

CD40-CD40 ligand

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Abstract: CD40 is a cell surface receptor that belongs to the tumor necrosis factor-R (TNF-R) family, and that was first identified and functionally characterized on B lymphocytes. Its critical role in T cell-dependent humoral immune responses was demonstrated by patients with the hyper-IgM syndrome, as well as by gene targeting in mice. However, in recent years it has become clear that CD40 is expressed much more broadly, including expression on monocytes, dendritic cells, endothelial cells, and epithelial cells. In addition, the CD40-ligand (CD40-L/CD154), a member of the TNF family, is also expressed more widely than activated CD4⁺ T cells only. Therefore it is now thought that CD40-CD40-L interactions play a more general role in immune regulation. Collectively these studies have culminated in pre-clinical and clinical studies that are in progress. This article reviews recent developments in this field of research, with main emphasis on (1) structure and expression of CD40 and its ligand; (2) CD40 signal transduction; (3) *in vitro* function of CD40 on different cell types; and (4) *in vivo* functions of CD40/CD40-L interactions. *J. Leukoc. Biol.* 67: 2–17; 2000.

Key Words: CD154 · lymphocyte activation · T cells · B cells · dendritic cells · inflammation · cytokines

INTRODUCTION

After it was first identified in 1985, research on CD40 was initially focused around the regulation of humoral immune responses. CD40 was identified as a molecule expressed during all stages of B cell development and differentiation, whereas its ligand, CD40L (CD154, gp39, T-BAM, or TRAP), was mainly expressed on activated CD4⁺ T cells. The pivotal role of CD40-CD40L in T cell-dependent B cell responses was proven by the finding that patients suffering from the X-linked hyper-IgM syndrome (HIGM), were characterized by mutations in their CD40L gene. Similar deficiencies in mounting immune responses were observed in genetically modified mice with inactivation of either the CD40 or CD40L gene.

After this breakthrough finding, research on CD40-CD40L has expanded in various directions. It was found that CD40 expression was much broader than initially thought. In addition, CD40L expression appeared not to be restricted to the

CD4⁺ T cells. One major observation has been the expression of CD40 on monocytes and dendritic cells (DC). On DC, CD40 seems to be a critical step in the final maturation of this cell into a fully competent antigen-presenting cell. As a consequence, both HIGM patients and CD40/CD40L knockout mice show abnormalities in priming of naive CD4⁺ T cells. Recently, CD40-CD40L interaction was also shown to be necessary for cross-priming of cytotoxic T lymphocyte (CTL) responses by DC. Next, CD40 is widely expressed on non-hematopoietic cells, including endothelial cells, fibroblasts, and epithelial cells. On these cells, CD40 is involved in the amplification and regulation of inflammatory responses. In line with the above-described results, using either knockout animals or blocking anti-CD40L antibodies, interference with CD40-CD40L interaction was shown to be beneficial in several disease models, including transplantation, autoimmunity, and infectious diseases.

Although CD40 and CD40L are both produced as cell-bound molecules, they have several characteristics that make them part of the cytokine network. CD40-CD40L interactions play an important role in the production of several cytokines, including interleukin-12 (IL-12). Furthermore, both molecules have been described in a natural soluble form, and therefore could act at distant sites. Finally, both CD40 and CD40L belong to emerging receptor-ligand families that include cytokines: the tumor necrosis factor-R (TNF-R) and TNF family. Families are characterized by structural homologies, clustered chromosome location, shared signal transduction pathways, and overlapping biological activities in processes such as cell growth, differentiation, and death.

Here we will review the major and most recent findings on the CD40-CD40L receptor-ligand pair. We will first deal with gene, protein, and expression of CD40L (cytokine), followed by the structural information on CD40 (receptor). Next, signal transduction and *in vitro* consequences of CD40 cross-linking will be dealt with, followed by the pathophysiological role of CD40-CD40L *in vivo*. Finally, we will discuss some future directions and (clinical) applications. Because of space limitations, we will essentially refer to the most recent publications. For more detailed information readers are referred to several extensive overviews [1–5].

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CD40L GENE

A cDNA coding for CD40-L was isolated from the murine EL-4 thymoma cell line after enrichment of cells binding to a CD40-Fc fusion protein [6]. The murine cDNA predicts a polypeptide of 260 amino acids (AA) consisting of a 22-AA cytoplasmic domain, a 24-AA transmembrane domain, and a 214-AA extracellular domain with four cysteines (Genbank no. X65453). Murine CD40-L is a type II membrane protein that lacks an amino-terminal signal peptide and presents an extracellular carboxy terminus.

A human CD40-L cDNA has been isolated by screening stimulated human blood T cell libraries with the murine CD40-L probe, or independently from an activated human T cell library [7, 8]. The cDNA for human CD40-L encodes a polypeptide of 261 AA consisting of a 22-AA cytoplasmic domain, a 24-AA transmembrane domain, and a 215-AA extracellular domain with five cysteines (Genbank nos. Z15017, X67878, S49392, L07414).

Using primers based on the human CD40L sequence, located in the 5' and 3' untranslated region, full-length CD40L could be amplified from activated cattle (*Bos taurus*) peripheral blood lymphocytes (Genbank no. Z48469). The cDNA for cattle CD40-L encodes a polypeptide of 261 AA consisting of a 22-AA cytoplasmic domain, a 23-AA transmembrane domain, and a 216-AA extracellular domain with five cysteines.

The gene for human CD40-L is located at the X-chromosome, position Xq26.3-Xq27.1. The human CD40-L gene spans 12–13 kb of chromosomal DNA and consists of five exons (Fig. 1) [9]. The first exon codes for the intracellular, transmembrane, and a small portion of the extracellular region, whereas exons II–V code for the rest of the extracellular domain (Genbank no. D31793-D31797). This sequence also includes ~2000 bp of 5' promoter sequences. Establishment of the chromosomal location was instrumental for the identification of CD40-L mutations in patients suffering from X-linked Hyper IgM syndrome. Genomic clones containing murine CD40L have been isolated to generate CD40L knockout mice [10, 11]. The murine CD40L gene was reported to contain five exons, but no specific DNA sequences are available.

Northern blot analysis has demonstrated the presence of two mRNA species of 2.1 and 1.4 kb in activated human T cells [8], which differ in the length of the 3' untranslated ends. CD40-L mRNA has been detected in activated CD4⁺, CD8⁺, and $\gamma\delta$ T cells. In addition, Northern blot analysis has demonstrated

specific hybridization signals, including alternative sizes of 3.7 and 1.7 kb, in several other cell types such as monocytes, DC, B cells, mast cells, basophils, and eosinophils [3]. Finally, mRNA expression of CD40L has been demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) in natural killer (NK) cells, endothelial cells, and the megakaryoblastic lines MEG-01 and UT-7 [12, 13].

CD40L PROTEIN

Although initial studies suggested a molecular mass of 39 kDa, hence gp39, on most cell types the molecular mass of CD40L is 32–33 kDa. The amino acid backbone predicts a protein of 29 kDa, which suggest that posttranslation modifications take place. Indeed, human-, murine-, and cattle-CD40L have conserved a single N-linked glycosylation site (human: Asn240) in the extracellular region. Comparison of human CD40L (Swissprot no. P29965) and mouse CD40L (Swissprot no. P27548) show that both sequences exhibit 78% AA identity. There is 75% identity in the extracellular domain, 96% between the transmembrane region, and 81% between the cytoplasmic domains.

The structure of the TNF-like region of human CD40L (a soluble form spanning from Gly116 to Leu261) has been resolved by X-ray crystallography at a resolution of 2 Å [14]. CD40L is a sandwich of two β sheets with jelly-roll topology and forms a threefold symmetric homotrimer (Brookhaven no. 1ALY). The three-dimensional organization is similar to that described for the TNF- α and LT α proteins and as predicted by initial modeling studies (Fig. 2).

Although CD40L is produced as a type II transmembrane protein, CD40L may be expressed on the cell surface as a heteromultimeric complex [15]. Apart from its 33-kDa form, the molecule is associated with two shorter versions of the protein of 31 and/or 18 kDa [16, 17]. These shorter soluble forms of CD40L retain their ability to form trimers, to bind CD40, and to deliver biological signals, thus indicating that CD40L might also act as a bona fide cytokine [16–18]. Qualitative differences between the soluble and membrane-bound forms may yet exist.

