the importance of CEACAM1 in the development of T cells.

Hence, the work of Jennifer Farrah, a previous student of our laboratory, revealed that CEACAM1 is not involved in the development nor in the migration of T cells to peripheral lymphoid organs, as enumeration of thymic (Fig. 1-7A-C) and splenic T cells

Figure 1-6: The differentiation of helper T cells into Th1 or Th2 types.

In the presence of IL-12, helper T cells called Th0 precursors differentiate into Th1 cells, whereas their exposure to IL-4 results in Th2 differentiation. Two transcription factors, T-bet and GATA3, respectively enable differentiated Th1 cells to synthesize IFNγ, and Th2 cells to produce IL-4.

Figure 1-6

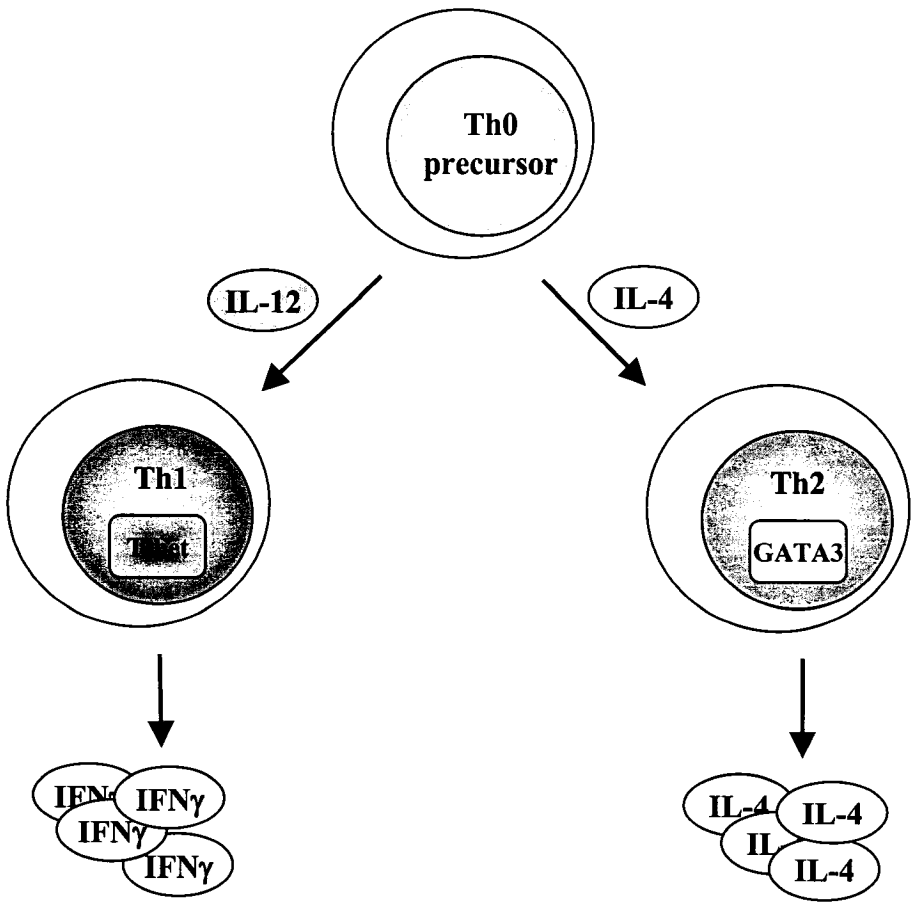

Th0
precursor
IL-12
IL-4
Th1
Th2
GATA3
IFNγ
IFNγ
IFNγ
IL-4
IL-4
IL-4

Figure 1-7: Enumeration of thymic and splenic T cells of WT and *Ceacam1*-/- mice.
(A-C) Thymi from 4- and 10- week-old wild type (WT) and *Ceacam1*-/- mice were disrupted into single cell suspensions, and lymphocytes were recovered and stained for various T cell markers. The percentage cell surface expression of each marker was determined by flow cytometry following the generation of two-color density plots, and is presented here as the mean values of 12 WT and 11 *Ceacam1*-/- 4-week-old mice, and 6 WT and 7 *Ceacam1*-/- 10-week-old mice. The error bars correspond to the standard deviation of the mean, and *, $P<0.05$. (A) Single positive expression of CD4, CD8, CD3, and CD69. (B) Double positive expression of CD4 and CD8 representing immature thymocytes. (C) Double positive expression of CD3 and CD69 representing the activation state of $CD3^+$ T cells. (D) Similar analyses were performed using splenic lymphocytes isolated from 10 week-old mice, using 9 WT and 11 *Ceacam1*-/- mice. Single positive expression of CD3, and CD4, are shown here, alongside that of IgM, a B cell marker, in order to assess if the abundance of B cells in the spleen was affected by the ablation of CEACAM1. Lymphocyte populations from the thymus (E) and spleen (F) of 10-week-old WT and *Ceacam1*-/- mice were stained with Annexin V to determine by flow cytometry whether the latter are more prone to apoptosis.
Adapted from the Masters' thesis of Jennifer Farrah, 2003.

Figure 1-7

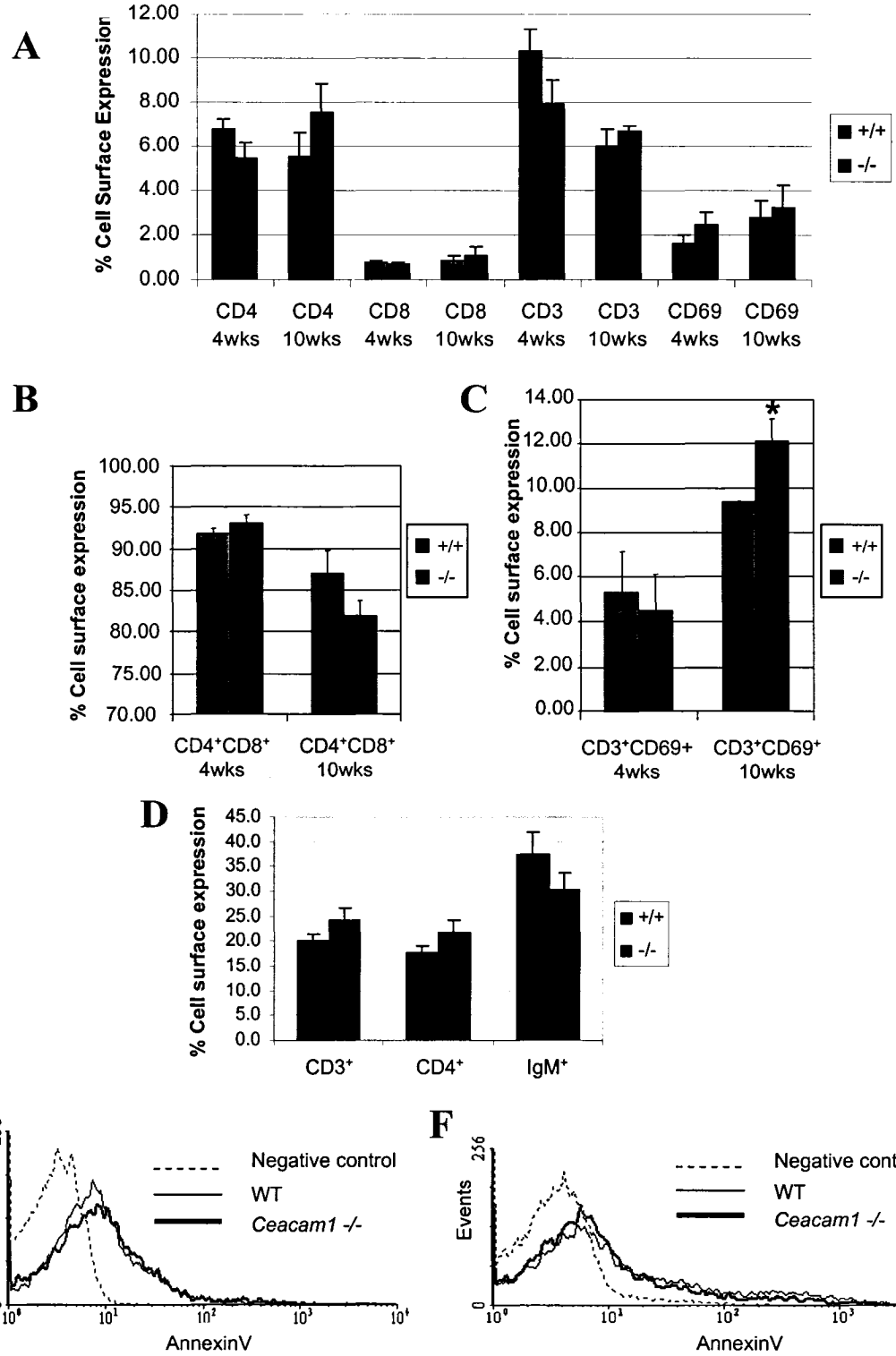

A
% Cell Surface Expression
12.00
10.00
8.00
6.00
4.00
2.00
0.00
CD4 4wks
CD4 10wks
CD8 4wks
CD8 10wks
CD3 4wks
CD3 10wks
CD69 4wks
CD69 10wks
+/+
-/-
B
% Cell surface expression
100.00
95.00
90.00
85.00
80.00
75.00
70.00
CD4+CD8+ 4wks
CD4+CD8+ 10wks
C
% Cell surface expression
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
*
CD3+CD69+ 4wks
CD3+CD69+ 10wks
D
% Cell surface expression
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
CD3+
CD4+
IgM+
E
Events
256
0
Negative control
WT
Ceacam1 -/-
AnnexinV
F
Events
256
0
Negative control
WT
Ceacam1 -/-
AnnexinV

Figure 1-8: *In vitro* proliferative response of splenic T cells from WT and *Ceacam1*-/- mice.

Purified T cells isolated from the spleens of 8-week-old WT and *Ceacam1*-/- littermates were stimulated with plate-bound anti-CD3 Abs (A), or ConA (B) for 48h, after which tritiated thymidine was added for an additional 16h-incubation to assess proliferation through its incorporation at the end of this period. Splenic T cells were also stimulated with plate-bound anti-CD3 in combination with anti-CD28 Abs (data not shown), which yielded similar profiles. The proliferation assays were done in triplicate, and the error bars correspond to the standard deviation of the mean. The results presented here are from a representative experiment, with averaged results from 9 WT and 11 *Ceacam1*-/- mice shown underneath. *, $P<0.05$; , $P<0.005$; *, $P<0.0005$; , $P<0.0001$.
Adapted from the Masters' thesis of Jennifer Farrah, 2003.

Table 1-2: Summary of the T and B cell markers studied and referred to in this work.

References:

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M. *Immunobiology: The immune system in health and disease.* 5th Edn. Garland Publishing, New York 2001.

Delves and Roitt, N. Engl. J. Med. **343**, 37 (2000)

Delves and Roitt, N. Engl. J. Med. **343**, 108 (2000)

Davidson *et al.* Immunity **21**, 707 (2004)

Kelly *et al.* Eur. J. Immunol. **23**, 3318 (1993)

McGargill *et al.* Nat. Immunol. **1**, 336 (2000)

Figure 1-8

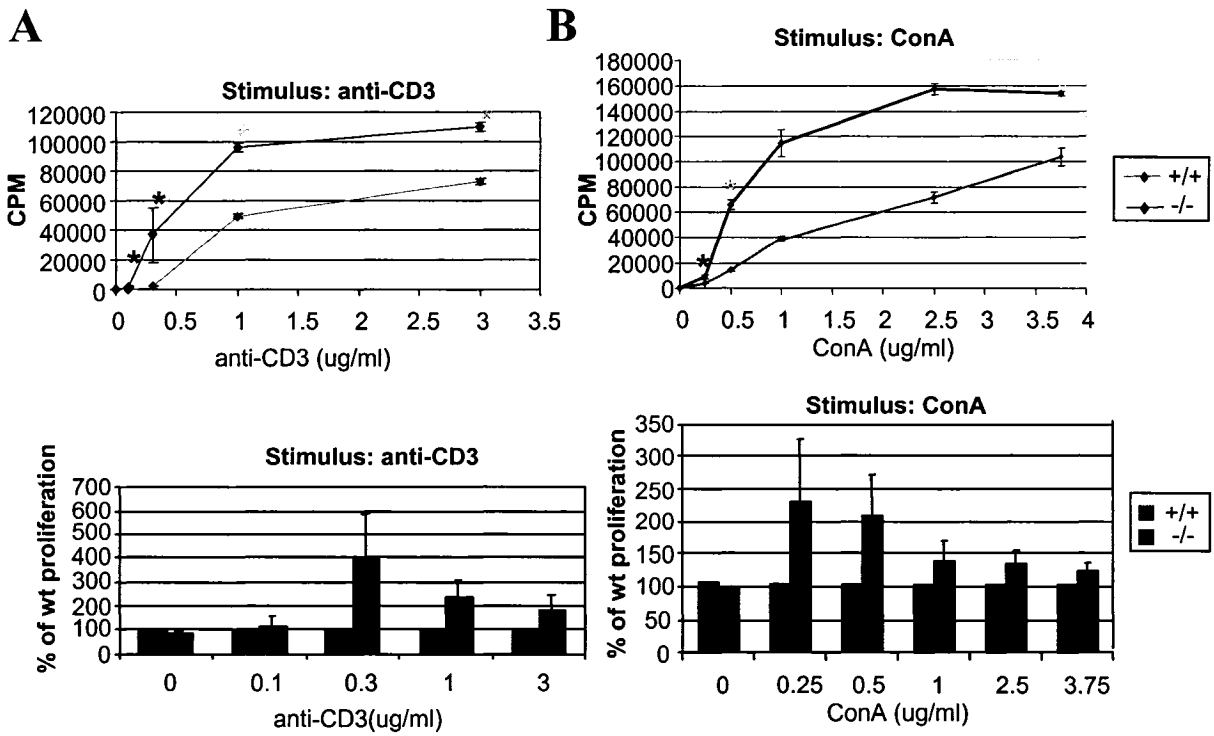


Table 1-2

Context	Marker	Description/Function
T cells	CD3	Part of the signalling complex associated with the TCR
	CD28	T cell costimulatory receptor
	CD4	Coreceptor of helper T cells; interacts with MHC class II molecules
	CD8	Coreceptor of cytotoxic T cells; interacts with MHC class I molecules
	CD69	Early activation marker.
	CD44	Adhesion molecule; highly expressed on memory T cells.
	CD62L	Also called L-selectin; highly expressed on naïve T cells.
	Vα2	TCR α chain expressed by OT-1 TCR Tg cytotoxic T cells
	Vβ5	TCR β chain expressed by OT-1 TCR Tg cytotoxic T cells
B cells	IgM	First Ig expressed and secreted
	IgD	Membrane-bound Ig expressed by mature naïve B cells

(Fig. 1-7D) from WT and *Ceacam1*-/- mice revealed no significant difference in the expression of the different T cell markers (summarized in Table 1-2). Furthermore, there were no differences in their apoptotic profiles (Fig. 1-7E, F). However, CEACAM1-devoid T cells are hyperproliferative compared to their WT counterparts when stimulated *in vitro* with anti-CD3 Abs with or without additional co-stimulation with anti-CD28 Abs, as well as Con A (Fig. 1-8). This behaviour was observed repeatedly with T cells from a great number of mice of both genotypes (at least 9 for each stimulus and genotype), and thus hinted at a co-inhibitory role of CEACAM1 in T cell functions.

