Review

CD40/CD40L Dyad in the Inflammatory and Immune Responses in the Central Nervous System

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CD40 and its cognate ligand (CD40L) are a pair of regulators of pro-inflammatory and immune responses. In the central nervous system (CNS), CD40 is expressed on a variety of cells, including vascular endothelial cells, smooth muscle cells, astrocytes and microglia (the brain macrophages, being the most sensitive cell type to respond to CD40 ligand). Interaction between CD40 on microglia and CD40L presented by infiltrating T lymphocytes and other resident CNS cells triggers a series of intracellular signaling events that promote the production of a wide array of cytokines, chemokines and neurotoxins. Thus, both molecules serve as amplifiers of pro-inflammatory and immune responses in the CNS and constitute important molecular targets for therapeutic intervention of diseases. ***Cellular & Molecular Immunology*****. 2006;3(3):163-169.**

Key Words: CD40, CD40 ligand, signal transduction, amyloid β, mFPR2, Alzheimer's disease

Introduction

CD40 is a member of the tumor necrosis factor (TNF) receptor family that is expressed on the surface of immune cells, including B cells, monocytes, and dendritic cells, as well as non-immune cells such as endothelial cells, epithelial cells, mesenchymal cells (fibroblasts, synoviocytes, stellate cells), platelets and malignant tumor cells (1-3). CD40L (CD152) is a 39 kD type II transmembrane protein of the TNF superfamily and is expressed preferentially by activated $CD4^+$ T cells and platelets, although its expression has been detected on monocytic cells, natural killer cells, B cells, $CD8^+$ cells, mast cells, and basophils (4). CD40 forms a trimer and after interaction with CD40L transduces a complex signaling cascade that involves the activation of various protein tyrosine kinases and transcription factors. CD40 bearing cells thus activated produce multiple bioactive molecules whose ultimate effects depend on the differentiation state of the cells, the level of receptor and ligand expression, and the tissue microenvironment where CD40 cross-linking occurs (5). In immune system, CD40-CD40L interaction affects some key processes, i.e., immune cell activation, differentiation, proliferation, and apoptosis. CD40-CD40L interaction also upregulates costimulatory molecules (ICAM-1, VCAM-1, E-selectin, LFA-3, B7.1, B7.2, class II MHC, and CD40 itself), Fas ligand, and the production of cytokines IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, MIP-1α (CCL3), and MCP-1 (CCL2). More recently, ligation of CD40 was shown to promote the production of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) by monocytic phagocytes (6).

In the brain, microglia, the resident macrophages, express CD40 (6-9). However, under resting conditions, the level of CD40 on microglia is relatively low but is markedly increased upon challenge with pro-inflammatory stimuli such as interferon-γ (IFN-γ), TNF-α and lipopolysaccharide (LPS) (10-12). Thus, it seems that CD40 on microglia serves as an amplifier of inflammatory responses in the CNS. This assumption is supported by the fact that CD40-CD40L interaction on microglia leads to the production of TNF-α, IL-12, nitric oxide (NO), metalloproteinase (MMP)-9, MCP-1 (CCL2), IP-10 (CXCL10), and neurotoxins with yet unknown identity (6). In the serum of patients with Alzheimer's disease (AD), there is a significant increase in

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; CD40L, CD40 ligand; mFPR2, mouse formylpeptide receptor 2; MS, multiple sclerosis.

