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IMMUNE REGULATION BY CD40-CD40-L INTERACTIONS - 2; Y2K UPDATE

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1. ABSTRACT

CD40 is a cell surface receptor, which belongs to the TNF-R family, and which was first identified and functionally characterized on B lymphocytes. However, in recent years it has become clear that CD40 is expressed much broader, including expression on monocytes, dendritic cells, endothelial cells and epithelial cells. Therefore it is now thought that CD40 plays a more general role in immune regulation. The present paper reviews recent developments in this field of research, with main emphasis on CD40 signal transduction and on *in vivo* functions of CD40/CD40-L interactions.

2. INTRODUCTION

After the identification of CD40 in 1985, research has for a long time been focussed around the regulation of humoral immune responses. CD40 was identified as a molecule expressed during all stages of B cell development and differentiation, which plays a pivotal role in T cell-dependent B cell responses. The in vivo relevance was proven by the finding that patients suffering from the X-linked hyper-IgM syndrome (HIGM), were characterized by mutations in the gene coding for the ligand of CD40 (CD40L or CD154).

After this breakthrough finding, research on CD40-CD40L has expanded in various directions. It was found that both CD40 and CD40L expression is much broader than initially thought. For instance, it has become clear that on dendritic cells (DC) CD40 is one of the critical steps in its final maturation into a fully competent antigen presenting cell. CD40 is also widely expressed on non-hematopoietic cells, including endothelial cells, fibroblasts and epithelial cells, where it seems involved in the amplification and regulation of inflammatory responses. Interference with CD40-CD40L interaction might represent a new target to treat patients with unwanted immune-activation such as autoimmunity or transplant rejection, and has resulted in respective phase II clinical trials.

CD40 and CD40L belong to the TNF-R and TNF family, an emerging group of receptor-ligand pairs. 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. In the present review we will mainly concentrate on two aspects of CD40 biology: i) mechanisms of signal transduction via the CD40 receptor, since this integrates the receptor with other members of the TNF-R family and might provide clues how this receptor can have such diverse effects; ii) the in vivo role of CD40-CD40L and the implications for its use in therapeutic settings. Other subjects will be briefly summarized and for more detail readers are referred to the 1996 version of this review (1), or to several extensive overviews (2-9).

3. IMMUNE REGULATION BY CD40-CD40-L INTERACTIONS

3.1. Structure and expression of CD40 and its ligand

The CD40 antigen is a 45-50 kDa glycoprotein of 277 AA, which belongs to the Tumor Necrosis Factor Receptor family (10). The extracellular segment of CD40, in particular the 22 cysteine residues, are homologous to other members of the TNF-R family, which at the moment includes at least 20 members (11, 12). Initially it was suggested that these cysteins 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 5 different forms of modules being identified (13). Although the structure of CD40 has not been resolved, a model has been built using homology modeling, mutagenesis, X-ray structures of TNF-R and allignment of the TNF-R family (14). The mouse CD40 gene, composed of nine exons that span a 16.3 kb of genomic DNA, is located on the distal region of chromosome 2 which is syntenic to human chromosome 20q11-q13 where the human CD40 gene is located.

CD40 is expressed by multiple cell types. In the hematopoietic system, it is expressed on $CD34^+$ hematopoietic progenitors, B cell progenitors, mature B Iymphocytes, plasma cells, monocytes, dendritic cells, eosinophils, basophils and on some T Iymphocytes. CD40 is also expressed on non-hematopoietic cells such as endothelial cells, fibroblasts and epithelial cells (6, 9).

Expression cloning using a CD40-Fc fusion protein allowed the isolation of a CD40-ligand (CD40-L) from activated T cells (15). Human CD40-L is a polypeptide of 261 AA including a 215 AA extracellular domain with five cysteins. 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 2 Å resolution (16). CD40L is a sandwich of two beta sheets with jelly roll topology and forms a 3-fold symmetric homotrimer. The three-dimensional organization is similar to that described for the TNF-alpha and LT-alpha proteins, and as predicted by initial modelling studies. The gene for CD40-L is located on the X-chromosome at position q26.3-q23.1. It spans over 12-13 kb and consists of five exons.