Furthermore, to study the importance of the Tyr residues present in the ITIM motifs of CEACAM1-4L, and to try and provide an intracellular signalling mechanism for the behaviour of these T cells, J. Farrah generated a Tg mouse model, the CD2-CC1-L mouse, in which the CEACAM1-L cDNA is placed downstream of the human CD2 promoter. This would result in an overexpression of CEACAM1-4L in the thymus of these animals, and consequently on their T cells, which should be hypoproliferative compared to their WT counterparts according to her previous observations.

5. Objectives of this thesis

The objectives of this thesis are to deepen the knowledge already acquired by our group regarding the function of CEACAM1 in murine T cells using our unique *Ceacam1* knockout and Tg mouse models, more precisely at the developmental and functional levels. Hence, I looked at the effect of the deletion of CEACAM1 on the ratios of naïve and memory $CD4^+$ and $CD8^+$ T cells. I also studied the consequences of the ablation of CEACAM1 on CTL proliferation and cytokine production by means of a Tg model resulting from the mating of our *Ceacam1*-/- mouse with Tg mice expressing a transgenic TCR on their CTLs, which recognizes a specific peptide: MHC class I complex presented by APCs, threby constituting a more physiological model. Moreover, I characterized the CD2-CC1-L Tg mouse model generated by J. Farrah by confirming their expected phenotype, as well as by performing *in vitro* proliferation assays and quantifying the resulting production of cytokines. I also generated a dominant negative Tg mouse model similar to that generated by J. Farrah, with the difference that both Tyr residues contained within the ITIMs of CEACAM1-4L were mutated to Phe. Finally, the results presented

here are but a part of the work which will be published subsequently, and this project has been taken up by Anne-Marie Charbonneau-Allard, a new graduate student in our laboratory.

CHAPTER 2: MATERIALS AND METHODS

1. Mice

All experiments carried out used age- and gender-matched littermates of the C57BL/6 background (ages are specified in the appropriate experiments). *Ceacam1*-/- mice were described in [137, 187]. Two lines, termed 2D2 and 11H11, were produced, and animals of the 6th (2D2 line) and 7th (11H11 line) backcrosses were used for the experiments. OT-1 TCR transgenic mice on a C57BL/6 background were purchased from The Jackson Laboratory. OT-1:*Ceacam1*-/- mice were generated by first breeding OT-1 mice with *Ceacam1*+/- mice, and then breeding the OT-1:*Ceacam1*+/- progeny with *Ceacam1*-/- mice. The CD2-CC1-L and CD2-FF mice were generated by the McGill University Transgenic Core Facility by microinjecting their respective constructs into C57BL/6 oocytes. J. Farrah previously generated the construct for the CD2-CC1-L Tg mice, whereas I generated that for the CD2-FF mice, using a common strategy. Briefly, the WT CEACAM1-4L cDNA (for the former), or a version harbouring the Y488,515F mutation (for the latter) were excised with EcoRI (New England Biolabs) from the pLXSN vectors they had been previously cloned into, and inserted into the sole EcoRI site of the VAhCD2 vector (gift of Dr A. Veillette, Institut de Recherches Cliniques de Montréal). DNA prepared from selected positive clones, identified following BamHI (New England Biolabs) digestion, was sequenced at the Service de biologie moléculaire of the Institut de Recherches Cliniques de Montréal. The constructs were then freed from the Bluescript bacterial plasmid component with NotI and KpnI (New England Biolabs), and the resulting linearized constructs were used for microinjections as per standard operating procedures. Finally, all mice were housed in specific pathogen-free conditions at the McGill Animal Resources Center, and the procedures were approved by the McGill Animal Care Committee.

2. Isolation of mouse genomic DNA

Mouse genomic DNA was isolated from tail samples from 3-week-old pups either using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol (except that the DNA was eluted in a final volume of 85 µl of elution buffer); or the tail pieces were boiled in 50 mM NaOH for 15 min, neutralized with 1 M Tris pH, 8.0, and the supernatants containing the genomic DNA collected after centrifugation. DNA obtained

from the first method was exclusively used for Southern blotting, whereas DNA obtained by either method was used for PCR genotyping.

3. PCR genotyping of Ceacam1-/- mice

Genotyping of *Ceacam1*-/- mice was performed as described in [137] using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems), and oligonucleotides were synthesized by Integrated DNA Technologies. OT-1:*Ceacam1*-/- mice were also genotyped by this method to confirm that they were *Ceacam1*-/-.

4. PCR genotyping of OT-1 and OT-1:Ceacam1-/- TCR transgenic mice

The following protocol was adapted from the one developed by The Jackson Laboratory. A 300 bp region resulting from the presence of the transgene was amplified in 10 µl reactions containing: 1X complete Taq buffer containing 2.5 mM $MgCl_2$ (Bioron), 192 µM dNTPs, 0.03 U/µl Taq DNA polymerase (Bioron), and the primer pair oIMR0675 (5'-AAggTggAgAgAgACAAAggATTC-3') and oIMR0676 (5'-TTgAgAgCTgTCTTC-3') at a final concentration of 1.4 µM (Integrated DNA Technologies). The reaction conditions were: an initial denaturing step at 94°C for 3 min, followed by 30 cycles of the following: a denaturing step at 94°C for 30 sec, an annealing step at 52°C for 60 sec, and an elongation step at 72°C for 15 sec; and a final elongation step of 2 min at 72°C.

5. PCR genotyping of CD2-CC1-L and CD2-FF transgenic mice

I designed the following primers, and both were synthesized at the Sheldon Biotechnology Centre (McGill University): CyT2 (5'-CTGGCTCCTTCTGACAACTCTC-3'), annealing within the cyt domain of the CEACAM1-4L and CEACAM1-4L-Y488,515F cDNAs inserted in the VAhCD2 vector, and CD2A2 (5'-CAGGAGGGCAGAAATCCACAGT-3') which anneals within the VAhCD2 vector. The resulting 320 bp product was obtained following its amplification in 15 µl reactions composed of 3X Vent DNA polymerase buffer (New England Biolabs), 267 µM dNTPs, 0.04 U/µl Vent DNA polymerase (New England Biolabs), and this

primer pair at a final concentration of 1 ng/μl. The reaction conditions were: an initial denaturing step at 94°C for 3 min, followed by 30 cycles of the following: a denaturing step at 94°C for 40sec, an annealing step at 66°C for 30 sec, and an elongation step at 72°C for 30 sec; and a final elongation step of 10 min at 72°C.

6. Southern blot genotyping of CD2-CC1-L and CD2-FF transgenic mice

This method was developed by J. Farrah to genotype CD2-CC1-L Tg mice. Due to the similarity of the constructs used in their creation, it could also be used for CD2-FF mice. Briefly, this method was adapted from the one developed for the genotyping of *Ceacam1*-/- mice described in [187], with the major difference being that a 1.3 kb fragment served as a probe. This fragment resulted from the excision of the CEACAM1-4L cDNA from the 58-9 (SK) BgpD plasmid with a combination of EcoRI and StuI enzymes (the StuI site is located ~400 bp from the 3' end of this cDNA). Other modifications were: genomic DNA obtained from CD2-CC1-L and CD2-FF pups as described above was digested with EcoRI; and DNA was transferred to Hybond-XL membranes (GE Healthcare).

7. Antibodies and flow cytometric analyses

Flow cytometric analyses were performed using a FACScan (BD Biosciences); the Cell Quest program (BD Biosciences) was used for data acquisition, and the WinMDI program was used for the analyses. One microliter (stock concentrations ranged from 0.2 mg/ml to 0.5 mg/ml) of the following anti-mouse Abs, all from BD Biosciences, were used, except where noted otherwise: FITC-conjugated anti-CD3ε, anti-CD4, anti-CD8a, anti-IgM, anti-Vα2; PE-conjugated anti-CD4, anti-CD8a, anti-IgD, anti-CD44; biotinylated anti-CD3ε, anti-Vβ5.1,5.2, anti-CD69, anti-CD62L (0.5 μl) (see Table 1-2 for a description of these markers). Streptavidin-Quantum Red (Sigma) or Streptavidin-PE-Cy5 (BD Biosciences) were used as secondary Abs in reactions containing the biotinylated Abs (4 μl of 1/10 dilution for the latter). To detect the presence of CEACAM1, the polyclonal Ab 2457 (5^{th} bleed, generated in our laboratory; 5 μl) was

used in conjunction with a FITC-labelled goat anti-rabbit secondary Ab (Cappel), and normal rabbit serum (NRS; 5 μl) from the same bleed was used as negative control.

The staining protocol was the following: $1x10^6$ cells (either purified total or cytotoxic T cells; or originating from single cell suspensions from thymi, lymph nodes (LN) or spleens, with the latter depleted of red blood cells), were incubated in a 1/300 dilution (or 1/200 for CEACAM1 staining) of anti-CD16/CD32 Mouse BD FcBlock reagent (BD Biosciences; stock concentration: 0.5 mg/ml) in PBS containing 2% FBS (PBS-2% FBS) for 5 min on ice to prevent non-specific Ab binding by FcR prior to staining. After increasing the reaction volume to 50 μl, primary or biotinylated Abs were added for 25 min on ice. Then, the cells were washed with PBS-2% FBS and conjugated Abs were added for a 25 min incubation on ice in the dark. After a final wash, cells were resuspended in 500 μl PBS-2% FBS for analysis. The anti-CD44, anti-CD62L, anti-Vα2, and anti-Vβ5.1,5.2 Abs were kindly provided by Dr S. Fournier (Dept. of Microbiology and Immunology, McGill University).

8. Preparation and pulsing of antigen-presenting cells

APCs were obtained from the spleens of two OT-1 mice as follows: spleens were disrupted in complete RPMI-1640 medium (i.e. supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco), 50 μM 2-mercaptoethanol (Fisher Scientific)) by rubbing them in between the frosted ends of two presterilized microscope slides. The resulting single cell suspension was treated for 5 min with an ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH, 7.2-7.4) to lyse red blood cells. T cells were then depleted by first incubating the remaining cells for 25 min on ice with supernatants from the anti-CD4 hybridoma GK1.5 and anti-CD8 hybridoma 3.155 (gifts of Drs A. Veillette, and S. Fournier, respectively), followed by treatment with a 1/20 dilution of Low-Tox-M rabbit complement (Cedarlane Laboratories) in RPMI medium without serum for 45 min at 37°C. The remaining cells were then washed with complete RPMI medium, filtered over a 70 μm nylon cell strainer (Falcon), and irradiated at 3000 rad with a Gammacell 1000 γ-irradiator. T cell depletion was assessed by flow cytometry using an FITC-labelled anti-mouse CD3ε Ab (BD Biosciences). APCs were then pulsed for 2 hours at 37°C in 10-fold serial dilutions of

$OVA_{257-264}$ peptide (SIINFEKL; synthesized at the Sheldon Biotechnology Centre, McGill University) ranging from 10^{-4}M to 10^{-11}M, and washed twice with RPMI medium before plating (see below).