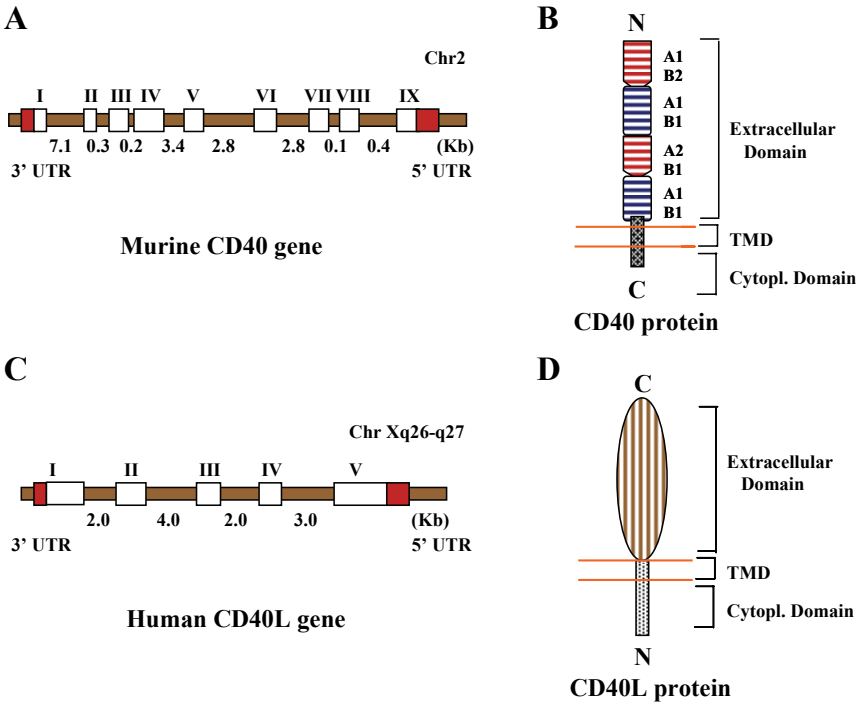

Figure 1. Structure of CD40 and CD40 ligand. (A) Structrure of murine CD40 gene located on chromosome 2. The mouse CD40 gene consists of 9 exons, with exon I coding for the leader sequence, exons II-VI for the extracellular region, exon VII for the transmembrane region, and exons VIII-IX for the intracellular region. (B) Structure of mouse CD40 protein. CD40 is a type I transmembrane receptor, with an extracellular region containing four cysteine-rich domains, each domain divided into two cysteine modules (A1, A2, B1, and B2). (C) Human CD40L gene located on chromosome Xq26-q27. The gene consists of 5 exons, with exon I coding for the intracellular and transmembrane regions, and exons II-V for the extracellular region. (D) Human CD40L protein. Human CD40L is a type II transmembrane protein with an intracellular amino terminus and an external carboxy terminus. The extracellular region of CD40L shares structural homology with other members of the TNF family.

the soluble CD40 with concomitant decrease in TGF-β1 as compared with healthy individuals (13). The source of CD40L in the brain remains a debating issue. Under conditions in which there is no obvious infiltration of T cells in the CNS, a major source of CD40L could be astrocytes, since copious quantity of CD40L is detectable in these cells *in vivo* after brain injury or in AD (14). Therefore, in the CNS, CD40 on microglial cells may be activated without contact with T cells.

CD40 and CD40L

Mouse CD40 gene is located in the distal region of chromosome 2, which is syntenic to human chromosome 20q11-q13. The gene consists of nine exons and spans a segment of 16.3 kb genomic DNA (Figure 1A). The mouse CD40 gene is expressed as two mRNA species of 1.7 and 1.4 kb, while the human CD40 gene is in a form of a single 1.5-kb mRNA (15, 16). The mouse CD40 protein has 305 amino acids (aa) with a 193-aa extracellular region, a 21-aa leader sequence, a 22-aa transmembrane domain, and a 90-aa intracellular region (Figure 1B). Human and murine CD40 proteins share 62% identity at the aa level in overall 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 extracellular cysteine residues are conserved, suggesting that both mouse and human CD40 fold into a very similar configuration. Because of the similarity in the extracellular segment, and in particular, its 22 cysteine residues, CD40 is considered as a member of the tumor necrosis factor receptor (TNF-R), TNF/nerve growth factor (NGF) receptor super-family (15, 16). CD40 is expressed as a 45-50 kD cell surface molecule.