CD40-L is expressed on activated mature T cells but not on resting T cells, as demonstrated with either CD40-Ig fusion proteins or specific monoclonal antibodies. CD40-L can be induced on Th0, Th1, and Th2 cells and is primarily restricted to CD4⁺ T cells, although a small population of CD8⁺ T cells

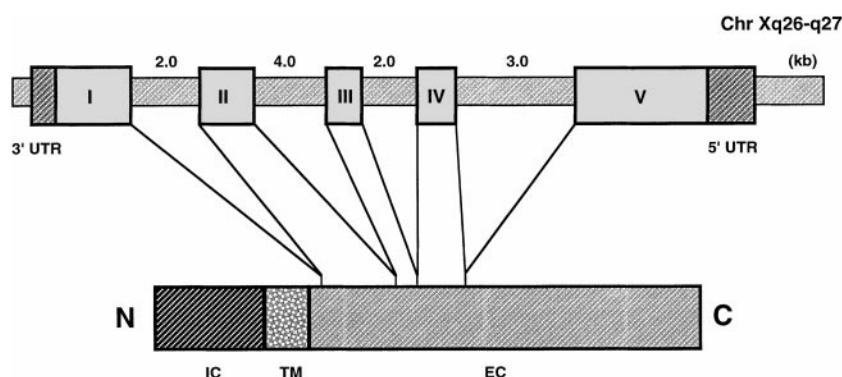
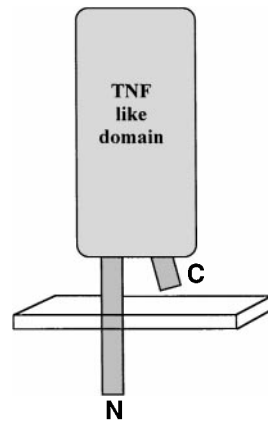
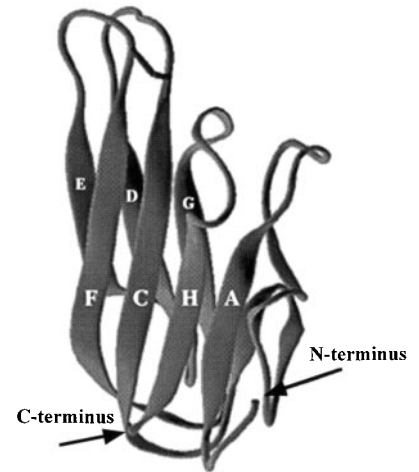


Fig. 1. Gene structure of human CD40L. Schematic representation of the chromosomal organization of the human CD40L gene located at chromosome Xq26-q27. The gene consists of 5 exons, with exon I coding for the intracellular (IC) and transmembrane (TM) region, and exons II–V for the extracellular (EC) region. The size (in kb) of the intervening introns is given on top.

A

Fig. 2. Structure of the CD40L protein. (A) Schematic representation of the CD40L protein, which is a type II transmembrane protein with an intracellular amino terminus and an external carboxy terminus. The extracellular region of CD40L shows structural homology with other members of the TNF family. (B) Representation of the extracellular part of a single CD40L molecule as determined by X-ray diffraction. Crystallization of the protein showed that the molecule forms a homotrimer. The structure of the molecule can be looked at in three dimensions (Brookhaven #1ALY).

**B**

also expresses CD40-L. CD40-L is also expressed on activated CD4⁺CD8⁺γδTCR⁺ T cells, but with lower expression levels than for αβTCR⁺ T cells.

Expression of CD40-L has been detected on cells other than T lymphocytes. Both primary cells and cell lines of mast cells, basophils, and eosinophils stain positive with CD40-Ig or CD40L antibodies. Similarly, expression of CD40L has been demonstrated on B cells and B cell lines, NK cells, monocytes/macrophages, and DC under certain conditions [12, 19–21]. Finally, pre-formed CD40L was demonstrated to be present in intracellular stores of thrombocytes [13].

Because secondary immune responses are induced in lymphoid organs, and the hyper-IgM syndrome has proven the pivotal role of CD40L within this process, one would expect CD40L expression at these anatomical locations. However, expression of CD40L in lymphoid organs has been controversial. Immunohistochemistry has demonstrated that human CD40-L is expressed on CD3⁺ CD4⁺ T lymphocytes of the germinal center light zone of secondary follicles in all peripheral lymphoid tissues and the interfollicular T cell-rich areas [22, 23]. In contrast, in murine spleen isolated 3–4 days after immunization with the thymus-dependent antigen KLH, CD4⁺ CD40-L⁺ T cells are found in and around the terminal arterioles, and on the periphery of the outer periarteriolar lymphoid sheath, but not in germinal centers [24]. It can be hypothesized that expression of CD40L should be tightly regulated, especially within lymphoid organs, to prevent unwanted bystander activation. Therefore, subtle differences might contribute to the pattern of CD40L expression observed. It is interesting that investigation of CD40L in other tissues showed that, especially under inflammatory conditions, a very strong CD40L expression can be found in atherosclerotic lesions and renal allograft rejection (**Fig. 3**).

REGULATION CD40L EXPRESSION

The expression of CD40-L on activated T cells is transient and tightly regulated. CD40-L expression can be seen on a subpopulation of CD4⁺CD45RO⁺ T cells, as early as 5–15 min after anti-CD3 activation [23]. Such surface expression does not

require *de novo* protein synthesis and probably consists of preformed CD40-L. A second wave of CD40-L expression, which does need protein synthesis and is preceded by increased mRNA levels, can be detected on all CD4⁺ T cells. Expression occurs early (1–2 h) after activation, is maximal after 6–8 h, and is followed by a gradual loss. Apart from this intrinsic transient expression, additional mechanisms operate to insure that CD40-L expression remains restricted in time: CD40-CD40-L interaction leads to (1) receptor-mediated endocytosis of CD40L and lysosomal degradation; (2) release of sCD40, which binds to CD40-L; (3) proteolytic cleavage of CD40-L; (4) down-regulation of CD40-L mRNA.

Multiple modes of polyclonal T cell activation have been shown to induce CD40-L expression *in vitro*, including phytohemagglutinin (PHA), phorbol myristate acetate (PMA) plus ionomycin, anti-CD3, and anti-CD2 antibodies. In addition, CD28 engagement further augments anti-CD3-induced CD40-L expression and subsequently stabilizes this CD40L expression [25]. Pretreatment of T cells with cyclosporin A or glucocorti-

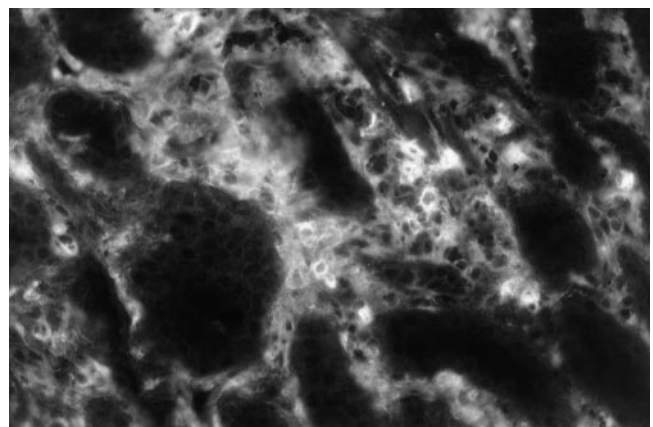


Fig. 3. Expression of CD40L during renal transplant rejection. Immunofluorescence staining of cryosections of human kidney biopsies taken during rejection after allograft transplantation. Sections were stained with specific anti-CD40L monoclonal antibody. Strong staining of a significant proportion of the lymphocytes infiltrating the graft can be seen. No staining was found on control normal renal tissue or with an isotype-matched control antibody (original magnification ×100).

coids prevents *in vitro* induction of CD40-L expression [26]. However, transplant patients receiving such immunosuppressive drugs still show immunostaining of CD40L in grafts undergoing rejection.

Less information is available on the physiological signals involved in CD40L expression on other cell types. Most studies have used PMA plus ionomycin for the stimulation of B cells, mast cells, and basophils. It is important that thrombin activation of human platelets resulted in CD40L surface expression within 1 min [13].

CD40 GENE AND PROTEIN

A cDNA encoding human CD40 was isolated by expression cloning from a library of the Burkitt lymphoma Raji [27]. The mature molecule is composed of 277 AA with a 193-AA extracellular domain, including a 21-AA leader sequence, a 22-AA transmembrane domain, and a 62-AA intracellular tail (Genbank no. X60592). Therefore, CD40 has the structure of a typical type I transmembrane protein. The human CD40 gene is expressed as a single 1.5-kb mRNA species.