9. T cell proliferation assays involving OT-1 and OT-1:Ceacam1-/- transgenic mice

Mandibular, brachial, axillary, inguinal, iliac and posterior gastric LN were isolated from three OT-1 and three OT-1:*Ceacam1*-/- mice in each experiment, and reduced to single cell suspensions in complete RPMI medium. CTLs were then isolated by treatment with Lympholyte-M (Cedarlane Laboratories) followed by loading on $CD8^+$ T cell negative selection immunocolumns (Cedarlane Laboratories); or using the EasySep negative selection $CD8^+$ T cell enrichment cocktail (StemCell Technologies). Flow cytometric analyses of the obtained CTLs showed >85% purity. Then, $1x10^5$ APCs pulsed with each of the different $OVA_{257-264}$ dilutions (see above) were plated per well, in triplicate, in flat-bottom 96-well plates (Nunc), and co-cultured with the same number of purified OT-1 or OT-1:*Ceacam1*-/- $CD8^+$ T cells in complete RPMI medium. As controls for baseline proliferation, triplicates containing $1x10^5$ $CD8^+$ T cells, $1x10^5$ unpulsed irradiated APCs, or both, were included on a separate plate to avoid contaminating T cells, sensitive for $OVA_{257-264}$. After 48h, 75 µl from each well of the triplicates were removed and pooled for subsequent analysis of cytokine profiles by ELISA. The wells were replenished with the same volume of medium. Then, 0.5 µCi of tritiated thymidine (PerkinElmer) in RPMI medium were added per well. Sixteen hours later, the cells were harvested with a FilterMate Harvester onto a UniFilter GF/C-96 plate, and thymidine incorporation was determined with a TopCount NXT liquid scintillation counter (all from PerkinElmer).

10. In vitro *T cell proliferation assays*

Single cell suspensions obtained from the spleens of WT and CD2-CC1-L Tg mice, and T cells were recovered after treatment either: with Lympholyte-M (Cedarlane Laboratories), and then loading on negative selection T cell immunocolumns (Cedarlane Laboratories); or with ACK buffer prior to purification with the EasySep negative selection T cell enrichment cocktail (Stem Cell Technologies). Preparations obtained

from the first method showed >75 % purity, whereas the second method yielded >85% purity as determined by flow cytometry. Then, $5x10^4$ T cells/well were plated in triplicate in flat-bottom 96-well plates (Nunc) in the presence of 0, 0.1, 0.3, and 3 μg/ml of anti-CD3ε Ab (2C11 clone, prepared in our lab or purchased from eBiosciences), previously coated in the wells according to standard procedures. This stimulation was done with or without the addition of soluble anti-CD28 Ab at a concentration of 1μg/ml (37.51 clone, prepared in our lab or purchased from eBiosciences). Other stimuli included Con A (Sigma) at concentrations of 0, 0.25, 0.5, and 2.5 μg/ml; and 100 ng/ml PMA plus 1 μM ionomycin (Sigma). Again, all cultures were done in complete RPMI medium. To assess proliferation, 1 μCi/well of tritiated thymidine was added after 48h of stimulation, and after 16h, incorporation was determined as mentioned above. Supernatants were collected from separate plates after 96h of stimulation for subsequent determinations of cytokine production by ELISA.

11. ELISAs

Cell culture supernatants collected at various time points were analysed for IL-2, IL-4 or IFNγ production using ELISA module sets (Bender Medsystems) as detailed in their accompanying protocols. Assays were performed in duplicate using flat-bottom 96-well Easy Wash EIA/RIA plates (Corning). In addition, the recommended tetramethylbenzidine (TMB) microwell peroxidase substrate system (KPL) was used for color development according to the manufacturer's instructions, and the reactions stopped with 1M H_3PO_4. Absorbances were determined with a Microplate Reader 3550 (Bio-Rad) at the recommended wavelength of 450nm. Data was acquired using the Microplate Manager software (Bio-Rad).

12. Western blots

Thymi and spleens from 5- and 8-week-old age-matched WT and CD2-CC1-L littermates aged were removed, immediately frozen on dry ice, and subsequently reduced to a fine powder with a mortar and pestle. The crushed organs were then resuspended in 400 μl lysis buffer (50 mM Tris pH, 8, 1% Nonidet P-40, 5 mM EDTA, 100 mM NaCl,

0.4 mM Na_2HPO_4, and complete inhibitors (Roche)). Following a 5-10 min incubation period on ice, samples were centrifuged for 10 min at 13 000 rpm at 4°C, and supernatants were collected. Protein concentration was then determined using the Bio-Rad protein assay, and 50 μg of proteins were loaded onto denaturing 10% polyacrylamide gels for SDS-PAGE. Following transfer to Immobilon-P membranes (Millipore), the presence of CEACAM1 was detected with the polyclonal 2457 anti-CEACAM1 Ab (1/2000 dilution). An anti-actin Ab (1/1000 dilution; Sigma) was used as a control for equal loading. Following the addition of a horseradish peroxidase-conjugated anti-rabbit secondary Ab (1/2000 dilution; GE Healthcare), proteins were visualized by enhanced chemiluminescence using the ECL detection system (GE Healthcare). Blocking and Ab dilutions were made in 5% non-fat milk in TBS-0.1% Tween.

13. Statistical analyses

Statistical significance was determined using the Student's t test computed with the Microsoft Excel software. The minimal condition for statistical significance was set at $P<0.05$.

CHAPTER 3: RESULTS

1. Naïve and memory T cell populations in wild-type and Ceacam1-/- mice

Previous analyses of WT and *Ceacam1*-/- T cell populations have revealed that CEACAM1 does not affect the development of T cells, nor their migration to peripheral lymphoid organs. We then decided to further investigate the impact of the ablation of CEACAM1 by examining the abundance of naïve and memory T cells in different organs. Memory T cells arise as one of the outcomes following the activation and proliferation of naïve T cells: upon recognizing the peptide:MHC complex for which their TCR is specific on the surface of an APC, naive T cells become either effector T cells possessing a short lifespan, or memory T cells with a long lifespan and able to respond rapidly and more efficiently should that Ag manifest itself again [203]. Hence, spleens and LN from 8-week-old WT and *Ceacam1*-/- mice were reduced to single cell suspensions, stained with a combination of anti-CD4, anti-CD44 and anti-CD62L Abs for helper T cell populations; or anti-CD8, anti-CD44 and anti-CD62L for cytotoxic T cell populations. During the acquisition, we first gated on live cells and the $CD4^+$ or $CD8^+$ populations. Then, two-color dot plots for CD44 and CD62L were generated, allowing for the identification of naïve T cells, which display a $CD44^{lo}CD62L^{hi}$ phenotype, and memory T cells, which possess a $CD44^{hi}CD62L^{lo}$ phenotype [213].

From the representative profile shown here, no significant differences between the naïve and memory $CD4^+$ T cell populations from the LN (Fig. 3-1A) or the spleen (Fig. 3-1B) of WT and *Ceacam1*-/- mice were noticed. This was also the case for $CD8^+$ T cells, whether originating from the LN (Fig. 3-1C) or the spleen (Fig. 3-1D) of these animals. This experiment was performed once with two pairs of mice, and in all instances, $P>0.05$ (Table 3-1). In addition, from Table 3-1, it can be seen that irrespective of their origin or genotype, as expected, more $CD4^+$ memory T cells were present compared to $CD8^+$ memory T cells. Another interesting point to note is that, irrespective of genotype, more memory T cells were present in the spleen than the LN. This could be observed for both the $CD4^+$ and $CD8^+$ T cell subsets (Dr S. Fournier, personal communication). Thus, CEACAM1-deficiency does not seem to affect the amounts of naïve and memory T cells from the $CD4^+$ and $CD8^+$ T lymphocyte subsets present in the LN and the spleen of *Ceacam1*-/- mice.

Figure 3-1: Comparison of naïve and memory T cell populations in WT and *Ceacam1*-/- mice.

Lymph nodes and spleens from 8-week-old WT and *Ceacam1*-/- siblings were reduced to single cell suspensions, and triple-stained with anti-CD44, anti-CD62L, and either anti-CD4 or anti-CD8 to identify helper or cytotoxic T cells, respectively, by gating during flow cytometric analysis. Two-color dot plots were then generated for CD44 and CD62L to identify populations of naïve ($CD44^{lo}CD62L^{hi}$; black) and memory ($CD44^{hi}CD62L^{lo}$; red) $CD4^+$ T cells present in the LN (A) and spleen (B). Similar analyses revealed the naïve and memory $CD8^+$ T cells populations of the LN and spleen, shown in (C) and (D), respectively. This experiment was performed once with 2 WT and 2 *Ceacam1*-/- mice, and a representative profile is shown here.

Table 3-1: Distribution of naïve and memory $CD4^+$ and $CD8^+$ T cell populations present in the lymph nodes and spleen of WT and *Ceacam1*-/- mice.

Compiled data obtained from the two-color dot plots for the CD44 and CD62L markers generated as described in Fig. 3-1, from 2 WT and 2 *Ceacam1*-/- littermates, shown as mean values accompanied by their standard deviations.

Figure 3-1

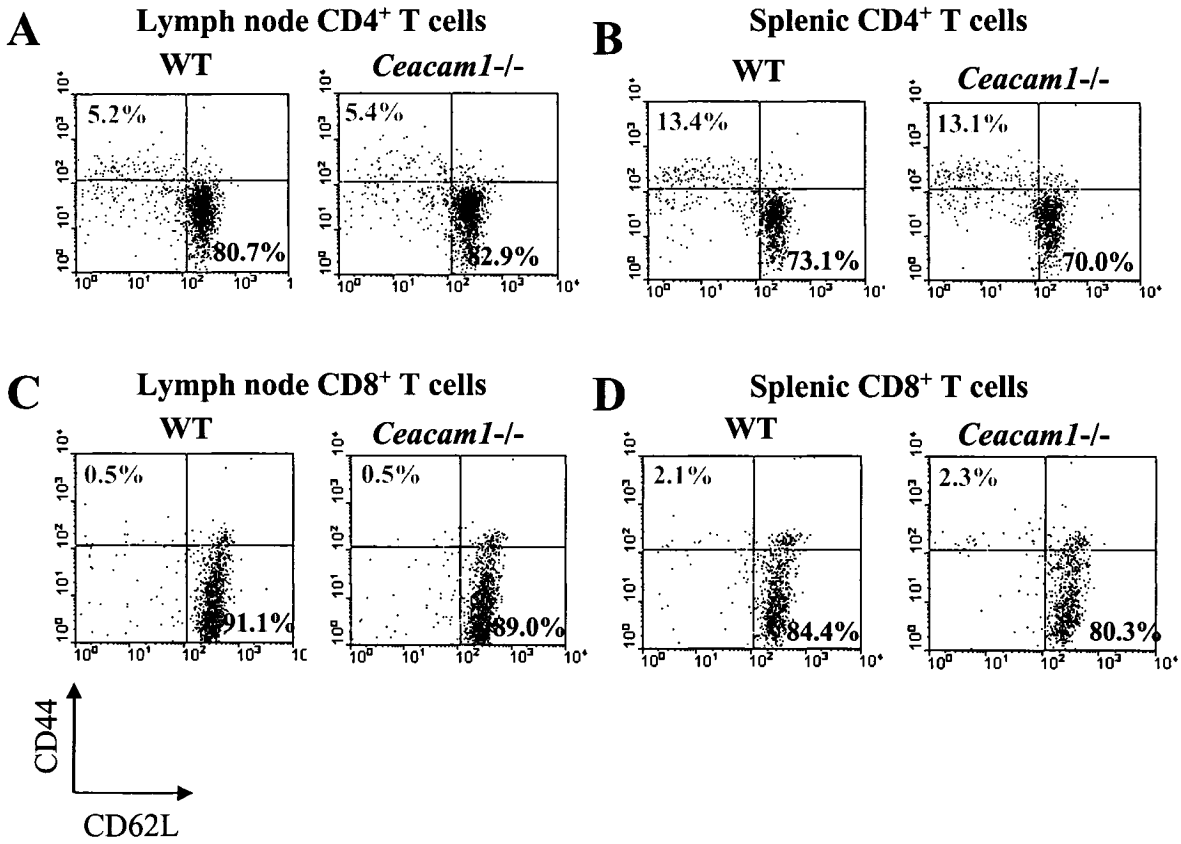


Table 3-1

T cell subset	Origin	Naïve T cells (%)		P value	Memory T cells (%)		P value
		WT	*Ceacam1 -/-*		WT	*Ceacam1 -/-*	
$CD4^+$	LN	78.8 ± 2.7	82.2 ± 0.9	0.15	7.1 ± 2.8	6.1 ± 0.9	0.34
	Spleen	69.8 ± 4.6	70.5 ± 0.7	0.44	16.0 ± 3.8	14.7 ± 2.2	0.36
$CD8^+$	LN	88.4 ± 3.7	89.1 ± 0.2	0.42	0.61 ± 0.16	0.59 ± 0.16	0.46
	Spleen	84.7 ± 0.5	82.4 ± 3.0	0.24	2.3 ± 0.3	2.8 ± 0.6	0.24

2. Assessment of the role of CEACAM1 in cytotoxic T cell functions

Our laboratory previously observed that CEACAM1-deficient T cells are hyperproliferative compared to their WT counterparts when stimulated *in vitro* with Abs against the TCR/CD3 complex with or without co-stimulation with anti-CD28 Abs, or with Con A. We then chose to further confirm these observations by using a more physiologically relevant model involving Ag presentation, and at the same time dissect the effect of CEACAM1-deficiency on the CTL subset. To achieve this, *Ceacam1*-/- mice were bred with OT-1 TCR transgenic mice. Almost all CTLs from these mice express a transgenic TCR that is specific for complexes formed when the chicken ovalbumin-derived peptide containing amino acids 257-264 ($OVA_{257-264}$; SIINFEKL) is bound to the H-$2K^b$ MHC class I molecule of APCs [214, 215]. Thus, CTLs from the resulting OT-1:*Ceacam1*-/- mice express the TCR specific for this peptide:MHC class I complex but are devoid of CEACAM1 on their surfaces.