The cognate ligand of CD40, CD40 ligand (CD40L/CD154, CD152), is typically found on the surface of activated T lymphocytes ($CD4^+$, $CD8^+$, and γδ T cells), although other cells have also been found to express CD40L. The gene for human CD40L is located in the X-chromosome at the position Xq26.3-Xq27.1 and spans 12-13 kb with five exons (Figure 1C). Murine CD40L gene also contains five exons yet no DNA sequences are available in the gene bank so far. Human and mouse CD40L exhibit 75% identity at the aa level in the extracellular domain, 96% in the transmembrane region, and 81% in the cytoplasmic domain (Figure 1D). CD40L is a type II membrane protein with a molecular mass of 32-33 kD, that lacks an amino-terminal signal peptide and its carboxy terminus is located extracellularly. The 33-kD form of CD40L is associated with its two shorter versions of

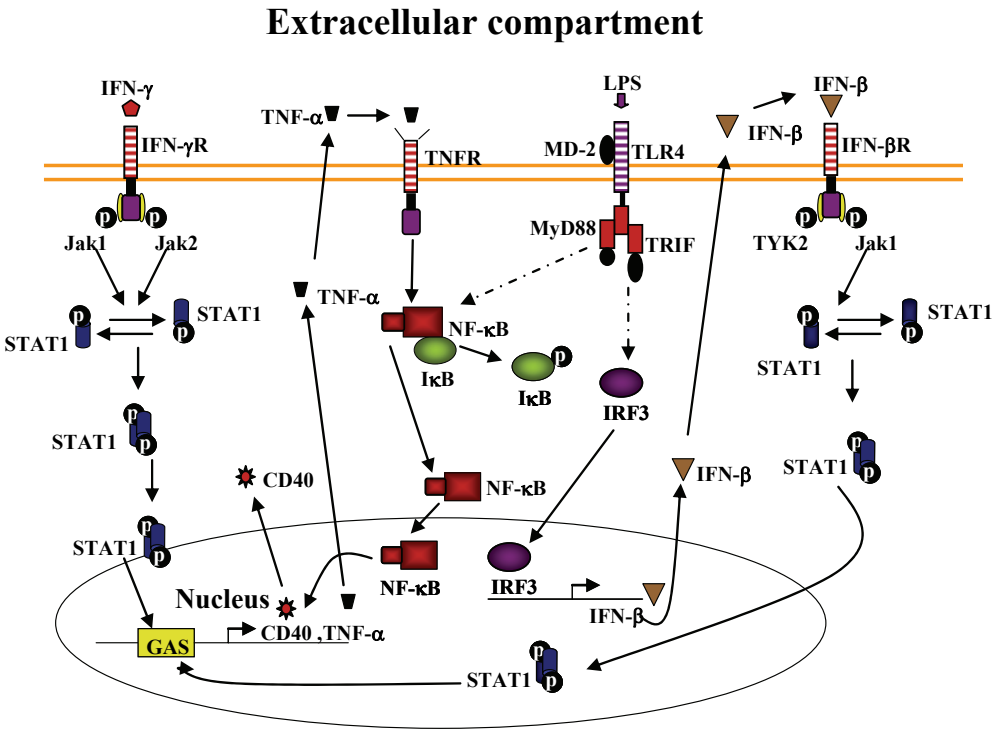

Figure 2. The mechanistic basis of CD40 induction by IFN-γ and LPS. IFN-γ-activated STAT-1 binds to the distal and medial activated sequence elements in human CD40 promoter. Concurrently, IFN-induced TNF-α activates NF-κB, which binds to three NF-κB binding sites (dNBS, mNBS, and m2NBS) in the CD40 promoter. The cooperation between STAT-1 and NF-κB promotes optimal IFN-γ-induced CD40 expression in microglia/macrophages. On the other hand, LPS activates NF-κB, which translocates into nucleus and binds to NF-κB elements in the CD40 promoter. LPS also activates IRF-3, which induces IFN-β and the subsequent activation of STAT-1. Dimerized STAT-1 translocates into the nucleus and binds to GAS elements in the CD40 promoter. This pathway has a delayed kinetics as compared with direct NF-κB p65 and p50 binding to the CD40 promoter. LPS additionally modifies H3 and H4, which recruit RNA Pol II. The subsequent binding of transcription factors and Pol II to the CD40 promoter in conjunction with permissive histone modifications results in transcriptional activation of CD40 gene.