The murine cDNA was cloned from lipopolysaccharide (LPS) + IL-4-stimulated murine B cells by cross-hybridization with the human cDNA probe [28]. The mouse CD40 protein is composed of 305 AA with a 193-AA extracellular region, including a 21-AA leader sequence, a 22-AA transmembrane domain, and a 90-AA intracellular region (Genbank no. M83312). Human and murine CD40 molecules share 62% amino acid identity in the complete coding sequence and 78% identity in the intracellular extensions. The last 32 carboxyl-terminal AA of human CD40 are completely conserved in the mouse sequence. In addition 22/22 extracellular cysteine residues are conserved, suggesting that both mouse and human CD40 fold into the same protein domains. Due to alternative polyadenylation, the mouse CD40 gene is expressed as two mRNA species of 1.7 and 1.4 kb.

Using degenerate primers, a partial cDNA has been isolated

from activated peripheral blood mononuclear cells (PBMC) of *B. taurus* (Genbank no. U57745). This cDNA codes for the complete extracellular and transmembrane region, but lacks approximately 30 bp of the 3' intracellular region. Also, bovine CD40 has conserved the extracellular cysteine residues important for the structure of the protein.

The CD40 murine gene consists of nine exons and spans a total region of 16.3 kb genomic DNA (**Fig. 4**) [29]. For murine CD40, the signal peptide, the transmembrane region, and the cytoplasmic tail are coded for by exons separate from the cysteine repeats. However, the cysteine repeat domains are not coded for by separate exons. The mouse CD40 gene is located on the distal region of chromosome 2, which is syntenic to human chromosome 20q11-q13 [29]. Accordingly, the human CD40 gene was mapped to chromosome 20 by using human-rodent somatic cell hybrids and to 20 q11-20q13-2 by *in situ* hybridization.

The CD40 antigen is a phosphorylated glycoprotein that migrates in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as a 48-kDa polypeptide under both reducing and non-reducing conditions. It is a hydrophobic molecule with an acidic pI of 3.2. The molecule has two (Asn153, Asn180) conserved N-linked glycosylation sites (Swissprot no. P25942). The intracellular region of CD40 does not betray a close relationship to any other characterized molecule.

The extracellular segment of CD40, in particular the 22 cysteine residues, are homologous to other members of the TNF-R family. Initially it was suggested that they form 4 predicted protein domains of about 40 AA. More recently the modularity of the TNF-receptor family has been refined, suggesting that every domain is built of two modules, with five different forms of modules being identified (**Fig. 5**) [30]. Although the structure of CD40 has not been resolved, a model has been built through the use of homology modeling, mutagenesis, X-ray structures of TNF-R, and alignment of the TNF-R family [31]. Combined with the structure of CD40L, it has been suggested that the interaction between the CD40 and CD40L molecules are based on the following charged residues: CD40

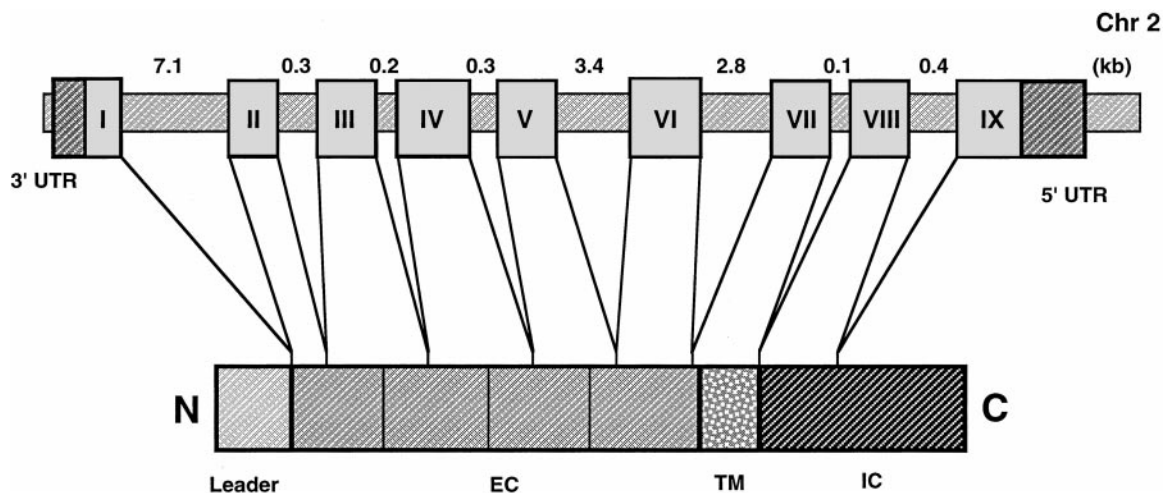


Fig. 4. Gene structure of murine CD40. Schematic representation of the chromosomal organization of the murine CD40 gene located at chromosome 2. The gene consists of 9 exons, with exon I coding for the leader sequence, exons II–VI for the extracellular region (EC), exon VII for the transmembrane (TM) region, and exons VIII–IX for the intracellular region. The size (in kb) of the intervening introns is given on top.

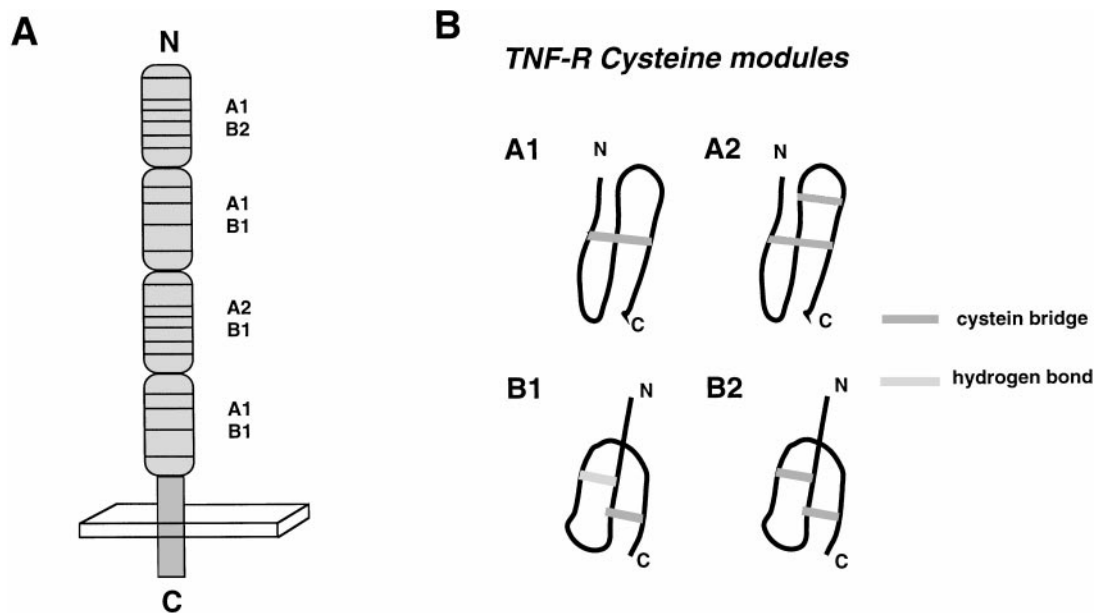


Fig. 5. Structure of CD40 protein. (A) Schematic representation of the CD40 protein, which is a type I transmembrane receptor. The extracellular region of CD40 is cysteine-rich (20 residues), as indicated by the horizontal lines. Initially, four cysteine-rich domains were identified. Subsequent studies (see text) have suggested that every domain can be divided into two cysteine modules (A1, A2, B1, B2), as indicated. (B) Model of the four different cysteine modules that form the building blocks of the TNF-R homologous region of the CD40 molecule. Chains are connected by one or two cysteine bridges as indicated in red, whereas in the B1 module, an additional hydrogen bond (in green) is formed. For details see review by Naismith and Sprang [30].

(acidic D84, E114, E117) and CD40L (basic K143, R203, R207). These results are mostly compatible with earlier mutagenesis studies identifying the following residues important for CD40-CD40L interactions: CD40 (E74, Y82, D84, N86, E117) and CD40L (K143, Y145, Y146, R203, Q220) [32, 33]. Further insight in the structural requirements for CD40L will be obtained by the databank that collects the natural mutations found in hyper-IgM patients [34] (<http://expasy.hcuge.ch/www/cd40lbase.html>).