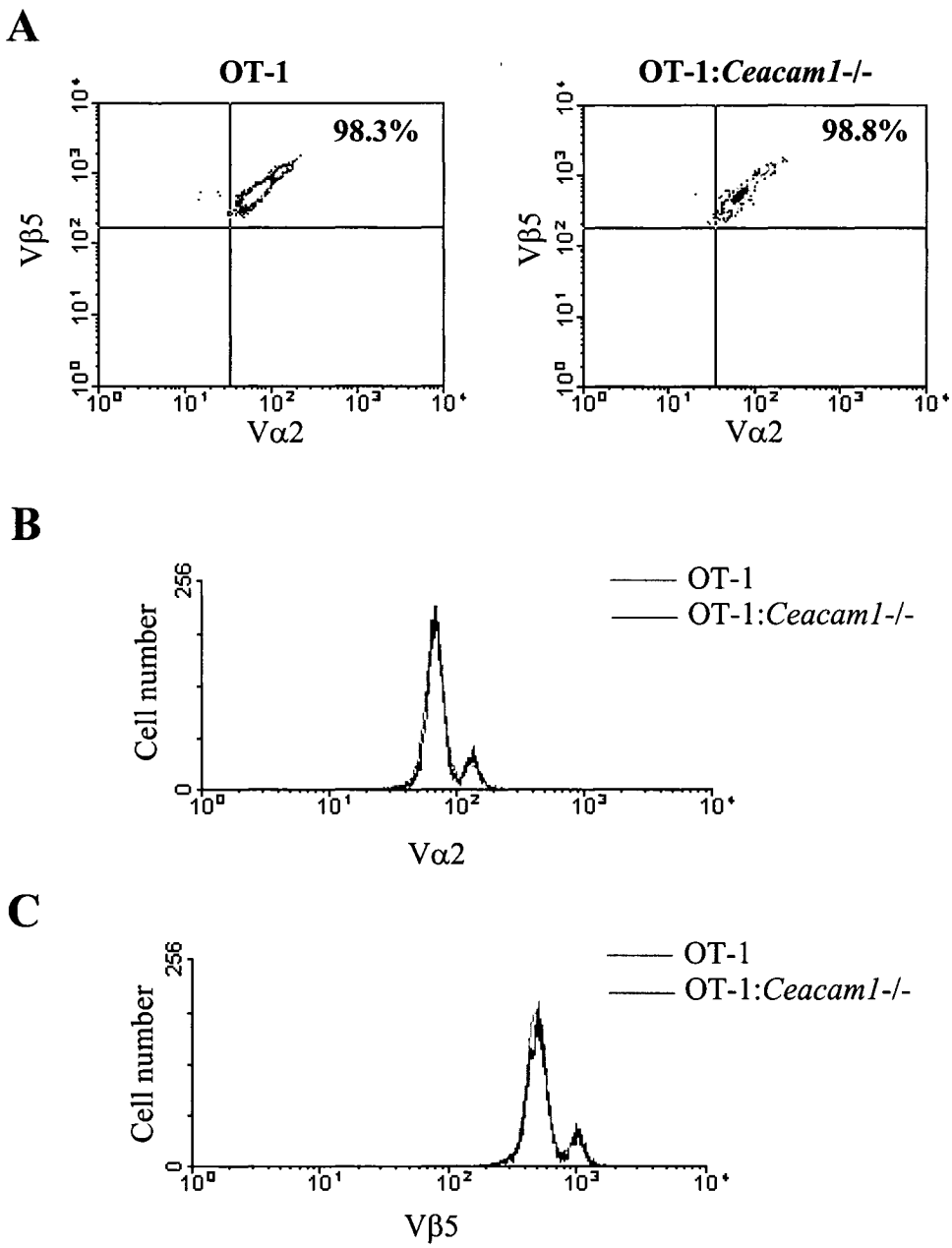
The transgenic TCR expressed by OT-1 CTLs is composed of an α chain and a β chain termed Vα2 and Vβ5, respectively [215, 216]. In order to determine if breeding of OT-1 mice with *Ceacam1*-/- mice affected the expression of this receptor, flow cytometric analyses were performed on purified CTLs for the expression of these two molecules by triple-staining with a combination of anti-CD8, anti-Vα2, and anti-Vβ5.1, 5.2 Abs. After gating on live $CD8^+$ T cells, two-color density plots were generated (Fig. 3-2A), revealing that 98.3% of OT-1, and 98.8% of OT-1:*Ceacam1*-/- CTLs expressed the transgenic TCR, as expected. Moreover, histograms were generated from the gated $CD8^+$ populations to examine the expression profiles of the Vα2 (Fig. 3-2B) and Vβ5 (Fig. 3-2C) chains more closely. Since no major differences were observed, it can be concluded that breeding with *Ceacam1*-/- mice did not alter the expression of the transgenic TCR in OT-1:*Ceacam1*-/- mice.

Since OT-1 and OT-1:*Ceacam1*-/- CTLs did not differ in their expression of the $V\alpha2V\beta5^+$ TCR, the effect of CEACAM1-deficiency on their proliferation and cytokine production could now be studied. Briefly, $CD8^+$ T cells were purified from LN of 8- to 10-week-old OT-1 and OT-1:*Ceacam1*-/- mice. After confirmation by flow cytometry

Figure 3-2: Expression of the Vα2Vβ5$^+$ transgenic TCR in OT-1 and OT-1: *Ceacam1*-/- cytotoxic T cells

(A) Purified CD8$^+$ T cells isolated from the LN of OT-1 and OT-1:*Ceacam1*-/- mice were subjected to triple-staining with anti-CD8, anti-Vα2, and anti-Vβ5.1, 5.2 Abs for flow cytometric analyses. After gating on viable CD8$^+$ cells, two-color density plots for the Vα2 and Vβ5 chains of the transgenic OT-1 TCR were obtained. The corresponding histograms for the expression of the Vα2 and Vβ5 chains are shown in (B), and (C), respectively. A representative profile of six is shown here.

Figure 3-2


A
OT-1
98.3%
OT-1:Ceacam1-/-
98.8%
Vβ5
Vα2
B
Cell number
256
0
OT-1
OT-1:Ceacam1-/-
Vα2
C
Cell number
256
0
OT-1
OT-1:Ceacam1-/-
Vβ5

that the obtained CTLs did not differ in their purity (anti-CD4/anti-CD8 double-staining), activation state (anti-CD3/anti-CD69 double-staining) and residual B cell content (anti-IgM/anti-IgD double-staining), they were cultured for 48h with gamma-irradiated APCs pulsed with concentrations of $OVA_{257-264}$ ranging from 10^{-4}M to 10^{-11}M. APCs (B cells and macrophages [217]) were obtained from single cell suspensions of spleens collected from OT-1 mice by red blood cell lysis followed by complement-mediated elimination of T cells bound by anti-CD4 and anti-CD8 Abs. T cell depletion was assessed by flow cytometry by comparing CD3 expression before and after this treatment. Spleens from these animals were used as the source of APCs, for only their T cells are Tg and that their APCs express WT CEACAM1 protein. In addition, APCs were irradiated to prevent their proliferation, as confirmed by the inclusion of controls where only unpulsed irradiated APCs were plated, as well as CTLs plated with or without unpulsed irradiated APCs.

The effect of deleting CEACAM1 on the proliferation of these cells was determined by tritiated thymidine incorporation following an additional incubation of 16h with this compound. As it can be seen from Fig. 3-3A, there was a dose-dependent response of CTLs of both genotypes, with the cells proliferating the most when co-cultured with APCs pulsed with 10^{-4}M of $OVA_{257-264}$. Even at very low concentrations of $OVA_{257-264}$ (10^{-10} and 10^{-11}M), there was a significant response, which was greater than the background counts provided by the controls (data not shown). This also revealed the sensitivity of these transgenic T cells for this peptide:MHC class I complex [218]. Most importantly, OT-1:*Ceacam1*-/- CTLs were hyperproliferative compared to their OT-1 counterparts at all peptide pulse concentrations. In the representative experiment of seven shown here, this increase in proliferation was of the order of 1.6 to 2-fold (Fig. 3-3A; $P<0.05$). Thus, CEACAM1 seems to exert an inhibitory role on the proliferation of CTLs.

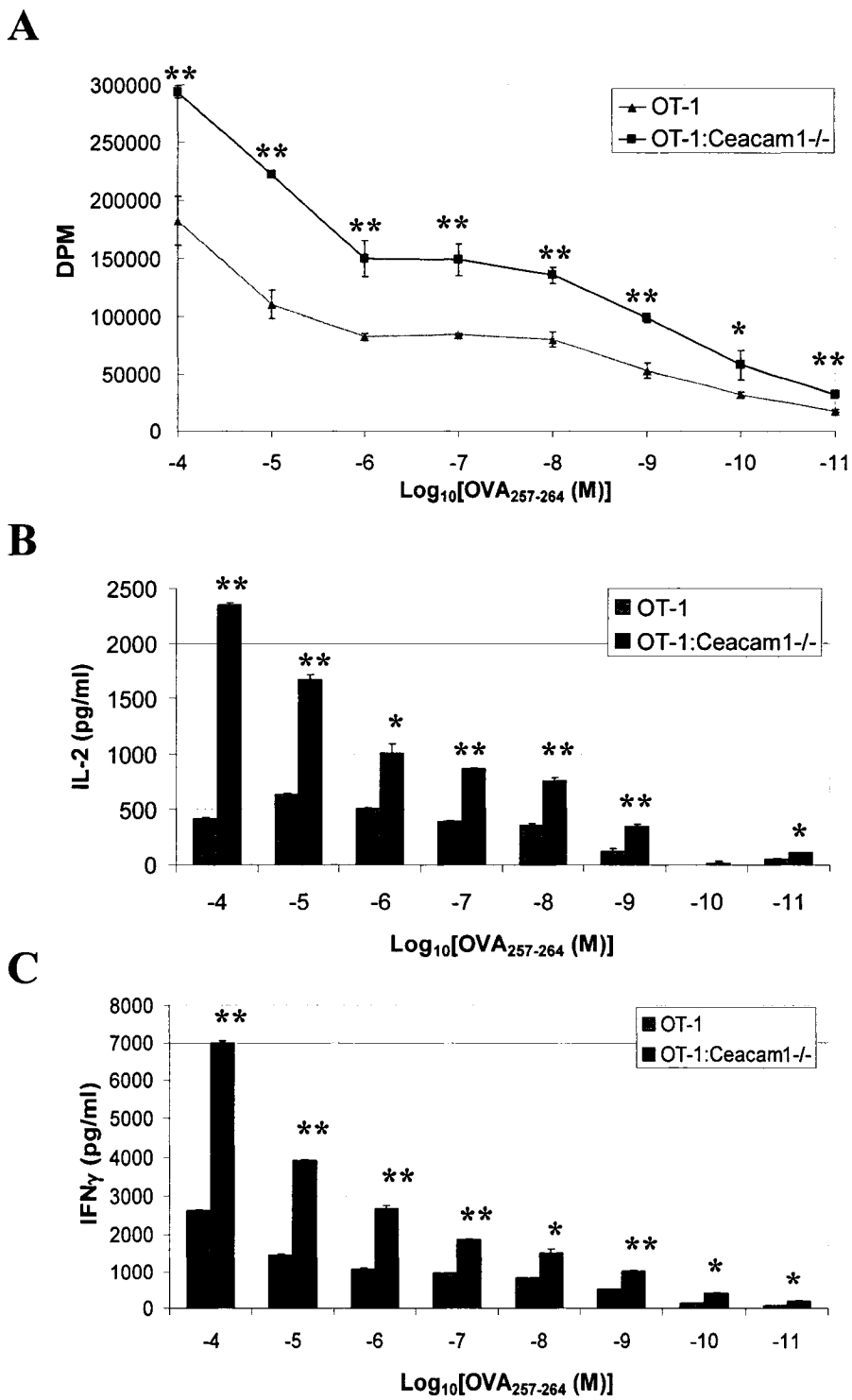
Furthermore, the production of IL-2 and IFNγ, two important cytokines produced by CTLs, was determined by ELISA from cell culture supernatants collected after 48h of co-culture with the peptide-pulsed APCs. As can be seen from the representative experiment shown here, OT-1:*Ceacam1*-/- CTLs produced more IL-2 than their OT-1 counterparts (Fig. 3-3B; 2- to 5.5-fold increase) in a dose-dependent manner which mirrored the increase in proliferation seen by tritiated thymidine incorporation (Fig. 3-3A). This increased production was statistically significant ($P<0.05$) except when they

Figure 3-3: Proliferative response and cytokine profiles of OT-1 and OT-1:*Ceacam1*-/- cytotoxic T cells.

Purified $CD8^+$ T cells from the lymph nodes of 8- to 10-week-old OT-1 and OT-1:*Ceacam1*-/- mice were co-cultured for 48h with gamma-irradiated APCs pulsed with $OVA_{257-264}$ peptide at concentrations ranging from 10^{-4} to 10^{-11}M (shown as Log_{10} values). (A) Proliferation was assessed by tritiated thymidine incorporation after an additional 16h-incubation with this compound. Production of IL-2 (B) and IFNγ (C) was determined by ELISA from cell culture supernatants collected at the 48h timepoint. Proliferation assays were done in triplicate, whereas ELISAs were done in duplicate; the error bars correspond to the standard deviation of the mean. One representative experiment of seven is shown here, and for each experiment a pool of 3 mice per genotype was used.

*, $P<0.05$; **, $P<0.01$.