31 and 18 kD (15, 16), which are soluble forms of CD40L retaining the ability to form trimers, to bind CD40, and to elicit signals, indicating that CD40L also may act as a bona fide circulating cytokine.

CD40 expression and regulation on microglia

Microglia are brain mononuclear phagocytes with functions similar to tissue macrophages, including phagocytosis and production of cytokines, eicosanoids, complement components, matrix MMPs, oxidative radicals, and nitric oxide (17). In the normal brain, microglia is in a quiescent phenotype. However, these cells are highly sensitive to pro-inflammatory and injurious insults and rapidly become activated. In addition, because activated microglia express class II MHC and accessory molecules, they are considered as potential antigen presenting cells (APC) in the CNS. Meanwhile, microglia may act as immune effector cells in CNS diseases such as multiple sclerosis (MS) that results from damage to the myelin sheath and oligodendrocytes. In AD, microglia is in a highly activated state and contribute to neuronal damage by the production of pro-inflammatory cytokines and neurotoxis in response to the amiloid β peptides overproduced in the AD brain tissue (6, 10, 18, 19).

Unlike peripheral blood monocytes, microglia in a non-activated state express relatively low levels of CD40 (20, 21), with a marked increase when the cells are stimulated by pro-inflammatory cytokines (12, 20, 22), among which IFN-γ is one of the most potent inducer. For instance, IFN-γ at low concentration was able to up-regulate the expression of CD40 on microglia by 20 fold (Chen et al, unpublished observation). TNF-α also promotes CD40 expression, but compared with IFN-γ, its effect was moderate. Interestingly, one report showed that TNF-α and IL-1β in combination slightly augmented IFN-γ-induced CD40 expression, but each failed to exhibit activity alone on microglial cells (23).

The IFN-γ receptor consists of an α-chain (IFN-γRI) with high ligand binding affinity, and a β-chain (IFN-γRII), which is necessary for signaling. The IFN-γ receptor is constitutively associated with Janus kinases (JAKs), the β-chain with JAK1 and the β-chain with JAK2. Binding of IFN-γ to its receptor activates JAK1 and JAK2, resulting in the phosphorylation of