SIGNAL TRANSDUCTION VIA CD40

Kinases

Studies on CD40 signal transduction have resulted in a complex picture of the different mediators and pathways involved. Although CD40 has no kinase domain, CD40 ligation activates several second messenger systems. Among the earliest detectable events after CD40 activation are the activation of protein tyrosine kinases (PTK; including *lyn*, *syk*, and *Jak3*), activation of phosphoinositide-3 kinase (PI-3 kinase) and phospholipase C γ 2. Activation of protein kinase A (PKA) has been controversial, but it has been shown that cAMP can modulate both positive and negative CD40-induced responses [35]. In recent years many studies have concentrated on the involvement of serine/threonine kinases: stress-activated protein kinase/c-jun amino-terminal kinase (JNK/SAPK), p38 MAPK, and extracellular signal-regulated mitogen-activated protein kinase (ERK). Contrasting results have been obtained regarding JNK, p38, or ERK activation [36–39]. However, general conclusions should be taken with care because these studies often have used different cellular models. For instance activation of different B cell subsets results in opposite

biological effects, depending on the differentiation/activation stage of the cells, which is probably a reflection of different routes of signal transduction.

TRAFs

Coupling of the CD40 receptor to different signaling pathways has been better understood by the identification of a new family of associated proteins: TRAF (TNF-R associated factor). With the two-hybrid system, such protein-protein interactions have been demonstrated for several members of the TNF-R family. At the moment six different members of the TRAF family have been identified. It is interesting that, although there is no cross reactivity between the extracellular ligands of the TNF-R family (with the exception of TNF and LT), the intracellular ligands seem to be much more promiscuous and form a complex network of homo- and heterodimers. Mutagenesis of the intracellular part of CD40 has provided more insight into the coupling to different signal transduction pathways (Fig. 6).

The first member identified as a protein associated with CD40 has been TRAF3, also identified under the name CRAF1, CD40bp, LAP1, and CAP1 [40]. TRAF3 is a 62-kDa intracellular protein that is expressed in almost all cell types. The protein contains several functional domains involved either in signal transduction (RING finger domain, isoleucine zipper, zinc finger) or in protein-protein interactions either to associate with CD40 or to form homo-/heterodimers. TRAF3 knockout mice show an apparently normal signaling in B cells, whereas T cell activation is impaired, already pointing at the redundancy of the TRAF molecules [41].

CD40 also associates with TRAF2, a molecule that interacts with TNF-R2. Induction of NF- κ B activation after CD40 cross-linking can in large part be attributed to TRAF2 signaling. Both a dominant-negative mutant of TRAF2, lacking an

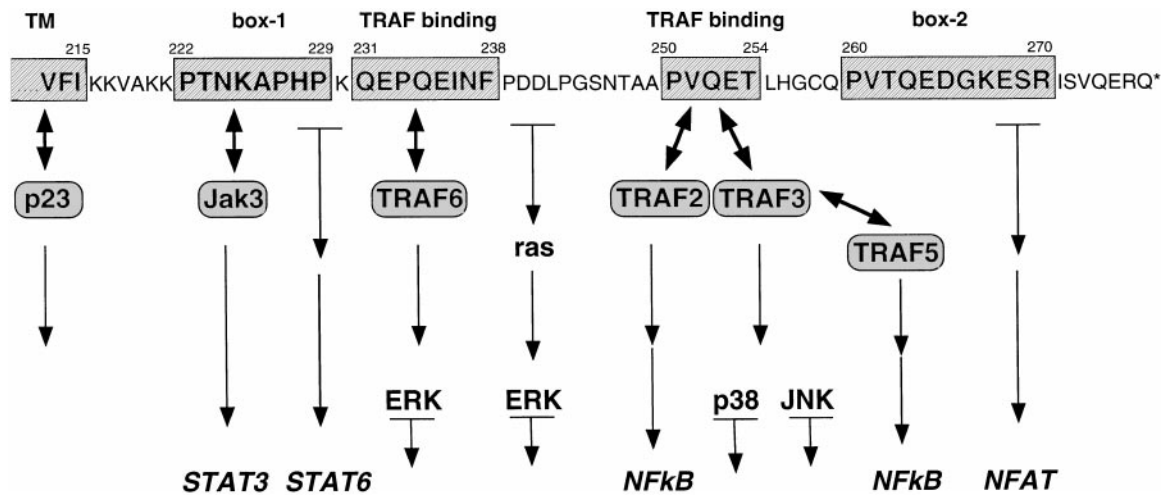


Fig. 6. Signaling pathways coupled to the CD40 intracellular region. Schematic representation of the intracellular region of the human CD40 molecule in one-letter amino acid code. The numbering of the amino acid given on top are according to Stamenkovic et al. [27] (Genbank no. X60592). The carboxy-terminal part of the transmembrane region (TM) is boxed in blue and intracellular recognition sites that were identified by various groups (see text), have also been boxed. Direct protein-protein interaction (<->) between CD40 and p23, Jak3, and different TRAF members is shown. The different pathways of kinases and transcription factors, which become subsequently activated, are schematically drawn.

amino-terminal RING finger domain, or a natural inhibitor protein called I-TRAF, can prevent NF- κ B activation [42, 43]. Crystallography of the TRAF domain of TRAF2 showed a spontaneous trimeric self-association, suggesting that the need for receptor trimerization might be based on the avidity for the TRAF2 trimer [44, 45]. Finally, two other TRAF proteins, TRAF5 and TRAF6, were also demonstrated to associate with the CD40 receptor [46, 47]. Inactivation of the TRAF5 gene resulted in hampered B cell proliferation and up-regulation of various surface receptors [48].

Early mutagenesis studies already suggested a critical role for the Thr residue at position 254 (complete coding sequence), and this residue was implicated in TRAF3 binding [49]. Deletion mutants of the intracellular region indeed showed association of TRAF2, TRAF3, and TRAF5 to one region (residue 246–269), whereas TRAF6 associated with a separate domain (residue 230–245) [47]. In accordance, pepscan analysis identified PVQET (residue 250–254) as the minimal unit for TRAF1, TRAF2, and TRAF3 binding, and QEPQEINF (residue 231–238) as the minimum for TRAF6 binding [50]. In this study, TRAF5 binding could only be demonstrated indirectly via TRAF3.

The various deletion mutants have been instrumental in unraveling the network of molecules associated, kinases activated, and genes induced. Using wild-type CD40 and a mutant only containing the binding site for TRAF6 (CD40 δ 246), it was demonstrated that CD40 activates ERK both by a ras-dependent pathway and a ras-independent pathway involving TRAF6 [51]. The various data have resulted in the model depicted in Figure 6.

TRAF3 was also cloned by screening for proteins that can interact with the Epstein-Barr virus (EBV) transforming gene product LMP1 (latent infection membrane protein-1) [52]. Physiologically, TRAF-mediated signals will be launched by trimerization of surface receptors. After EBV transformation, it is likely that the oligomerization of these molecules is obtained by the spontaneous aggregation capacity of the LMP1 molecule.

This indicates that EBV utilizes the CD40 signaling pathway to activate and immortalize B lymphocytes. Continuous expression of LMP1 is essential for the proliferation of the EBV-immortalized B cells [53]. Generation of chimeric proteins suggest that CD40-induced and LMP1-induced pathways are completely overlapping [54].

In conclusion, models have been generated based on experiments of induced overexpression or protein-protein interactions in the test tube. However, most data have to be confirmed in the natural CD40-expressing cells. A direct and constitutive association with CD40 has been described for Jak3, TRAF2 [55, 56]. It is interesting that CD40 activation seemed to diminish the amount of TRAF2 associated with CD40 [55], whereas the relative recruitment of TRAF2 versus TRAF3 is determined by concomitant signals [57], introducing another level of complexity.

GENE ACTIVATION

After early biochemical changes, these signals are translated into the activation of specific transcription factors that drive further gene activations. Cross-linking of CD40 on B cells and other cells results in activation of NF- κ B and NF- κ B-like transcription factors. Gel retardation assays showed the presence of, at least, the NF- κ B family members p50, p65 (relA), and c-Rel. Activation of NF- κ B has been described as a downstream effect of various members of the TRAF family (TRAF2, 5, 6) [58]. CD40-induced NF- κ B activation is at least partially mediated by a dramatic decrease in the half life of both I κ B- α and I κ B- β in a proteasome-dependent manner [59].

Next to NF- κ B, CD40 cross-linking results in the expression/activation of other transcription factors. CD40 activation results in nuclear expression of AP1 and NF-AT. It is interesting that NF-AT induction after CD40 ligation is CsA/FK506 resistant, which is different from the CsA/FK506-sensitive NF-AT induction after anti-Ig triggering. CD40 cross-linking was demon-

strated to activate members of the Jak-STAT pathway, a route important for signaling of various cytokines. CD40 is associated with Jak3, and activation leads to phosphorylation of STAT3 [56]. In addition, CD40 triggering can activate the STAT6 transcription factor [60].