Figure 3-3


A
300000
250000
200000
150000
100000
50000
0
DPM
OT-1
OT-1:Ceacam1-/-
-4
-5
-6
-7
-8
-9
-10
-11
Log10[OVA257-264 (M)]
B
2500
2000
1500
1000
500
0
IL-2 (pg/ml)
OT-1
OT-1:Ceacam1-/-
Log10[OVA257-264 (M)]
C
8000
7000
6000
5000
4000
3000
2000
1000
0
IFNγ (pg/ml)
OT-1
OT-1:Ceacam1-/-
Log10[OVA257-264 (M)]

were co-cultured with APCs pulsed with 10^{-10}M $OVA_{257-264}$. A similar trend was observed for the secretion of IFNγ, and this enhanced secretion was statistically significant ($P<0.05$) at all peptide pulse concentrations (Fig. 3-3C; 1.9- to 3.1-fold increase). Thus, not only does CEACAM1 play an inhibitory role on the proliferation of CTLs, but it also inhibits the production of cytokines associated with the activation and functions of these cells.

3. Generation of the CD2-CC1-L and CD2-FF transgenic mouse models

To further confirm the potential role of CEACAM1 as an inhibitor of T cell functions, two Tg mouse models were generated based on a common strategy: overexpressing either the CEACAM1-4L cDNA or a mutant harbouring the Y488,515F mutation on the surface of T cells. The rationale behind this design is that if CEACAM1 is an inhibitory co-receptor in T cells, then overexpression of CEACAM1-4L on their surface would lead to hypoproliferative responses compared to their WT counterparts upon stimulation. On the other hand, overexpression of the Y488,515F mutant would create a dominant negative model through the association of mutated CEACAM1-4L with WT protein present on the surface of T cells. An effect similar to that of the ablation of CEACAM1 would then be expected, since the Tyr residues present in both ITIMs are mutated. This would also provide further insight on the importance of these residues for downstream signalling following CEACAM1 ligation and confirm/disrepute previous findings [64].

J. Farrah generated the construct for the first Tg model, termed CD2-CC1-L. Briefly, the CEACAM1-4L cDNA was inserted downstream of the human CD2 (hCD2) promoter contained in the VAhCD2 vector at its unique EcoRI site (Fig. 3-4A). This vector has been used previously to create Tg mice overexpressing constitutively a protein of interest in T cells, and the level of expression it confers is independent of where it integrates in the mouse genome, but dependent on the number of copies integrated [219]. Following the removal of the Bluescript bacterial plasmid component, this construct was microinjected in C57BL/6 oocytes that were subsequently implanted in C57BL/6 surrogate mothers. I used the same approach to generate the CD2-FF Tg mouse, the difference being that the CEACAM1-4L-Y488,515F cDNA was cloned in the VAhCD2

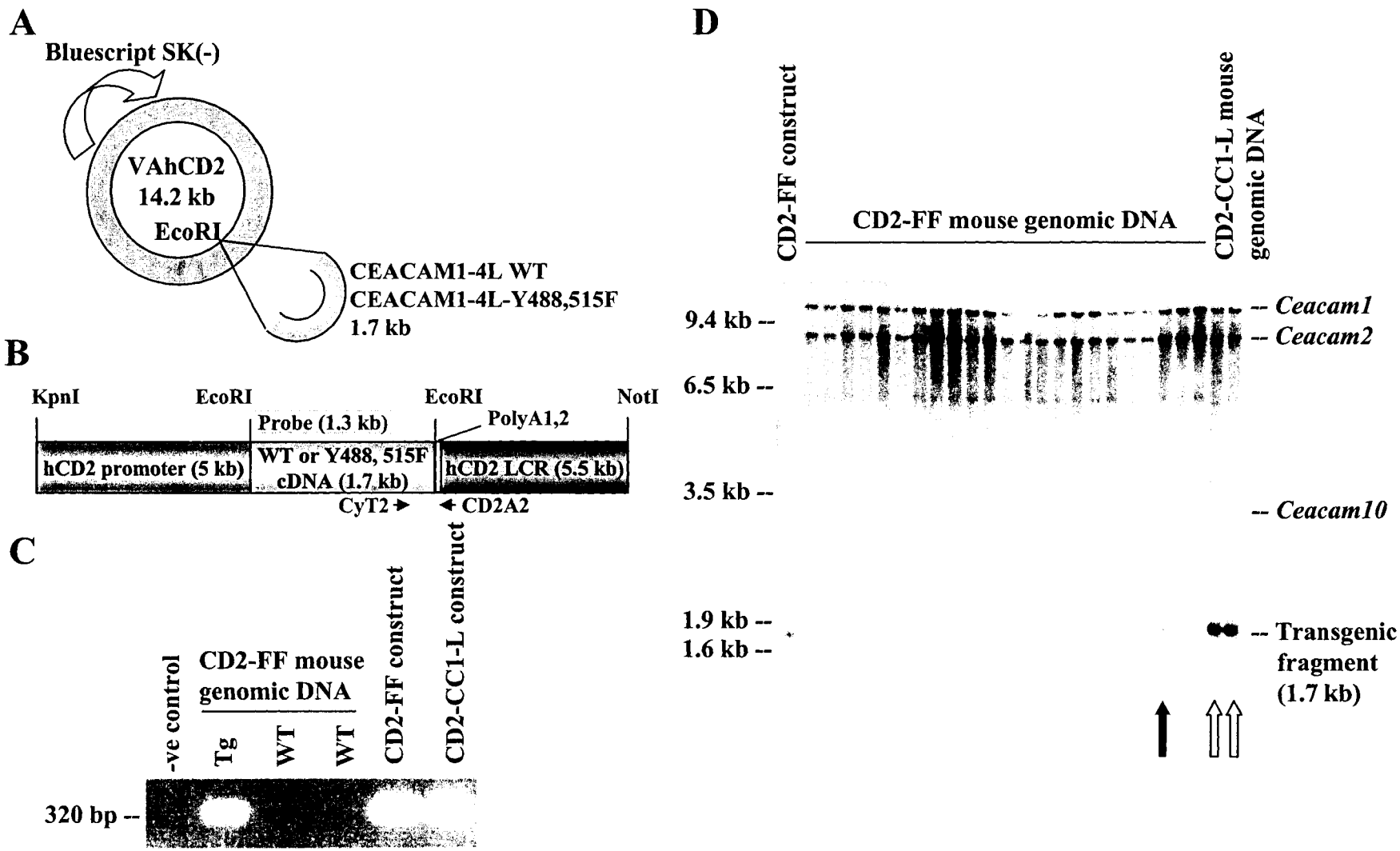
Figure 3-4: Generation of the CD2-CC1-L and CD2-FF transgenic mice.

(A) The constructs used in the generation of the CD2-CC1-L and CD2-FF transgenic (Tg) mouse models were obtained by inserting, respectively, the WT CEACAM1-4L cDNA or a version harbouring the Y488,515F mutation, into the sole EcoRI site contained within the VAhCD2 vector. Two polyadenylation sites (PolyA1,2) are provided in the vector. (B) The linearized construct, freed from the Bluescript SK(-) bacterial plasmid component, was subsequently microinjected into C57BL/6 oocytes. Arrows represent the primers (CyT2 and CD2A2) designed to amplify a 320 bp region to identify Tg mice by PCR. The probe used for Southern blot genotyping is also shown. Its subsequent hybridization with the CEACAM1-4L WT or Y488,515F cDNA following the digestion of Tg genomic DNA with EcoRI would lead to the appearance of a 1.7 kb band.

hCD2: Human CD2; LCR: Locus control region

(C) Identification of the CD2-FF founder by PCR through the detection of the 320 bp amplification product, which also appeared when CD2-CC1-L Tg mice were genotyped by this method. Negative control: PCR mix only. (D) Identification of the CD2-FF founder (black arrow) by Southern blot genotyping. Genomic DNA from the Tg progeny of the CD2-CC1-L founder (white arrows) was included for comparison. All Tg mice displayed the expected 1.7 kb band. In addition, the probe recognized the endogenous *Ceacam1* gene, as well as the highly homologous *Ceacam2* gene. The *Ceacam10* gene was also weakly detected.

Figure 3-4


A
Bluescript SK(-)
VAhCD2
14.2 kb
EcoRI
CEACAM1-4L WT
CEACAM1-4L-Y488,515F
1.7 kb
B
KpnI
EcoRI
EcoRI
NotI
Probe (1.3 kb)
PolyA1,2
hCD2 promoter (5 kb)
WT or Y488, 515F
cDNA (1.7 kb)
hCD2 LCR (5.5 kb)
CyT2
CD2A2
C
-ve control
CD2-FF mouse
genomic DNA
Tg
WT
WT
CD2-FF construct
CD2-CC1-L construct
320 bp --
D
CD2-FF construct
CD2-FF mouse genomic DNA
CD2-CC1-L mouse
genomic DNA
9.4 kb --
6.5 kb --
3.5 kb --
1.9 kb --
1.6 kb --
-- Ceacam1
-- Ceacam2
-- Ceacam10
-- Transgenic
fragment
(1.7 kb)

vector (Fig. 3-4A).

Since the only difference between these transgenes resides in the Tyr mutations present in the CD2-FF construct, common PCR and Southern Blot methods were used to identify mice having integrated their respective constructs in their genomes (Fig. 3-4B). For the first method, two primers were designed such that one (CyT2) annealed within the cyt domain of the CEACAM1-4L (WT or Y488,515F) cDNA, whereas the other (CD2A2) annealed within the VAhCD2 vector between the two polyadenylation (polyA) sites, resulting in the generation of a 320 bp amplification product (Fig. 3-4B). For the second method, Tg mice were identified by the presence of a 1.7 kb fragment corresponding to the CEACAM1-4L WT or mutated cDNAs following digestion of genomic DNA with EcoRI and subsequent hybridization with a ^{32}P-labelled probe binding to CEACAM1 (Fig. 3-4B). In addition, this probe detected the endogenous *Ceacam1* gene, as well as the endogenous *Ceacam2* gene due to the high degree of homology existing among these genes. Weak detection of the *Ceacam10* gene also occurred (Fig. 3-4D).

Three founder CD2-CC1-L mice were identified, but only one gave rise to progeny carrying the transgene (Fig. 3-4D, white arrows). This founder generated the line which was subsequently characterized, and which will be described in more details in the next section. As for the CD2-FF Tg mouse, out of the 23 pups that were born, one was identified to carry the transgene by PCR (Fig. 3-4C) and Southern blotting (Fig. 3-4D, black arrow) but never gave rise to Tg progeny. Finally, due to the inherent difficulties of generating Tg mice on the C57BL/6 background [220], the microinjections were not pursued and this aspect of the project was abandoned.

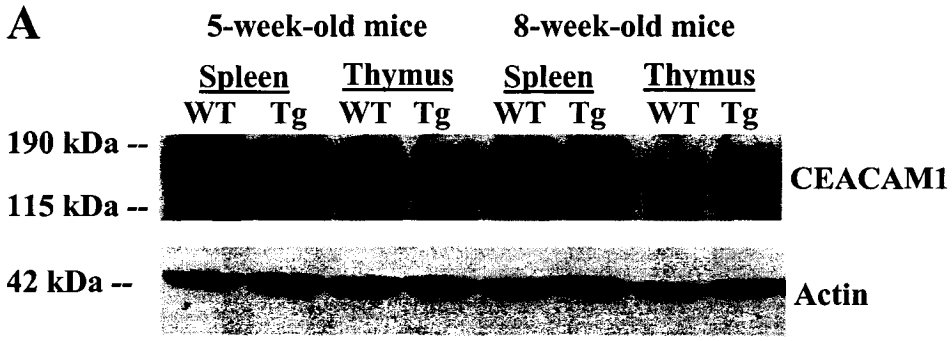
4. Characterization of the CD2-CC1-L mouse model

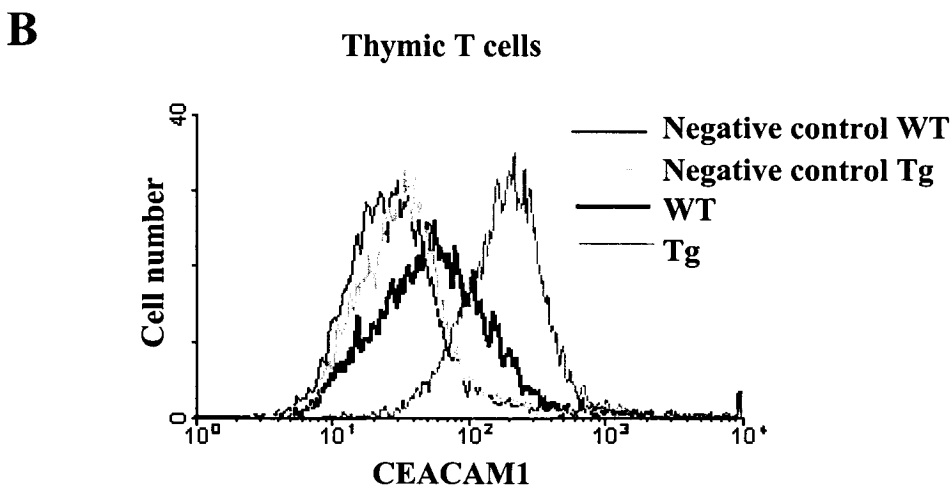
Since we identified one founder of the CD2-CC1-L lineage and its progeny to carry the transgene by PCR and by Southern blotting, the next step was to determine if its integration in their genome led to an actual overexpression of CEACAM1-4L in T cells. This was first examined by Western blotting using the polyclonal anti-CEACAM1 Ab 2457 on thymus and spleen lysates from 5 and 8-week-old WT and Tg littermates (Fig. 3-5A). To gain insight as to the expression of this transgene on thymocytes and mature peripheral T cells [221, 222], we analyzed thymic and splenic lysates for CEACAM1

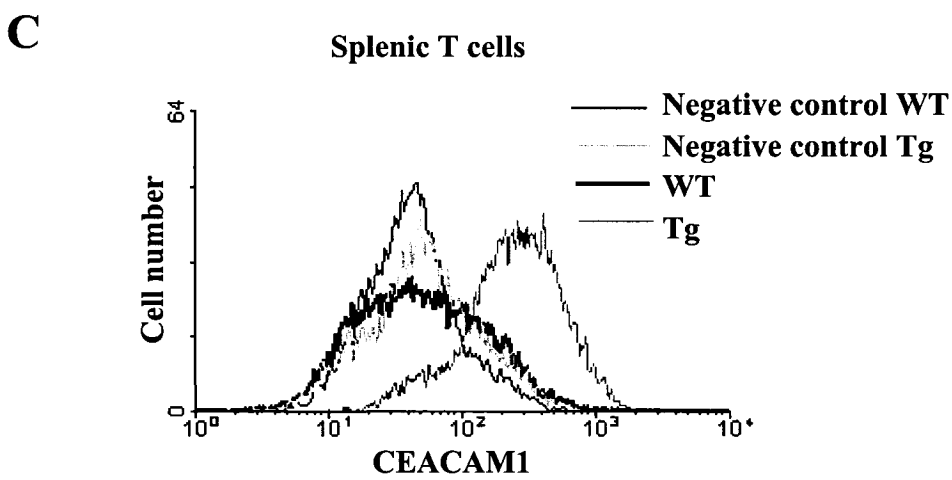
Figure 3-5: Expression of CEACAM1 in CD2-CC1-L transgenic mice.