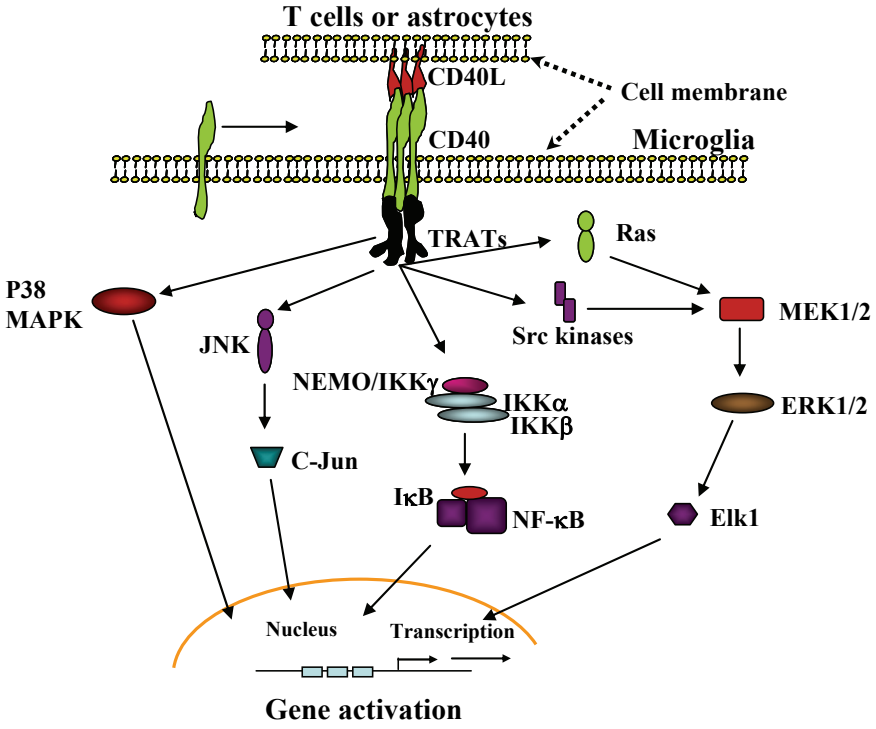

Figure 3. Signaling pathways coupled to CD40-CD40L interaction. CD40 trimerizes, binds trimeric CD40L and activates TRAFs. The proto-oncogene Ras and the downstream kinases are also activated. These kinases catalyze a cascade of signaling events that lead to gene transcription.

tyrosine residue 440 of the α-chain, followed by recruitment of the signal transducer and activator of transcription (STAT)-1α and its phosphorylation at tyrosine residue 701. The STAT-1α homodimer then translocates into the nucleus where it binds to γ activation sites (GAS), also termed STAT binding element (SBE), in the promoters of many IFN-γ inducible genes including the IFN regulatory factor-1 (IRF-1) and ICAM-1. In microglia, IRF-1 and STAT1 transactivate multiple genes encoding CD40, IL-12, IP-10 (CXCL10), and iNOS (6, 24). It has also been reported that TNF-α may play a critical role in the up-regulation of CD40 gene by IFN-γ in microglia (25), since co-treatment of microglia with IFN-γ and neutralizing antibodies to TNF-α attenuates the capacity of IFN-γ to induce CD40 mRNA expression. Further evidence for the involvement of TNF-α in IFN-γ induction of CD40 expression in microglial cells was provided by results from TNF-α deficient mice in which primary microglia after stimulation with IFN-γ only express a moderate level of CD40 (25). TNF-α produced by IFN-γ-activated microglia promotes the binding of NF-κB to promoters important for CD40 gene transcription (25) (Figure 2). On the other hand, CD40 expression on microglia can be inhibited by anti-pro-inflammatory cytokines, neurotrophins, neuropeptides, and statins (6). It has been reported that TGF-β inhibits IFN-γ- induced CD40 and protein expression by destabilizating CD40 mRNA (23). IL-4 also potently inhibits IFN-γ-induced CD40 gene transcription in microglia. The effect of IL-4 is mediated by competitive binding of STAT-6 to the CD40 promoter, which interacts with IFN-γ-activated transcription factors (26). Moreover, neuropeptides such as adenylate cyclase intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) inhibit IFN-γ- induced CD40 gene expression in microglia by disrupting IFN-γ signal transduction cascades, specifically STAT-1α phosphorylation and its subsequent binding to GAS elements in the CD40 promoter (20). LPS as a potent activator of innate immunity also induces CD40 gene and protein expression in microglia (11). The effect of LPS involves activation of multiple transcription factors. For example, it directly activates NF-κB in microglia and induces production of IFN-β, which in turn promotes the nuclear translocation of STAT-1α and initiation of CD40 gene transcription (Figure 2) (11). Thus, in microglial cells, CD40 is tightly regulated by both pro- and anti-inflammatory cytokines produced under pathophysiological conditions in the CNS.