Activation of CD40 has important effects on the biological function of the various cells expressing CD40. Therefore expression of key molecules involved in these biological responses is modulated by CD40 triggering. Especially in B cells, CD40 activation also directly contributes to genetic modifications: isotype switching of immunoglobulin genes and introduction of somatic mutations. In conclusion, many response genes are induced, directly or indirectly, after launching the CD40 pathway. More compelling information on the wide variety of genes induced will most likely be obtained in the near future by the use of microchip cDNA arrays.

BIOLOGICAL EFFECTS *IN VITRO*

B lymphocytes

Extensive studies on CD40 activation of B cells *in vitro* have demonstrated that CD40 activation has major effects on many steps of the B cell natural history [1, 2, 4]. CD40 activates proliferation, differentiation, and Ig production of immature and mature B cell subsets. In addition, CD40 can induce re-expression of telomerase activity in memory B cells, thereby contributing to an expanded lifespan of these cells [61]. CD40 activation guides the B cells through their differentiation program, including rescue from apoptosis, differentiation into

germinal center cells, isotype switching, selection, and maturation into memory cells. However, CD40 prevents the terminal differentiation of activated mature B cells into plasma cells [62, 63]. Most of these processes take place in specialized anatomical structures: germinal centers. *In vitro* studies of these different steps have been greatly facilitated by the characterization and isolation of discrete B cell subsets that recapitulate the essential steps of T cell-dependent B cell maturation (**Fig. 7**) [64].

In vitro studies have shown direct effects of CD40 activation on cytokine production (IL-6, IL-10, TNF- α , LT- α), expression of adhesion molecules and costimulatory receptors (ICAM, CD23, B7.1/CD80, B7.2/CD86), and increased expression of MHC class I, MHC class II, and TAP transporter by B lymphocytes [65]. These molecules, which all contribute to the biological function of the B cells, have also provided the tools to study signal transduction pathways in more detail. For most processes, CD40 acts in concert with either cytokines or other receptor-ligand interactions.

An important example of such cooperation is the process of isotype switching, which is initiated by CD40, but for which the specificity of the isotype is determined by cytokines. The development of switch substrate retroviruses will allow a more detailed analysis of the switching process [66]. In humans, IL-4/IL-13 induce the switch to IgE and IgG4, which is fine-tuned by other cytokines. IL-7 (via the induction of IL-9 and sCD23) might enhance class switching to IgE and IgG4 [67], whereas addition of IL-10 inhibits IgE, but promotes IgG4 production [68]. Switch to IgG1 and IgG3 is induced by IL-10, whereas IgG2 is induced by an as yet unidentified T cell factor. Finally, the switch to IgA production is promoted by a

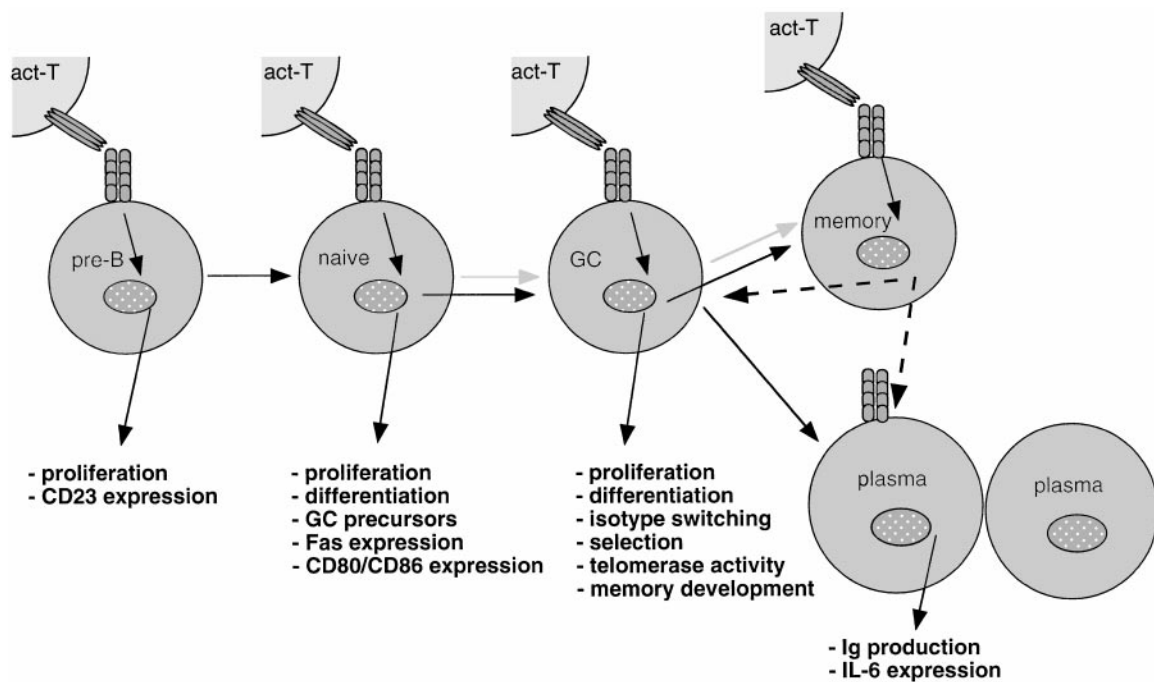


Fig. 7. Biological effects of CD40 activation on different stages of B cell development. This figure indicates the consequences when CD40L-expressing T cells interact with pre-B cells, naive B cells, germinal center (GC) B cells, or memory B cells. Transition from naive to GC and from GC to memory (green arrows) is thought to be promoted by CD40 activation. CD40 activation of memory B cells can either lead to a new round of germinal center reaction and/or an accelerated differentiation into Ig-producing plasma cells. Although both CD40⁺ and CD40⁻ plasma cells have been identified, the relationship between these two cells is not firmly established.

combination of IL-10 plus transforming growth factor β (TGF- β). More recently, it has become clear that other molecular interactions, like interaction with DC, also might enhance the level of Ig production [69], or even directly promote the switch to a specific isotype (IgA) [70]. In contrast, CD30, another member of the TNF-R family, seems to be a CD40-inducible negative regulator of isotype switching [71].

For the regulation of selection of high-affinity memory B cells, a complex network of interactions exists involving CD40, Fas, B cell antigen receptor (BCR), and cytokines (**Fig. 8**). CD40 cross-linking induces Fas expression and sensitivity for Fas-mediated apoptosis. However, the simultaneous triggering of the BCR increases the resistance to Fas-induced apoptosis [72]. In contrast, apoptosis of germinal center B cells can be induced by prolonged cross-linking of the BCR, an effect that can be prevented by the addition of IL-4 [73]. The interplay of these various signaling pathways has been confirmed *in vivo* using HEL transgenic mice [74]. It is interesting that BCR activation also seems to block the induction of CD30, thereby not only providing a safeguard to prevent selection, but also unwanted isotype switching of non-antigen-selected B cells [71].

Monocytes and DC

The expression of CD40 on antigen-presenting cells (APCs) like monocytes and DC is now well established [75, 76]. CD40 ligation on monocytes and DC results in: (1) an enhanced survival of these cells; (2) the secretion of cytokines (such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF- α , MIP-1 α) and enzymes such as matrix metalloproteinase (MMP); (3) enhanced monocyte tumoricidal activity; (4) NO synthesis. In addition, CD40

ligation considerably alters these APCs' phenotype by up-regulating the expression of costimulatory molecules such as CD54/ICAM-1, CD58/LFA-3, CD80/B7-1, and CD86/B7-2. Therefore the interaction between CD40 and CD40L has important consequences for both APC function and T cell function (**Fig. 9**). Activation of the CD40 receptors is one of the critical signals that allow the full maturation of DC into the most powerful APCs [77]. Although increased IL-12 secretion and CD80/CD86 expression will have an important impact on T cell activation, it is most likely that additional CD40 response genes contribute to the unique features of DC. Initial gene fishing techniques have already identified several new genes expressed after CD40 activation [78].

It is interesting that CD40 appears to contribute to the ontogeny of these cells because CD40 ligation of CD34⁺ hematopoietic progenitors induces their proliferation as well as differentiation into cells with prominent DC attributes [79].