(A) Western blot analyses were performed on lysates from whole thymi and spleens from 5- and 8-week-old WT and CD2-CC1-L Tg littermates with the polyclonal anti-CEACAM1 Ab 2457. Blotting with an anti-actin Ab was preformed as control for equal loading. This experiment was done twice. (B) The cell surface expression of CEACAM1 on thymic T cells from 8-week-old WT and CD2-CC1-L Tg siblings was determined by flow cytometry by double-staining thymic single cell suspensions with anti-CD3 and 2457, or anti-CD3 and normal rabbit serum from the same bleed as a control for non-specific Ab binding (this profile is designated negative control). Histograms revealing the expression of CEACAM1 were generated after gating on $CD3^+$ T cells. One representative experiment of four is shown here. (C) The expression of CEACAM1 on the surface of purified splenic T cells was determined similarly. One representative experiment of two is shown here.

Figure 3-5


A
5-week-old mice
8-week-old mice
Spleen
Thymus
Spleen
Thymus
WT Tg WT Tg WT Tg WT Tg
190 kDa --
115 kDa --
CEACAM1
42 kDa --
Actin


B
Thymic T cells
Negative control WT
Negative control Tg
WT
Tg
Cell number
40
0
10⁰
10¹
10²
10³
10⁴
CEACAM1


C
Splenic T cells
Negative control WT
Negative control Tg
WT
Tg
Cell number
64
0
10⁰
10¹
10²
10³
10⁴
CEACAM1

overexpression, respectively. Actin was used as a control to confirm equal protein loading. At both ages, CEACAM1 expression was increased in the thymus of CD2-CC1-L Tg mice compared to their WT counterparts. This overexpression was of the order of 1.6-fold and 3.4-fold, respectively, for 5-week-old and 8-week-old Tg mice. On the other hand, this overexpression was more modest in the spleen: 1.2-fold in 5-week-old Tg mice, and 1.1-fold in 8-week-old Tg mice. Thus, CD2-CC1-L Tg mice overexpress CEACAM1 in their thymus, suggesting that their T cells overexpress this protein as well.

Since the detection of CEACAM1 by Western blotting revealed the presence of total CEACAM1, irrespective of its location, flow cytometric analyses were performed in order to determine if there was indeed an increased cell surface expression of this protein in T cells from Tg mice. Briefly, single cell suspensions obtained from disrupting thymi from 8-week-old WT and Tg siblings, as well as purified splenic T cells from these mice, were stained with a combination of the polyclonal anti-CEACAM1 Ab 2457 (or NRS from the same bleed as a control for background staining) and anti-CD3. After gating on $CD3^+$ cells, histograms were generated to reveal the expression of CEACAM1. As it can be seen in the representative experiment shown in Fig. 3-5B, thymic T cells from Tg mice expressed more CEACAM1 on their surface compared to their WT counterparts. Based on the mean fluorescence intensity obtained from the profiles of four pairs of mice, this increase in expression was on average 3.2-fold and was statistically significant (P=0.03). The same analyses performed on purified splenic T cells (Fig. 3-5C) revealed an average 4-fold increased CEACAM1 expression on the surface of Tg T cells compared to their WT counterparts (statistical significance could not be assessed here). Thus, the integration of the CD2-CC1-L transgene led to an increased expression of CEACAM1 on the surface of both thymic and peripheral T cells.

Having confirmed this, the effect of the overexpression of CEACAM1-4L on their proliferative capacity and cytokine secretion was studied. To do so, T cells were isolated from the spleens of 8-week-old WT and Tg mice and stimulated *in vitro* for 48h with anti-CD3 Abs, with or without additional co-stimulation with anti-CD28 Abs. T cells were also stimulated with Con A, or a combination of PMA and ionomycin, respectively activating PKC, and influencing calcium fluxes [213, 223]. In a preliminary proliferation assay, where tritiated thymidine incorporation was assessed after an additional 16h of

culture with this compound, T cells from CD2-CC1-L Tg mice showed a statistically significant decrease in proliferation compared to their WT counterparts when stimulated with 3 μg/ml anti-CD3 or PMA and ionomycin (Fig. 3-6A; $P<0.05$ and $P<0.01$, respectively). In both instances, this decrease was approximately 2-fold. This trend was also observed when the cells were stimulated with both anti-CD3 and anti-CD28 Abs, as well as with Con A (data not shown). However, we have had some difficulty in reproducing these results in subsequent experiments. Thus, it is with some reserve that we conclude from this preliminary experiment that CD2-CC1-L transgenic T cells exhibit a decreased proliferative capacity compared to their WT counterparts.

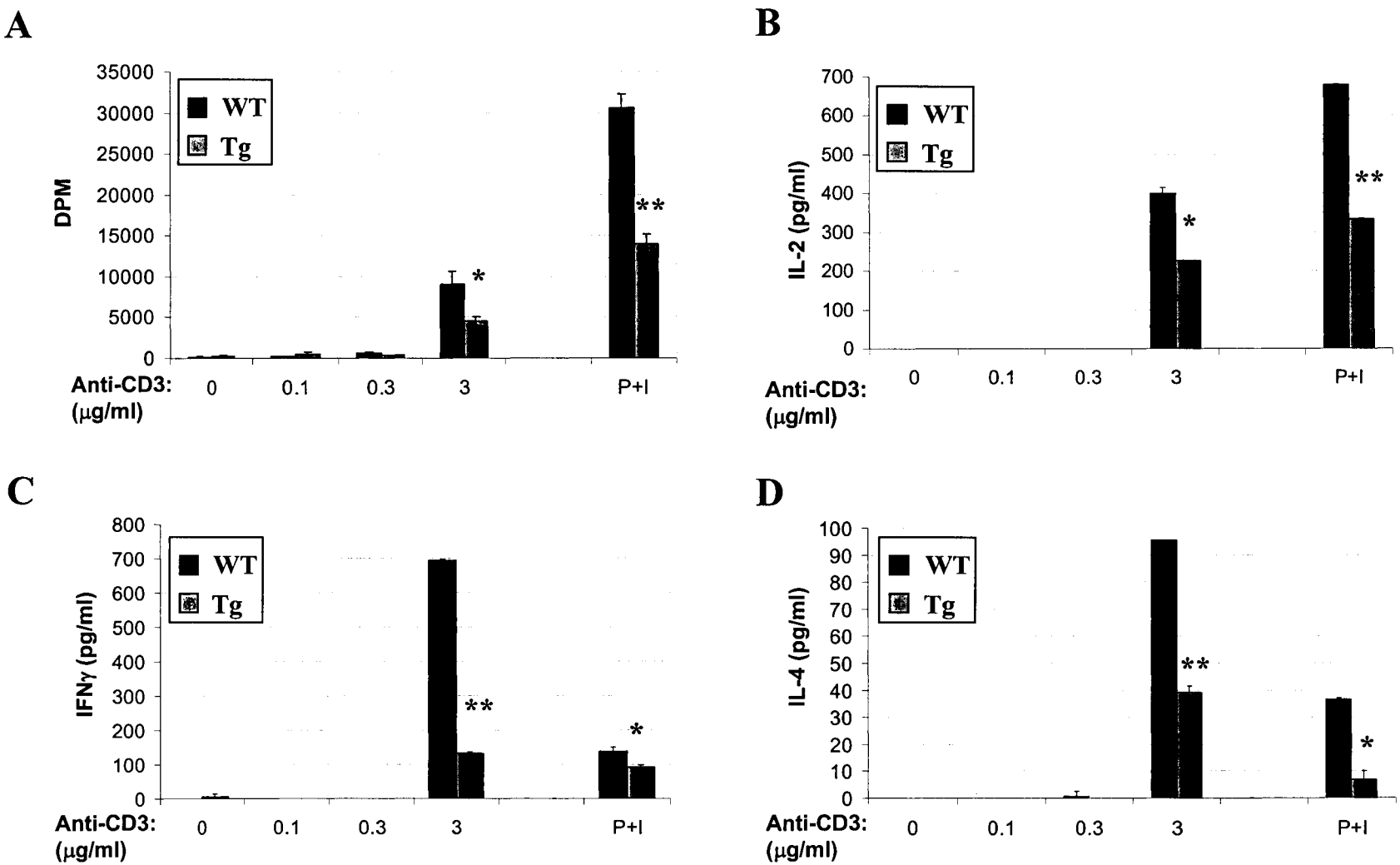
In order to further assess the impact of the overexpression of CEACAM1-4L, cell culture supernatants were collected after 96h of stimulation to examine the secretion of the Th1 cytokines IL-2 and IFNγ, and of the Th2 cytokine IL-4 by ELISA. Comparison of Tg T lymphocytes with their WT counterparts revealed a statistically significant reduction in IL-2 secretion (Fig. 3-6B) after stimulation with 3 μg/ml anti-CD3 (1.75-fold; $P=0.03$), or with PMA and ionomycin (2-fold; $P=0.0004$). A similar trend was observed regarding the production of IFNγ by CD2-CC1-L T cells (Fig. 3-6C): stimulation with 3 μg/ml anti-CD3 led to a 5.2-fold reduction ($P=0.00002$), whereas stimulation with PMA and ionomycin led to a 1.5-fold reduction ($P=0.04$). Interestingly, the production of IL-4 (Fig. 3-6D) by Tg T cells was also decreased by 2.4-fold with 3 μg/ml anti-CD3 ($P=0.009$), and 5-fold with PMA and ionomycin ($P=0.02$). However, this needs to be further confirmed, as the cytokine profiles obtained from stimulation with both anti-CD3 and anti-CD28 Abs, or Con A, were not as consistent. Thus, it is with some caution that it can be inferred that overexpressing CEACAM1-4L on the surface of T cells leads to a reduction in the production of both Th1 and Th2 cytokines.

Figure 3-6: Proliferative response and cytokine profiles of WT and CD2-CC1-L transgenic T cells.

Purified T cells isolated from the spleens of 8-week-old WT and CD2-CC1-L transgenic (Tg) littermates were stimulated with plate-bound anti-CD3 Abs, or PMA and ionomycin (P+I). (A) After 48h, tritiated thymidine was added for an additional 16h-incubation to assess proliferation through its incorporation at the end of this period. (B-D) Cell culture supernatants were collected after 96h of stimulation in order to quantify the secretion of IL-2 (B), IFNγ (C), and IL-4 (D) by ELISA. The proliferation assay was done in triplicate, whereas ELISAs were done in duplicate; the error bars correspond to the standard deviation of the mean. The results presented here are from a preliminary experiment involving one mouse from each genotype.

*, $P<0.05$; **, $P<0.01$.

Figure 3-6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Anti-CD3: (μg/ml)
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CHAPTER 4: DISCUSSION

1. CEACAM1 does not affect the development of memory T cells

Previously, J. Farrah observed that CEACAM1 did not affect the development or the migration of T cells to peripheral lymphoid organs, as developing T cell populations of the thymus, and mature ones of the spleen of WT and *Ceacam1*-/- mice displayed similar expression of the T cell markers studied. In concordance with these results, the flow cytometric experiments described above revealed that there are no differences in the presence of helper and cytotoxic memory T cells, respectively characterized by $CD4^{+}CD44^{hi}CD62L^{lo}$, and $CD8^{+}CD44^{hi}CD62L^{lo}$ phenotypes [213], in the LN and the spleen of *Ceacam1*-/- mice compared to their WT counterparts. Conversely, no significant differences were observed in levels of naïve $CD4^{+}$ and $CD8^{+}$ T cells.

Furthermore, these observations entail that the differences observed in the cytokine profiles of WT and *Ceacam1*-/- T cells (currently under study), and by extrapolating, those of OT-1 and OT-1:*Ceacam1*-/- CTLs, are not influenced from an initial imbalance between the ratios of naïve and memory T cells. Indeed, it has been reported *in vitro* that memory T cells differ from naïve T cells during an immune response to a previously encountered Ag in that they produce more cytokines earlier and faster than do naïve T cells, which, on the other hand, proliferate more effectively [224]. Memory T cells also produce a broader cytokine spectrum compared to their naïve counterparts [225, 226], hence the importance of assessing if CEACAM1-deficiency altered their ratios.