CD40-CD40L signaling pathways in microglia

Despite a wealth of data aimed at discerning CD40 associated intracellular signaling pathways, the understanding of these pathways remains incomplete. One of the widely accepted models of CD40 signaling attributes the initiation of the cascade to its trimerization. It is believed that only trimerized CD40 is able to bind CD40L, which is also in a trimeric form

(9). Since the cytoplasmic C-terminus of the CD40 molecule lacks intrinsic enzymatic activity, the signaling *via* CD40 is mediated through interaction with a family of proteins known as tumor necrosis factor receptor-associated factors (TRAFs) (27). TRAFs themselves also have no intrinsic activity, but they act as adaptor proteins promoting the recruitment of signaling molecules into a complex. The TRAF family consists of 6 members, of which 2, 3, and 6 each binds directly to the cytoplasmic tail of CD40 through their C-terminal domains. On the other hand, TRAF1 and TRAF5 may interact with CD40 indirectly through the formation of hetero-oligomers with TRAF2 and TRAF3. These adaptors link CD40 to multiple downstream pathways that include phosphoinositide 3-kinases (PI3K), phospholipase Cγ (PLC-γ), mitogen-activated protein kinases (MAPKs), and NF-κB (Figure 3) (10, 28).

In mouse primary microglia, CD40 ligation by CD40L results in the phosphorylation and activation of p44/42 MAPK without significant effect on p38 MAPK phosphorylation (14), although this kinase has been shown to participate in CD40-mediated cytokine production. Activation of CD40 signaling in microglia results in the production of TNF-α and other toxins that potentially cause neuronal injury. Concomitant treatment of microglial cells with CD40L and TGF-β or IL-10 inhibits CD40-mediated activation of p44/42 MAPK and TNF-α production, thus reducing cell-mediated neurotoxicity (29).

The effect of CD40-CD40L interaction in microglia on the progression of Alzheimer's disease

Gene chip analyses revealed that CD40L stimulation of microglial cells leads to changes in mRNA expression of a number of genes encoding proteins that are involved in the proteolytic cleavage of amyloid precursor protein (APP) (30), which yields Aβ peptides a major component of the senile plaques in the AD brain. Cross-linking of CD40 on microglial cells increased the expression levels of the genes coding for the elements of the γ secretase complex, which is important for the metabolism of APP to release the pathogenic form of the $A\beta_{42}$ peptide fragments. By doing so, CD40 activation in microglia exacerbates the progression of AD. In addition, in CD40L stimulated microglia, the mRNAs for LRP and APOE are up-regulated. The protein products of these two genes are known to bind, thus may increase the pathogenic activity of, Aβ peptides. In fact, increased expression of LRP and APOE genes has been considered as genetic risk factors for AD.

Chemokines are a super-family of small cytokines that mediate leukocyte trafficking and homing (31). Microglia have been shown to be an important source of chemokines, whose production may play a key role in pro-inflammatory and immunologic CNS diseases (32, 33). It has been demonstrated that purified human microglial cells treated with IFN-γ and CD40L increase the production of several macrophage and T lymphocyte attracting chemokines such as MCP-1 (CCL2), IP-10 (CXCL10), MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) (8). Interestingly, IFN-γ and CD40L induction of MCP-1 (CCL2) was mediated by activation of the ERK1/2 MAPK pathway, whereas p38 MAPK pathway was crucial for IP-10 (CXCL10) (8), despite the claim by another study showing little activation of p38 by CD40 ligation (14).