Interrupting CD40/CD40-L interactions *in vitro* during T cell/DC cocultures results in reduced T cell proliferation. However, *in vivo* and *in vitro* studies have established that CD40/CD40L interactions can be bidirectional. Cross-linking CD40L *in vivo* contributes to the generation of helper function and germinal centers [80]. In accordance, *in vitro* ligation of CD40L on T cells considerably enhances their cytokine production [81]. Thus, inhibition of T cell responses can be the consequence of both altered CD40 signaling to the APCs and/or altered CD40-L signaling to the T cells.

Endothelial cells, epithelial cells, and fibroblasts

Apart from hematopoietic cells, the expression of CD40 has now been observed on many other cell lineages (**Table 1**).

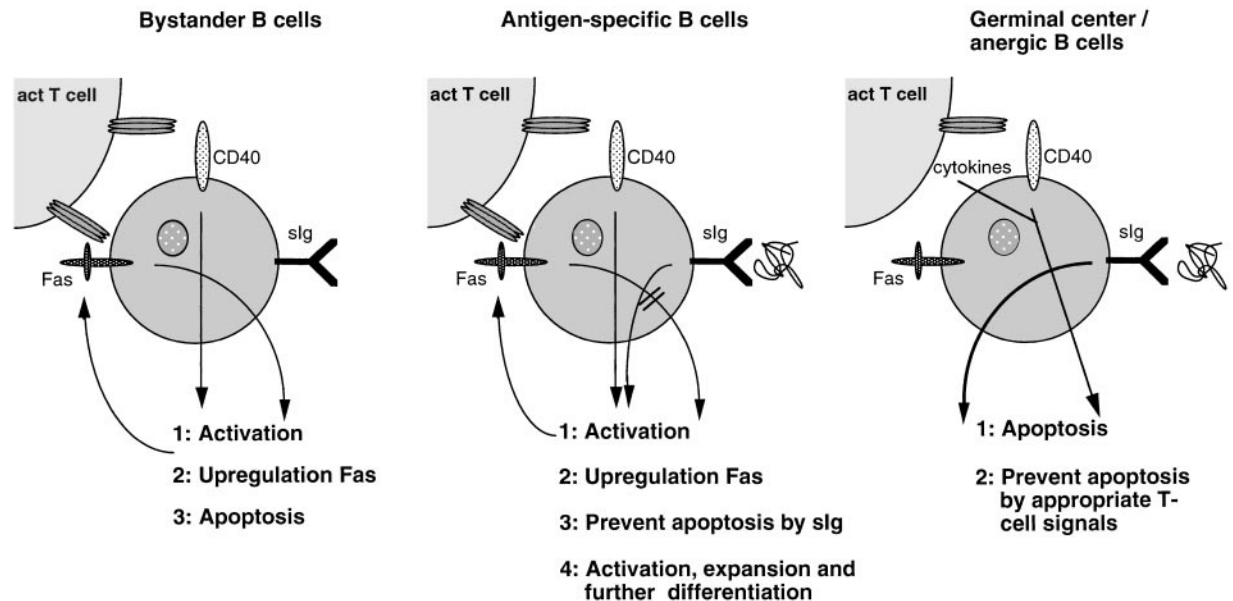


Fig. 8. Dual role of CD40 and surface Ig in B cell activation and selection. Model of the complex interactions between CD40, surface Ig (sIg), and Fas signaling and its differential effect on different subsets of B cells. (A) Activation via CD40 of antigen-nonspecific bystander B cells leads to cellular activation and induction/increase of Fas expression. Subsequent interaction with Fas-ligand on the activated T cell will induce apoptosis of the B cell and will thus terminate expansion of these B cells. (B) Antigen-specific B cells will be activated in a similar way, leading to cell activation and increased Fas expression. However, subsequent induction of apoptosis via Fas-FasL is prevented by a signal delivered by the surface Ig receptor (BCR), therefore leading to further activation and differentiation processes such as isotype switching. (C) Germinal center B cells, as well as anergic B cells obtained in different tolerance models, seem to go through a phase where cross-linking of the BCR results in an active induction of apoptosis. This induction of apoptosis seems to be independent of Fas-FasL. Appropriate T cell signals, such as a combination of CD40L and cytokines (IL-4) are able to prevent this form of apoptosis, thus allowing a further activation of these B cells.

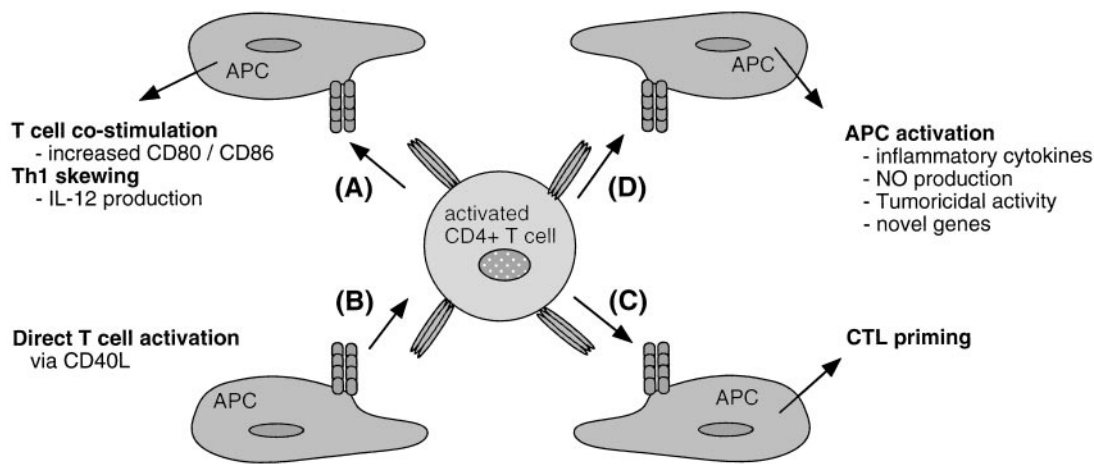


Fig. 9. Role of CD40-CD40L in the interaction between T cells and professional APC. (A, B) Activation of CD40 on APC results in strongly increased CD80/CD86 expression and induction of IL-12 production, thereby enhancing T cell costimulation and possible skewing of Th cells in the Th1 direction. On the other hand, CD40-CD40L interaction might also have a direct CD40L-mediated effect on T cell activation and T cell cytokine production via an as yet poorly defined signaling mechanisms. (C) In addition, it has been demonstrated that CD40 activation of APC/DCs is a crucial step in the process of cross-priming of CTL responses. At present it is not clear which molecular mechanisms are underlying this process. (D) CD40 activation of APC has additional effects, including production of inflammatory mediators, as well as several newly identified gene products with unknown function. These factors will be of importance for the local effector function of the APC.

Although the expression is generally low in normal tissues, the molecule is clearly up-regulated under various pathological conditions. Accordingly, expression can be observed on many cultured cell types including endothelial cells, epithelial cells, and fibroblasts. Increased expression of CD40 is observed after *in vitro* activation, notably after stimulation with IL-1 or interferon- γ (IFN- γ). The functional relevance of the CD40 molecule on these cells is relatively less investigated.

In patients suffering from the HIGM syndrome, as well as in CD40 and CD40L knock-out mice, no gross abnormalities are observed in organs that have been demonstrated to express CD40. However, it has been suggested that the inability to ligate CD40 on biliary epithelial cells may contribute to a defective defense against intracellular pathogens and a subsequent higher incidence of carcinomas in HIGM patients [82]. Alternatively, on some cell types, including carcinomas, it has been demonstrated that CD40 activation results in an induction of cell death [83–85].

Most likely, CD40 expression on non-hematopoietic cells might be relevant for inflammatory responses. Initial *in vitro* studies, using various cell types (Table 1), have demonstrated prominent effects of CD40 cross-linking on the production of cytokines and chemokines. However, other inflammatory mediators are also increased, including expression of adhesion molecules, production of matrix metalloproteinases (MMP), and procoagulant activity. The latter finding might be important in view of the observation that activated thrombocytes can express a preformed form of CD40L [13].

PATHOPHYSIOLOGICAL ROLES AND EFFECTS *IN VIVO*

CD40-CD40L: humoral immune responses

The demonstration of the critical role of CD40/CD40-L interactions *in vivo* came from the discovery that the hyper IgM

syndrome, an X-linked immunodeficiency, is due to a genetic alteration of the CD40-L gene [34]. This disease is characterized by a severe impairment of T cell-dependent antibody responses with no B cell memory, deficient induction of somatic mutations, and little or no circulating IgG, IgA, or IgE antibodies. The role of CD40L for B cell proliferation and germinal center formation was demonstrated *in vivo* by simultaneous tracking of antigen-specific T and B cells after immunization [86]. The generation of CD40 and CD40-L knockout mice revealed a phenotype comparable to that of the patients suffering from the hyper IgM syndrome [10, 11, 87].