One aspect of memory T cell immunity which was not addressed here is whether the ablation of CEACAM1 equally affects the immune responses specifically mediated by naïve or memory T cell subsets. For instance, Davidson *et al.* reported that although there was no significant difference in the ratios of naïve and memory helper T cells originating from mice lacking Fyn, one of the early protein Tyr kinases involved in TCR signalling, both subsets exhibited impaired proliferative responses, Th2 signalling and Th2 cytokine production [213]. Since T cells from *Ceacam1*-/- mice are hyperproliferative compared to their WT counterparts according to our previous observations, it would be interesting to determine whether this behaviour is reflected both in naïve and memory T cells.

2. CEACAM1 is a co-inhibitory receptor in cytotoxic T cell proliferation and cytokine production

The initial controversy surrounding the function of CEACAM1 in T cells has not been formally resolved, despite a majority of publications appointing it a co-inhibitory role. Indeed, an initial co-stimulatory role was ascribed to this molecule based primarily on *in vitro* experiments which involved engagement of CEACAM1 by anti-CEACAM1 Abs [40, 53]. This engagement also yielded opposite results in similar experiments, and opened the door to a co-inhibitory function [78, 210]. As mentioned by Nakajima *et al.*, in addition to not being physiological, these approaches cannot discriminate between an actual induction of co-inhibitory signals or a block of co-stimulatory signals transduced by CEACAM1 [78].

Subsequently, evidence for an inhibitory role was gained from models bypassing these limitations. These models were either more physiological by using natural ligands for CEACAM1, such as the inhibition of activation and proliferation of helper T cells induced by its ligation with the Opa_{52} proteins of *N. gonorrhoeae* [14], and the impairment in Th1 differentiation and IFNγ secretion due to binding of S of MHV [188]; or altogether bypassed the use of ligating Abs by stimulating decidual T cells with a superantigen in the presence of CEACAM1-transfected cells to mimic *in vivo* homophilic interactions with the EVT [63]. Transfection of CEACAM1 isoforms in T cell lines by themselves [47], or in conjunction with a luciferase reporter system has also been used [64]. Recently, considerable information regarding the role of CEACAM1 in murine T cells *in vivo* was gained through a model of colitis akin to human IBD, namely its negative regulation of pathways involved Th1 differentiation and Th1 cytokine production [188].

As more knowledge was acquired regarding the function of CEACAM1 in T cells, several comparisons were drawn with other ITIM-containing immune receptors in an attempt to explain its elusive behaviour. Indeed, similarities with the activating NKp44, and β chain of the IL-3R [40], or with CD5, CD72, and platelet-endothelial cell adhesion molecule 1 (PECAM-1), exerting both stimulatory and inhibitory functions in different settings [14], and signalling via SHP-1 and/or -2 [227], were suggested. Parallels were also drawn with CD22 [64], a transmembrane inhibitory B cell receptor brought into the

BCR signalling complex upon Ag binding, which then becomes Tyr phosphorylated on its ITIMs, recruits and activates SHP-1, thereby inhibiting BCR signalling [228]. So far, the most substantiated comparison is that with the important T cell co-inhibitor, CTLA-4. Indeed, both CEACAM1 and CTLA-4 appear to share a similar mechanism of expression, i.e. cytoplasmic retention of preformed molecules until cell activation via associations with the adaptor proteins 1 and/or 2 that are subject to the phosphorylation status of their cyt tail [78]. Also, both use SHPs to convey inhibitory signals: SHP-2 for CTLA-4 [228], and SHP-1 (and potentially SHP-2), for CEACAM1 [14, 47, 64, 78]. Finally, the latter was also compared to programmed death 1 (PD-1), similar to CTLA-4 [15, 229], and able to inhibit both Th1 and Th2 differentiation and cytokine production under specific conditions [188].

Furthermore, the elegant models mentioned above do not reveal the extent of the implication of CEACAM1 in T cell development and functions. Only the *Ceacam1*-/- mouse model renders this possible, with another key feature being that it allows us to assess the importance of CEACAM1 in mediating these functions under more physiological conditions: the murine colitis model shows this in a pathological context, and the levels of CEACAM1 expressed by the transfected T cell lines (also differing from primary T cells in that they have been immortalized) might not reflect the physiological ones. Hence, our previous observations that *Ceacam1*-/- T cells are hyperproliferative *in vitro* compared to WT counterparts confirm the co-inhibitory role of CEACAM1 in these cells with a model requiring minimal manipulations of the cells.

Moreover, the use of a more physiological model involving antigen-presentation, namely the use of OT-1:*Ceacam1*-/- CTLs, harbouring the $V\alpha2V\beta5^+$ transgenic TCR of OT-1 TCR transgenic mice but devoid of CEACAM1 on their surfaces, allowed us to specifically study the effect of CEACAM1 in this subset. Not only were OT-1:*Ceacam1*-/- CTLs hyperproliferative at all concentrations of $OVA_{257-264}$ peptide presented by MHC class I molecules of APCs compared to their OT-1 counterparts, thereby corroborating our previous findings supporting its co-inhibitory role, but they also produced more IL-2 and IFNγ. One might infer from the very similar expression profiles of the $V\alpha2V\beta5^+$ transgenic TCR in OT-1 compared to OT-1:*Ceacam1*-/- CTLs that CEACAM1 does not appear to be involved in the mechanisms regulating TCR expression.

In addition, these observations are in agreement with the predominantly co-inhibitory role exerted by CEACAM1 in T cell functions reported by others [47, 64], as ablation of all CEACAM1 isoforms resulted in enhanced proliferation and cytokine production by OT-1:*Ceacam1*-/- CTLs. This could occur through the elimination of homophilic interactions mediating its co-inhibitory effect in T cells [63, 64, 188], prevented between OT-1:*Ceacam1*-/- CTLs and APCs (still expressing CEACAM1), but leading to inhibition when OT-1 CTLs interact with APCs. This could be further examined by using APCs originating from the spleens of OT-1:*Ceacam1*-/- mice. Hence, we would expect even more pronounced hyperproliferative responses and increases in cytokine production when both CTLs and APCs lacking CEACAM1 on their surfaces are brought together. This has occurred in recent studies conducted by A.-M. Charbonneau-Allard in our laboratory.

In light of recent findings, this behaviour of OT-1:*Ceacam1*-/- CTLs might result from their deprivation of a significant negative regulator of stimulatory signalling pathways. Indeed, the absence of CEACAM1-L could prevent or reduce the recruitment of SHP-1 [14, 47, 64, 78] and/or SHP-2 [14] to the TCR/CD3 complex with which CEACAM1 might be associated [47], with SHP-1 appearing to be the most important of the two in conveying CEACAM1 inhibitory signals in T cell responses. Because it is recruited by and inactivates ZAP-70 by dephosphorylating it [228, 230], SHP-1 is an important conveyor of inhibitory signals during T cell activation. This is underlined in *motheaten* mice that are SHP-1-deficient and display exaggerated TCR-mediated proliferation, and IL-2 production [227]. Impaired recruitment of SHP-2, seemingly used by Opa_{52}^{+} gonococci after CEACAM1 binding [14] to convey inhibitory signals during T cell activation, might also be involved. For instance, this phosphatase is used by CTLA-4 to convey its co-inhibitory signals [228].

Further downstream, this impaired recruitment of SHP-1 caused by the absence of CEACAM1-L would prevent the inhibition of MAPK activation, as uncovered in CEACAM1-transfected Jurkat T cells [64]. MAPK pathways not only culminate with the activation of transcription factors involved in T cell proliferation [204], but also influence $CD4^{+}$ T cell differentiation. Indeed, JNK and p38 favour Th1 differentiation and production of IFNγ, and inhibit Th2 differentiation, instead governed by Erk1/2 [231,

232]. Their functions differ in CTLs: p38 mediates IFNγ secretion, but also induces apoptosis [231, 233]. Moreover, JNK1 favours the expression of the IL-2R α subunit on the surface of T cells, and its function dominates over that of JNK2, which inhibits IL-2 production [233]. Hence, based on studies involving Jurkat T cells which revealed that CEACAM1-L inhibits the activation of JNK [64], this could potentially render CTLs less sensitive to IL-2 [233] under normal conditions, with the opposite occurring in its absence. Also, despite the fact that CEACAM1-L did not affect the activation of p38 in this cell line [64], differences in MAPK expression kinetics arise in primary T cells [233], and hence p38 could still affect the proliferative response, and secretion of IFNγ by OT-1:*Ceacam1*-/- CTLs in the absence of CEACAM1 inhibition.

Additionally, the enhanced proliferation and IL-2 production of OT-1:*Ceacam1*-/- CTLs compared to their OT-1 counterparts are in agreement with findings that CEACAM1-L inhibits IL-2R signalling by affecting its cell-surface expression, IL-2 production, and proliferation [47]. In these studies, Chen *et al.* reached these conclusions in part following CEACAM1 ligation with anti-CEACAM1 Abs on human peripheral blood T cells, and CEACAM1-transfected Jurkat T cells. To further document these observations, they used Kit-225 T cells transfected with either CEACAM1-4L, or -4S, and found that -4L inhibited IL-2-dependent proliferation [47]. We have used a more physiological model, i.e. the stimulation of OT-1:*Ceacam1*-/- CTLs with $OVA_{257-264}$-pulsed APCs, and our results suggest that this same mechanism could be involved here.

This unique model would also allow us to confirm the effect of CEACAM1 (and of its absence) on downstream molecules involved in IL-2R signalling and T cell proliferation, namely Jak3 [47], and Jak1, whose phosphorylation and activation by Jak3 is essential for these processes *in vivo* [234]. This model would also enable us to determine the consequences of CEACAM1-deficiency on the activation of transcription factors by IL-2R signalling, such as STATs 1, 3, and 5, involved in the expression of genes whose protein products allow proliferation and convey anti-apoptotic signals [234]. Indeed, this could be determined from Western blot analyses comparing activated and "resting" OT-1 and OT-1:*Ceacam1*-/- CTLs, both cocultured respectively with APCs previously $OVA_{257-264}$-pulsed or not. Following recovery of CTLs of both genotypes through negative selection (much like when they were first isolated from murine LN),

probing of cell lysates with the appropriate Abs would allow us to assess effect of CEACAM1 on the levels of the aforementioned IL-2R signalling components. The overall phosphorylation status of both unstimulated and activated OT-1 and OT-1:*Ceacam1*-/- CTLs could also be gauged. This approach could also be used to verify/confirm the implication of SHP-1, SHP-2, JNK1/2, and p38. The levels of the transcription factors AP-1 and NF-kB [40] in nuclear extracts from OT-1 and OT-1:*Ceacam1*-/- CTLs [188] could define how these cells behave in the absence of CEACAM1.

Another player which might explain the elevated production of IFNγ by OT-1:*Ceacam1*-/- CTLs compared to OT-1 CTLs is the transcription factor T-bet. Indeed, studies by Iijima *et al.* of a murine model of hapten-induced colitis revealed an inhibitory role of CEACAM1 in Th1 responses primarily by reducing the production of IFNγ by modulating the levels of T-bet [188]. T-bet, crucial for the differentiation of Th1 cells and their secretion of IFNγ [212], is also important for the differentiation of naïve CTLs into effector $CD8^+$ T cells. Although OT-1: *T-bet*-/- CTLs proliferate normally, they kill target cells less efficiently than OT-1 CTLs, secrete less IFNγ, but more IL-2, IL-4, and IL-10, the latter two being unusual cytokines for this subset [235]. Hence, Western blot analyses of nuclear extracts from CTLs originating from our unique model could uncover a link between the higher IFNγ release and T-bet levels in the absence of CEACAM1.

One aspect which was not addressed here is the effect of CEACAM1 and of its absence on cytolytic responses of CTLs. So far, only contradictory conclusions were obtained regarding this. For instance, Morales *et al.* reported that in redirected killing assays, immortalized iIELs killed target cells less efficiently upon prior treatment with anti-CD3 Abs and anti-CEACAM1 Abs compared to iIELs only treated with anti-CD3 Abs [210]. On the other hand, Donda *et al.* did not observe any effect of CEACAM1 engagement by anti-CEACAM1 Abs on target cell killing by T cell clones, and suggested that these discrepancies arose due to the concentrations of anti-CEACAM1 Abs used as well as how they were used, i.e. soluble vs plate-bound [40].

Using CTLs from our OT-1:*Ceacam1*-/- mouse model to bypass these variations, we could study this *in vitro* by coculturing them (as well as OT-1 CTLs) with OVA-pulsed syngeneic target cells incubated with ^{51}Cr to measure cytolysis via its release [235].

Moreover, our model also enables us to do so *in vivo*: we could inject OT-1 and OT-1:*Ceacam1*-/- CTLs in C57BL/6 mice, then inject them with $OVA_{257-264}$ for priming, and after a few days, inject $OVA_{257-264}$-pulsed target cells labelled with the fluorescent dye CFSE, which would allow the quantification of unlysed target cells recovered from neighbouring LN by flow cytometry [235]. Thus, we could resolve this controversy by assessing if CEACAM1-deficiency leads to the expected enhanced target cell killing.