In the AD brain, there is a persistent chronic inflammation characterized by increased production of pro-inflammatory cytokines and acute-phase reactants in the senile plaque areas (18, 19). Microglia as a major immune cell type in the CNS play an important role in the inflammatory responses seen in AD by first infiltrating and surrounding Aβ peptide deposits followed by activation and release of neurotoxins (18, 19). A study of post-mortem human brain tissues from 18 AD patients reveals that reactive microglia were positive for CD40 staining yet the expression was not limited to microglia but also by cells of the vasculature in the lesion area (34). CD40 can be released from cell membrane to become a soluble form (Scd40) and the elevated Scd40 is detectable in the circulating blood of AD patients. For instance, sCD40 levels in the plasma of AD patients are significantly higher than the healthy controls, thus the plasma Scd40 may be used as a diagnostic marker for late-onset AD (13). In mouse models of AD, disruption of CD40 gene reduced the amyloid burden and the level of astroglial cell activation was considerably lower (35). These studies provide very convincing evidence for the effect of CD40 on exacerbation of the progression of AD. Another important evidence for the role of CD40/CD40L pair in AD progression is the increased production of CD40L in human AD brain and in animal models. In human AD, intense CD40L immunoreactivity is detected in hypertrophied astrocytes distributed throughout the frontal cortex. The majority of CD40L-positive astrocytes in the gray matter are located within or in the periphery of the plaques. In mouse AD models, the cortex and hippocampus contain numerous neuritic plaques and CD40L-positive astrocytes, which are not detected in normal mice (14). Similar to mice lacking CD40, the AD mice lacking CD40L gene show decreased astrocytosis and microgliosis in association with diminished Aβ plaque load. In addition, administration of an anti-CD40L antibody in AD mice alleviated AD pathology in the brain and improved cognitive performance of the animals (36). Thus, depletion of either CD40 or CD40L in mice markedly reduces the rate of AD progression.

The 42 aa form of the proteolytic product of APP, $A\beta_{42,}$ is a major causative factor in AD and interacts with microglial cell surface receptors (18, 19). One of such receptors is a G-protein coupled formylpeptide receptor FPRL1, or its mouse homologue mFPR2. Activation of FPRL1 and mFPR2 by $A\beta_{42}$ increases microglial chemotaxis and release proinflammatory mediators and neurotoxin (37, 38). Interestingly, microglia isolated from normal new borne mice express very low levels of mFPR2. However, after activation by TNF-α or agonists for Toll-like receptors, the expression of mFPR2 in microglial cells was markedly enhanced and the cells migrate in response to $A\beta_{42}$ (39-42). Further studies reveal that

Table 1. CD40-CD40L in CNS diseases

Expressing cells		Diseases
CD40	CD40L	
Macrophages/microglia	T-cells	Multiple sclerosis
Macrophages/microglia	T-cells	Experimental allergic encephalomyelitis
Macrophages/microglia	T-cells	Theiler's murine encephalomyelitis virus-induced demyelinating disease
Macrophages/microglia	Astrocytes	Alzheimer's disease
Monocytes	T-cells, platelets	Cerebral ischemia
Macrophages/microglia	?	HIV-1-associated dementia

activated microglia endocytose $A\beta_{42}$ *via* mFPR2 and in human macrophages, FPRL1-mediated $A\beta_{42}$ uptake causes the retention of the receptor/$A\beta_{42}$ complexes in the cytoplasmic compartment (37), followed by fibrillary aggregation. Our recent study additionally showed that the Th1 cytokine IFN-γ is also able to up-regulate the expression of mFPR2 as well as CD40 in mouse microglia and moreover, addition of soluble CD40L produces a synergistic effect with IFN-γ on increased expression of mFPR2 and its mediated $A\beta_{42}$ endocytosis (Chen et al., manuscript in preparation). Therefore, CD40 in microglia is an important mediator of pro-inflammatory responses in AD and by promoting the expression and function of mFPR2, may profoundly affect the course of AD pathogenesis.

Perspectives

CD40-CD40L interaction has been implicated in participating in a variety of pro-inflammatory and neurologic diseases in the brain. Aberrant expression of both CD40 and CD40L has been detected in HIV-1-associated dementia (8), MS (43), and AD. Table 1 lists some neurologic diseases in which the expression of CD40 and CD40L is increased (6). CD40 expression in macrophages/microglia in the CNS constitutes an important component of the neuroinflammatory responses. The fact that depletion of either CD40 or CD40L gene in mouse models of AD renders the animals more resistant to disease progression defines a detrimental role for these molecules in promoting inflammation in the CNS. Further studies are thus warranted to more thoroughly understand the role of CD40/CD40L in multiple CNS diseases and their potential as therapeutic targets.

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