In addition to patients with the X-linked form of hyper-IgM, who have mutations in CD40L and normal B cells, other patients have been described who have a similar immunodeficiency caused by deficiencies in CD40 signaling [88]. A perturbation of CD40L expression on T cells has also been suggested in B cell chronic lymphocytic leukemia [89], whereas others suggested excessive CD40L expression on B cells of this malignancy [90].

In view of the critical role of CD40-CD40L interactions in humoral immune responses, strategies have been developed to either decrease or increase these responses. Blocking anti-CD40L antibodies have been used in animal models to inhibit situations of unwanted antibody production. Anti-CD40L treatment inhibits production of autoantibodies in models of collagen-induced arthritis, systemic lupus erythematosus (SLE) nephritis, and experimental autoimmune encephalomyelitis (EAE). Furthermore, anti-CD40L treatment has been successfully used to prolong the expression of recombinant genes after gene transfer by preventing the development of neutralizing antibodies to adenoviral vectors. On the other hand, administration of agonistic anti-CD40 antibodies can result in strong, isotype-switched, antibody responses against pneumococcal polysaccharides [91]. Similarly, an expression plasmid containing the CD40L-trimer has been administered together with *Leishmania* Ag, and was successful in controlling infection with *Leishmania*

TABLE 1. Cells Expressing CD40 and Functional Consequences of *In Vitro* CD40 Activation

Cell type	Functional consequences
Pre-B cells	proliferation CD23 expression
Naive mature B cells	proliferation, differentiation isotype switch
Germinal center B cells	proliferation, differentiation Fas expression, selection
Plasma cells	IL-6 production
Monocytes/macrophages	cytokine secretion NO production production metalloproteinases monocyte procoagulant activity and tissue factor expression
Synovial monocytes	TNF secretion
Alveolar macrophages	high-level CD40 expression in sarcoidosis
Dendritic cells	growth and survival expression costimulatory molecules enhanced cytokine production see dendritic cells
Langerhans cells	proliferation
CD34 ⁺ precursors	development dendritic cells
T cells	proliferation, CD25 expression cytokine production
Eosinophils	enhanced survival GM-CSF production
Endothelial cells – HUVEC	up-regulation CD54, CD62E, CD106 increase tissue factor/thrombomodulin expression, and proagulant activity T cell costimulation increased production LIF, IL-6, GM-CSF
Microvascular endothelial	CD40 expression increased by HIV
Vascular EC (saphenous veins)	increased IL-1, IL-6, IL-8 production
Epithelial cells	
Thymic epithelial cells	GM-CSF production
Kidney epithelial cells	cytokine/chemokine secretion; IL-6, LIF, GM-CSF IL-8, MCP-1, RANTES
Keratinocytes	enhanced expression CD54, Bcl-x IL-8 secretion
Carcinomas and transformed epithelial cells	growth inhibition/apoptosis
Fibroblasts	
Synoviocytes	proliferation cytokine production (GM-CSF, MIP-1 α)
Lung fibroblasts	increased IL-6, IL-8, NF- κ B increased Cox-2, PGE2
Dermal fibroblasts	increased IL-6, IL-8, NF- κ B
Thyroid fibroblasts	increased IL-6, IL-8, NF- κ B
Gingival fibroblasts	increased IL-6, IL-8
Others	
Follicular dendritic cells	growth CD54 expression

TABLE 1. (Continued)

Cell type	Functional consequences
Vascular smooth muscle cells	increased MMP-1, MMP-3, MMP-9, MMP-2 activation ICE (IL-1 β converting enzyme) induction stromelysin-3 (MMP- 11)
Hepatocytes	induction of Fas-L expression

major [92]. Therefore, administration of CD40-stimulating agents might be used for future vaccination strategies against weakly immunogenic, T cell-independent antigens, or to boost immune responses in immunocompromised individuals.

CD40-CD40L: priming of T cell responses

Patients with hyper-IgM syndrome have also demonstrated an enhanced susceptibility to opportunistic infections, such as *Pneumocystis carinii* pneumonia and *Cryptosporidium* diarrhea. This indicates a role for CD40-CD40L interactions in cell-mediated immune responses. Indeed, CD40-L knockout mice display a considerable impairment of antigen-specific T cell priming and appear for instance particularly susceptible to *Leishmania* infection [93–95].

The contribution of CD40-CD40L interactions to the process of T cell priming, differentiation, and effector functions has been extensively reviewed [76, 96, 97]. The notion that CD40 is expressed on (professional) APCs has been a breakthrough in this field. Activation of CD40 has important effects on the expression of costimulatory molecules and the production of IL-12, thereby skewing Th1 vs. Th2 responses (Fig. 9). Cooperative effects between anti-CD40L and CTLA4-Ig have been observed *in vivo*, suggesting a positive loop between these two activation pathways. There has been some controversy as to whether antigen presentation in the absence of CD40-CD40L interaction leads to tolerance [98]. In some models, like the cellular immunity against LCMV or VSV virus, T cell priming seems to be independent of CD40L [99], although differences might exist between the induction of the CTL response and maintenance of CTL memory [100, 101]. The difference in CD40L dependence has not been fully explained, but factors like TCR occupancy, antigen persistence, and simultaneous inflammation have been proposed.

For a long time, it has been realized that in many cases, CD4⁺ T cells are required to induce efficient CD8⁺ cytotoxic T cell responses, a process termed cross-priming. Although it was clear that a third cell type, the APC, was involved in this process, it could not be explained how two rare, antigen-specific, T cells (CD4⁺ and CD8⁺), as well as an APC expressing the correct peptides, could meet simultaneously. Recently it has become clear that CD40-CD40L interaction between the CD4⁺ T cell and the professional APC (DC), stimulates the DC in such a way that it acquires the capacity to subsequently stimulate CD8⁺ CTL (Fig. 9) [102–104]. The molecular mechanism underlying this capacity of the APC is at present unknown. However, it seems that other signals, like TNF- α , LPS, or viral infection may substitute for the CD40L-

mediated signals, explaining the existence of CD4-independent CD8 T cell responses. Stimulating CD40 antibodies have been used to convert a tolerogenic peptide vaccine into strong CTL priming, and to show regression of established tumors [105–107].

CD40-CD40L: role in autoimmunity

The availability of either blocking anti-mouse CD40L antibodies and both CD40 and CD40L knockout mice have offered the possibility to test the role of CD40-CD40L interactions in several disease models. These include models for autoimmunity, infection with micro-organisms or transplantation tolerance (summarized in **Tables 2** and **3**).

Administration of blocking anti-CD40L has been demonstrated to be beneficial in several models of autoimmunity, including spontaneous diseases like lupus nephritis in SNF1 mice or diabetes in NOD mice or in experimentally induced forms of disease like collagen-induced arthritis or EAE [108]. A start has been made toward investigating the expression of CD40 and CD40L in different patients. In patients with SLE, an increased expression of CD40L was observed on circulating

TABLE 2. Impact of Genetic Modifications of CD40 or CD40L on Experimental Animal Models

Model	Impact
CD40 and/or CD40L knockout mice	
<i>Leishmania</i> infection	reduced resistance to <i>Leishmania major</i> and <i>Leishmania amazonensis</i> infection; more severe lesions
Anti-viral immunity	infection with LCMV results in severely compromised anti-viral Ab responses, normal primary CTL responses but lower memory CTL response
Experimental allergic encephalomyelitis	No disease development because of defected T cell priming and IFN- γ production
Murine Lyme disease	Normal protective Abs against <i>Borrelia burgdorferi</i>
Murine AIDS	CD40L-knockout not susceptible for LP-BM5 murine retrovirus
Inflammatory lung disease	reduced inflammation in CD40L-knockout mice
Mouse mammary tumor virus (MMTV)	defective viral replication in CD40L-knockout
<i>Histoplasma capsulatum</i>	resistance; accelerated disease with anti-IL-12
<i>Mycobacterium tuberculosis</i>	resistance; normal IL-2, TNF, and NO
CD40L overexpression/administration	
Lung inflammation	installation of exogenous CD40L induces pulmonary inflammation
<i>Leishmania</i> infection	anti-CD40 treatment (or CD40L trimer) stimulates IL-12 and IFN- γ and gives protection in BALB/c
Thymic architecture	disturbed architecture
T-dependent immune responses	increased antibody responses