3. Potential implication of CEACAM1 in anti-viral immune responses

One of the major functions of CTLs is the eradication of virus-infected cells via the cytolytic molecules perforin and granzyme [189]. IFNγ secretion by CTLs not only induces a state of resistance by blocking viral replication in nearby cells, but also renders infected cells better presenters of virus-derived peptides, and by the same token CTL targets, by increasing their expression of MHC class I molecules, TAP proteins, and proteasome constituents [235]. Hence, the fact that CEACAM1 plays an inhibitory role in CTL proliferation and secretion of IL-2 and IFNγ based on our findings with OT-1:*Ceacam1*-/- CTLs would make this molecule a choice target for viruses to spread and escape elimination. This could occur during infection with CMV, which induces the expression of a CEACAM1 ligand on the surface of infected cells, thereby possibly inhibiting CTL and NK cell responses central to its elimination [63]. This is also exemplified in the block of Th1 differentiation and IFNγ production induced by S of MHV after binding CEACAM1 [188], thereby undermining the important contribution of Th1 cells in CTL responses [236]. Maybe CEACAM1-binding by MHV also directly downregulates IFNγ production by CTLs; both scenarios are conducive to a reduced efficiency in the disposal of virus-infected cells. Thus, compounds blocking CEACAM1-virus interactions might prevent infection and inhibition of CTL responses crucial to virus elimination.

4. Potential implication of CEACAM1 in tumor immunosurveillance

Like NK cells, CTLs are also involved in the elimination of tumor cells [236]. An inhibitory role of CEACAM1 in CTL functions emerged from our studies of OT-1:*Ceacam1*-/- CTLs, notably in the production of IFNγ. This cytokine can restore or

enhance MHC class I expression in tumor cells which had decreased it in order to evade recognition and clearance by the immune system [229]. This again makes CEACAM1 a convenient target in this process. For instance, RCC cells having lost the expression of CEACAM1 can re-express it by means of the ISRE of its promoter [41] upon IFNγ release by CTLs which had infiltrated the tumor to eradicate it [93]. Hence, activated CTLs (and NK cells), expressing CEACAM1 on their surface, would be inhibited by the resulting upregulation of CEACAM1 on tumor cells, in turn allowing RCC cells to avoid cytolysis and reduce the release of IFNγ due to its potentially adverse effects on their survival [93]. Our observations could be extended further to iIELs, also cytolytic cells [210] and potential players in colon cancer immunosurveillance [229]. Since CEACAM1 can also be upregulated by IFNγ in human colorectal carcinoma cell lines [69], similar inhibition of iIEL function might occur, as in RCC. Finally, this utilization of CEACAM1 to corrupt immune responses has also been reported in malignant melanoma cell lines, where CEACAM1 [192] and CEA [194] expressed on the surface of tumor cells can inhibit the cytolytic functions of NK cells. Hence, a similar effect on CTL functions is not excluded, and might render CEACAM1 an interesting target in the development of cancer therapies.

5. Potential implication of CEACAM1 in cytotoxic T cell-mediated autoimmunity

CTLs also play a role in a number of autoimmune diseases, as presentation of self Ag in the context of MHC class I molecules, a normally tolerogenic process, can activate autoreactive CTLs, leading to the destruction of healthy cells [237]. This happens in a number of autoimmune diseases, such as thryroiditis, type I diabetes, multiple sclerosis, systemic lupus erythematosus, as well as diseases arising from the presentation of self Ag by specific MHC class I alleles to autoreactive CTLs, such as Behçet's disease and ankylosing spondylitis. For the latter, preliminary studies revealed that peptides derived from type II and type IV collagen presented by HLA-B27 MHC class I molecules activated CTLs, in turn potentially eliciting an autoimmune response to joint cartilage and causing this disease [237]. Bearing this in mind along with the co-inhibitory role of CEACAM1 in CTLs reported here, CEACAM1 might play a role in AS, as well as in other autoimmune diseases, by modulating CTL activity, as it appears to do for NK cells

during TAP2 deficiency [196]. Also, its implication in AS was recently hinted at, as NK cells of AS patients express high levels of CEACAM1 [193].

6. Insights gathered from the CD2-CC1-L transgenic mouse model

A Tg mouse model in which CEACAM1 is overexpressed specifically in T cells would further confirm the emergence of its co-inhibitory role from work by other groups, as well as previous results obtained by J. Farrah through studies of WT and *Ceacam1*-/- T cells, and mine obtained from the studies of OT-1 and OT-1:*Ceacam1*-/- CTLs. Hence, an hypoproliferative behaviour was expected from CD2-CC1-L Tg T cells compared to WT counterparts, accompanied by a reduction in IL-2, and IFNγ release, also perhaps occurring for IL-4 [64, 188]. In a preliminary experiment, we indeed observed that CD2-CC1-L Tg T cells proliferated less than WT T cells *in vitro* for all stimuli used, which was accompanied by decreased IL-2, IFNγ, and IL-4 production. The latter two were previously observed in Jurkat T cells transfected by CEACAM1-3L, hence its comparison to PD-1 [64]. However, these trends in cytokine production were clear only in two of the four stimuli used, and there was a lack of consistency when these experiments were repeated, as sometimes no differences or opposite results were observed.

This capricious behaviour might be explained by the fact that the obtained Tg founder did not overexpress a high enough level of CEACAM1, which was reflected in the cell surface expression of CEACAM1, only about ~3- to 4-fold higher than that of WT T cells. Hence, we hypothesize that only oocytes having integrated fewer copies of the CD2-CC1-L transgene were able to develop and come to term, as overexpressing CEACAM1 would inhibit their growth. This effect of CEACAM-L has been reported with CEACAM1-expressing NK cell clones, which grow slower than those which do not [194]. This could in part explain the difficulties we had in obtaining enough founders to generate different CD2-CC1-L Tg lines, in addition to the fact that these Tg lines were being produced in the C57BL/6 background, notorious for rendering this process more difficult, as foetuses arising from the microinjected oocytes show a higher death rate during embryonic development [220].

Moreover, it has been reported that $CD4^+$ T cells from a Tg mouse model overexpressing the Ly-6A.2 helper T cell inhibitory co-receptor were hyperproliferative

compared to their WT counterparts when stimulated *in vitro* with anti-CD3 and anti-CD28 Abs, whereas breeding these Tg mice with a TCR Tg model yielded helper T cells displaying the expected hypoproliferative behaviour, proposedly arising from the more physiological interactions taking place when APCs offered their specific Ag compared to generic stimulation with these Abs [217]. Hence, performing similar crosses with CD2-CC1-L mice with OT-1 or OT-2 TCR Tg mice (see below) to respectively study $CD8^+$ or $CD4^+$ T cells overexpressing CEACAM1 on their surfaces might "stabilize" their behaviour.

7. Future directions

A number of points remain to be addressed, a main one being the examination of the intracellular signals resulting from CEACAM1-L engagement. As proposed above to deepen our studies of OT-1 and OT-1:*Ceacam1*-/- CTLs, we could perform Western blot analyses of whole cell lysates (or nuclear extracts to study the levels of transcription factors) of resting and activated WT and *Ceacam1*-/- T cells with the aforementioned *in vitro* stimuli. Probing with the appropriate Abs would allow us to confirm the negative regulation observed for MAPKs, as well as the impact of the absence of CEACAM1-L on IL-2R signalling, and on the levels of SHP-1, SHP-2, of overall Tyr phosphorylation of the cells, and of the transcription factors AP-1 and NF-κB. Similarly, the effect of CEACAM1-4L overexpression on signalling in murine T cell functions could be studied, this time with the possibility to also carry out immunoprecipitation reactions of the components interacting with its cyt tail both in WT and CD2-CC1-L Tg T cells. Again, these two unique models would provide insight in this matter with minimal T cell manipulation. One of their limitations, however, is that they do not enable us to actually confirm/disrepute the reportedly opposite roles of CEACAM1-L and –S in these cells [64].

Another key point which remains to be addressed is the importance of the Tyr residues found within the ITIM motifs of CEACAM1-L in mediating its inhibitory effects. Had we been luckier, we would have done so with T cells derived from the CD2-FF Tg mice, designed to overexpress a dominant negative version of CEACAM1-4L in which both of the ITIM Tyr residues were converted to Phe. Due to the difficulties also

encountered with CD2-CC1-L Tg T cells, I performed retroviral infections in which WT and *Ceacam1*-/- $CD4^+$ T cells were infected with the bicistronic retrovirus pMIG [213], which encodes for GFP (used as subsequent cell-sorting marker), and in which I cloned either the WT or CEACAM1-4L-Y488,515F cDNA, with the following rationale: infection of *Ceacam1*-/- T cells with retroviruses containing WT CEACAM1-4L would "rescue" their phenotype, whereas that with retroviruses encoding for WT or mutated CEACAM1 in WT $CD4^+$ T cells would respectively "recreate" CD2-CC1-L and CD2-FF Tg T cells. Although a high number of $CD4^+$ T cells were infected with pMIG alone, retroviruses containing either cDNAs rarely infected more than 1% of them, resulting in poor T cell recovery after sorting. Similar retroviral experiments using the more readily infectable CEACAM1-devoid beef insulin (BI) hybridoma T cell line are currently under way to bypass this. Also, infections of these cells with a pMIG retrovirus containing the CEACAM1-4S cDNA, which I also cloned, would allow us to study the function of this isoform.

Moreover, our unique mouse models would allow us to delve into the role of CEACAM1 in Th1 and Th2 differentiation, as recent studies involving a murine model of colitis pointed out it modulated Th1 responses [188]. To do so, $CD4^+$ T cells from WT and *Ceacam1*-/- mice could be stimulated *in vitro* with anti-CD3 combined or not with anti-CD28, Con A, and PMA and ionomycin, to assess their proliferative response, and then quantify the release of IL-2, IL-4 and IFNγ by ELISA. Another approach is to carry out proliferation assays with OT-2:*Ceacam1*-/- $CD4^+$ T cells. This cross resulting from breeding OT-2 TCR transgenic mice, whose $CD4^+$ T cells recognize a peptide containing the residues 323-339 of chicken ovalbumin ($OVA_{323-339}$; ISQAVHAAHAEINEAGR) in the context of the MHC class II molecule $I\text{-}A^b$ [238, 239], with *Ceacam1*-/- mice is under way. Thus, not only this model enables us to study the effect of CEACAM1 specifically on this subset in a more physiological manner, but also to assess if CEACAM1 favours Th1 or Th2 polarization. Finally, Western blot analyses could be performed as described above with both the *Ceacam1*-/-, and OT-2:*Ceacam1*-/- $CD4^+$ T cells (and appropriate counterparts) to assess changes in Th1- and Th2-specific transcription factors levels, i.e. T-bet and GATA3 (Fig. 1-6) [212].

In addition, true *in vivo* experiments, which are lacking in the field, have to be performed to also further validate the observations we have acquired so far. In addition to the experiment described above involving the transfer of OT-1:*Ceacam1*-/- CTLs or their OT-1 counterparts into C57BL/6 recipients to assess how they would eliminate target cells also introduced in recipients, another approach would consist in injecting WT and *Ceacam1*-/- mice with an emulsion of chicken ovalbumin in complete Freund's adjuvant, and then isolating $CD4^+$ T cells from the neighbouring draining LN. These cells would then be cultured in the presence of different concentrations of chicken ovalbumin presented by APCs *in vitro*, and their proliferative response determined by tritiated thymidine incorporation, and the release of IL-2, IL-4, and IFNγ. assessed by ELISA. These experiments are also under way.

Finally, in light of our findings, a new avenue to explore would be the role of CEACAM1 in regulatory $CD4^+CD25^+$ T cells (Tregs), important inhibitors of both helper and cytotoxic T cell functions *in vitro* and *in vivo*. Accordingly, $CD4^+CD25^+$ Tregs have been implicated in the inhibition of immune responses during infections, as well as that of antitumor, and autoimmune responses [240]. Indeed, there are no reports to date regarding its expression and function in Tregs. Considering the evidence pointing at an inhibitory role of CEACAM1 in T cells, *trans* homophilic interactions between CEACAM1 on the surface of activated T cells and Tregs might represent a novel means by which they mediate their suppressor function. Moreover, the functions of CEACAM1 in NKT cells also deserves closer scrutiny, as it has previously been reported that it reduces the release of IFNγ by decidual NKT cells [63]. Considering that NKT cells are involved in pathogen clearance, tumor immunosurveillance, protection against autoimmunity, as well as in the exercise of regulatory functions in both innate and adaptive immunity [241], novel functions for CEACAM1 in these T cell subsets uncovered through studies of our *Ceacam1*-/- mouse model might potentially designate CEACAM1 as both an important therapeutic target in these processes and a key player in the regulation of immune responses.

8. Conclusion

Despite the original controversy, most recent studies have pinpointed a co-inhibitory role of CEACAM1 in T cell functions. Through the use of unique mouse models, namely *Ceacam1*-/- and OT-1:*Ceacam1*-/- mice, we gathered yet more evidence ascribing such a function to CEACAM1. Here, we report that CEACAM1 does not affect the development of memory T cells, and acts as a co-inhibitory receptor in CTL activation and proliferation, as well as in their production of IL-2, and IFNγ. We also characterized and carried out experiments with the CD2-CC1-L Tg mouse model, and report that as expected, T cells from these Tg mice overexpress CEACAM1 on their surfaces. Preliminary results revealed a hypoproliferative response of CD2-CC1-L Tg T cells *in vitro*, accompanied by a decrease in IL-2, as well as both IFNγ (Th1) and IL-4 (Th2) cytokine secretion compared to WT T cells. This was not always consistently reproducible in subsequent experiments. Thus, the results presented here and obtained so far by our group with these unique models hint at an inhibitory role of CEACAM1 in T cell functions, accompanied with possible implications in processes such as anti-viral responses, tumor immunosurveillance, and CTL-mediated autoimmunity. This would make CEACAM1, a molecule whose intercellular adhesion and signal regulatory functions are intertwined, a choice therapeutic target in these processes.

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APPENDIX