TABLE 3. Effect of Anti-CD40L Interference Studies in Experimental Animal Models

Model	Anti-CD40L treatment
Collagen arthritis	diminished joint inflammation; lower serum Ab levels; low level infiltration delayed disease onset with reduced incidence accelerates renal disease in lpr mice reduces established nephritis long-term inhibition by combined treatment with CTLA4-Ig
Graft-versus-host disease	inhibits donor allospecific Th cells
Experimental allergic encephalomyelitis	disease prevention and reduction of clinical signs
HgCl ₂ -induced autoimmunity	abrogated autoimmunity without affecting IL-4 production
Allergic contact dermatitis	long-lasting unresponsiveness when treated in combination with CTLA4-Ig
<i>Pneumocystis pneumonia</i>	increased susceptibility to <i>Pneumocystis carinii</i> infection
Murine AIDS	inhibits ongoing disease
Gene therapy	prolonged adeno transgene expression delay in anti-adeno response
Atherosclerosis	reduced disease in hyperlipidemic mice
Oxygen-induced respiratory distress syndrome	prevent lung injury reduced Cox-2 expression
NOD mice	prevent insulinitis and diabetes when treated at week 3–4
Murine thyroiditis	prevent priming of thyroglobulin-specific T cells
Tranplantation models	prolonged graft survival after treatment with CD40L, given either alone or in combination with donor splenocytes or CTLA4-Ig \rightarrow allografts or xenografts of heart, kidney, skin, aorta, pancreatic islets

lymphocytes [109, 110], and CD40L-positive cells could also be demonstrated in kidney sections of SLE patients [111]. Similarly, activated helper T cells expressing CD40-L surface protein are detected in multiple sclerosis patient brain sections where CD40 bearing APCs can be found [112]. Because most diseases will have a complex pathophysiology, the levels at which the anti-CD40L might potentially interfere are diverse (antigen presentation, Ig production, inflammatory responses). It is interesting that, both for lupus nephritis and EAE, it was demonstrated that anti-CD40L could also interfere with ongoing disease, confirming the role of CD40-CD40L in the effector phase of the disease [112–114].

CD40-CD40L: role in transplantation

CD40-CD40L interactions play a critical role in T cell priming and have been suggested to prohibit tolerance induction. Therefore, interference with CD40 activation has been extensively investigated to prolong the survival of experimentally transplanted organs. These models have been mostly limited to

murine models, since only blocking anti-murine CD40L antibodies are at present available, and include allografts of heart, skin, aorta, and pancreatic islets [115], as well as pancreatic islet, heart, and skin xenografts [116]. Although treatment with anti-CD40L alone prolongs survival, better results have been obtained by combined treatment, either with donor splenocytes or CTLA4-Ig. Simultaneous blocking of the CD40 and CD28 pathways resulted in long-term acceptance of skin and cardiac allografts [117].

The treatment regimen has been different in the various studies, ranging from once at the time of transplantation until continuous administration every 72 h to prevent chronic rejection in aortic allografts [118]. In the latter case, CD40L effector functions operating within the transplanted graft might also be of importance. Analogously, local expression of CD40L has been found in human renal (Fig. 3) and heart allografts, and expression seems to correlate with rejection [119, 120]. It is important that, also in a rhesus monkey model of kidney transplantation, beneficial effects of anti-CD40L treatment (5c8, anti-human CD40L) have been observed, either with or without CTLA4-Ig [121, 122]. These findings might open the way to new therapeutic intervention strategies to prevent transplant rejection and induce long-term tolerance.

CD40/CD40-L: role in inflammation

Long-lasting, chronic, inflammatory responses, which are characteristic of many diseases, ultimately lead to severe histological changes and loss of normal function. Generally, these situations, such as atherosclerosis or lung fibrosis, are characterized by the local presence of infiltrating mononuclear cells, including T cells, B cells, and monocytes [123]. Treatment with anti-CD40L antibodies was shown to reduce the development of atherosclerosis and reduce stromelysin-3 expression in hyperlipidemic mice [124, 125], and to prevent pulmonary inflammation and fibrosis as a consequence of oxygen-induced respiratory distress syndrome [126]. In view of the broad expression of CD40 and the various mediators produced after *in vitro* CD40 activation, it can be anticipated that a role for CD40-CD40L in chronic inflammation will involve both interaction of T cells with other immune cells, as well as the action on resident tissue cells.

USE IN DIAGNOSIS AND DISEASE INVOLVEMENT

The importance of CD40-CD40L for the hyper-IgM syndrome has been discussed. For diagnosis of deficiencies in CD40L expression, FACS protocols have been developed, which make use of sets of monoclonal antibodies recognizing functional epitopes of the CD40L protein, but with binding of CD40-Ig fusion proteins still as gold standard. CD40L expression is investigated on PMA plus ionomycin-stimulated peripheral blood T cells. Caution should be taken when using total PBMC, since the presence of B cells and monocytes during activation might diminish the detection of the CD40L protein. Naturally occurring mutations are collected in an on-line database [34] (<http://expasy.hcuge.ch/www/cd40lbase.html>), and have re-

vealed that mutations are very heterogeneous, but with hotspots at some positions (like Trp140). In some cases, the information of mutations and genomic structure has been used to perform prenatal diagnoses of individuals at risk [9, 127].

PRECLINICAL AND PHARMACOKINETICS

In vivo experiments have suggested that potentially two opposite strategies might be desired for the treatment of different diseases. On the one hand, one would like to augment CD40L-CD40 interactions in situations of immunodeficiency. In mice, an agonistic anti-CD40 antibody has been successfully used as a strong adjuvant for boosting of specific immune responses [91]. On the other hand, in situations of uncontrolled immune reactions, one would like to interfere specifically with CD40-CD40L interactions. Many encouraging results have also been obtained in different mouse models. It is important that beneficial effects of anti-CD40L treatment (5c8, anti-human CD40L) have also been observed in a rhesus monkey model of kidney transplantation [121].

Both a chimeric and a humanized form of 5c8 has been generated, and these antibodies have been used for pharmacokinetic and pharmacodynamic studies in cynomolgus monkeys [128]. Single-dose injection resulted in a half-life of 300 h, which was substantially prolonged after repeated-dose injection (500 h). A median concentration of 0.84 µg/mL was needed to inhibit the development of primary and secondary antibody titers against tetanus toxoid [128].

THERAPEUTIC APPLICATIONS AND CLINICAL RESULTS

Although several developments are now in a preclinical stage, and *in vivo* experiments in smaller animals suggest a broad application for interference with the CD40-CD40L pathway, there are at present no clinical results available.

LICENSED PRODUCTS

Various tools have been developed that can be used for studying CD40 and CD40L biology, such as expression, activation, and inhibition. Monoclonal antibodies against both human and mouse CD40 and CD40L have been raised, some of which are available through ATCC: 5c8, mouse IgG2a anti-human CD40L (no. HB-10916), G28-5, mouse IgG1 anti-human CD40 (no. HB-9110), and MR1, and hamster IgG anti-mouse CD40L (no. HB-11048). Finally, single-chain immunotoxins targeted to CD40 and recombinant soluble forms of CD40 and CD40L (fusion proteins and trimeric forms) have been generated. Readers are referred to the individual researchers for further details.

ASSAYS FOR DETECTION

Monoclonal antibodies are available for the detection of CD40 and CD40L expression, both in frozen sections and on *in vitro* cultured or isolated cells. Excessive CD40 and/or CD40L expression are indicative of immune activation, whereas ab-

sence of expression is associated with immunodeficiencies. Soluble forms of both CD40 and CD40L have been described and specific enzyme-linked immunosorbent assays for sCD40 and sCD40L have been developed, but the relevance of these molecules is at present unknown.

CONCLUDING REMARKS

In conclusion, experiments in the past have shown the complexity and importance of CD40-CD40L interactions. Starting as a receptor-ligand pair with a critical role in T cell-dependent humoral immune responses, as proven by the hyper-IgM syndrome, the molecules are now thought to have a much broader role. Especially under pathological conditions, a widespread expression of CD40 and CD40L has been demonstrated, suggesting roles in acquired cellular immune responses, as well as in innate immunity. The use of tools that interfere with CD40-CD40L interactions have shown its potential use in the treatment of various disease states. Clinical trials that are ongoing ultimately have to show under which conditions this is a feasible and useful strategy and under which conditions a further refinement of our understanding of the underlying mechanisms is necessary.

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