CD40-L is expressed on activated 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 also expresses CD40-L. Apart from T lymphocytes, both primary cells and cell lines of mast cells, basophils and eosinophils stain positive with CD40-Ig or CD40L antibodies (17). Similarly, expression of CD40L has been demonstrated on B cells and B cell lines, NK cells, monocytes/macrophages and dendritic cells under certain conditions (18-21). Finally, pre-formed CD40L was demonstrated to be present in intracellular stores of thrombocytes (22).

CD40L is produced as a type II transmembrane protein, but may be expressed on the cell surface as a heteromultimeric complex (23). The molecule can excist in two shorter versions of 31 kD and 18 kD (24, 25). 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 "cytokine" (24-26). Qualitative differences between the soluble and membrane bound forms may yet exist.

3.2. CD40 signal transduction

3.2.1. Kinases

Like all other members of the TNF-R family, CD40 has no kinase domain and no known consensus sequence for binding to kinases. Yet, CD40 ligation activates several second messenger systems, including activation of protein tyrosine kinases (PTK; *lyn*, *syk* and Jak3), protein kinase A (PKA), phosphoinositide-3 kinase (PI-3 kinase) and phospholipase C (6). In recent years many studies have concentrated on the involvement of serine / threonine kinases: stress activated protein kinase / c-jun aminoterminal kinase (JNK/SAPK), p38 MAPK and extracellular signal-regulated mitogen activated protein kinase (ERK) (27-30). Since contrasting results have been obtained regarding JNK, p38 or ERK activation, general conclusions should be taken with care.

An important reason for different experimental results might be that studies often have used different cell types or models of transfected cells. This might have important consequences for signal transduction pathways. For instance, although the NF-kB inducing kinase (NIK) was shown to be essential for B cell activation, it seems to be dispensable for activation of dendritic cells (31). Alternatively, TRAF6 (see below) is an intermediate in CD40-induced NF-kB activation in 293 epithelial cells, but not in B cells (32). Finally, comparing members of the NF-kB family upon CD40 ligation showed a difference in p50 (B cells and monocytes) or p65 (B cells only) induction (33). The same study showed that in both B cells and monocytes, CD40 is associated with JAK3, but only in monocytes CD40 triggering results in activation of STAT5a. This might contribute to the finding that CD40 signaling seems to be normal in monocytes of patients with non-X-linked hyper IgM syndrom (34), a B cell deficiency not based on the absence of functional CD40L.

3.2.2. TNF-R Associated Factors (TRAF)

In recent years it has been established that members of the TNF-R family associate intracellularly with different families of signalling molecules. This includes the 'death domain' family for receptors involved in apoptosis induction (12). For CD40, coupling of the receptor to different signaling pathways has been better understood by the identification of another family of associated proteins: TRAF (TNF-R Associated Factor)(for review (11)). Most of these factors have been identified by the technique of the two-hybrid system, and at the moment 6 members of the TRAF family have been cloned and identified. The TRAF proteins are well conserved during evolution and TRAF-like proteins have been found in C. elegans and Drosophila. The proteins contain several functional domains which

might be involved in signal transduction (RING finger domain, Isoleucine zipper, Zinc finger). In addition the TRAF-C domain plays a role in protein-protein interactions, either to associate with CD40 or other TNF-R members, or to form homo- / heterodimers. Crystallography of this TRAF-C domain of TRAF2, with peptides of either TNF-R2 or CD40, showed a spontaneous trimeric self-association (35, 36).

In general, there is little cross reactivity between the extracellular ligands and members of the TNF-R family, with the exception of TNF and LT, TRAIL and it's receptors and the interaction between BAFF/TALL-1 and TACI or BCMA (37). In contrast, the intracellular ligands seem to be much more promiscuous and form a complex network of homo- and hetero-dimers and interact with many members of the TNF-R family (11).

At least 4 TRAF molecules have been demonstrated to associate with the CD40 cytoplasmic tail (TRAF2, TRAF3, TRAF5, TRAF6), and three of these were cloned using CD40 as bait in the two-hybrid system. TRAF3 was the first identified CD40-associated TRAF molecule and was simultaneously found by several groups as CRAF1, CD40bp, LAP1 and CAP1 (38-41). TRAF3 is a 62 kD intracellular protein which is expressed in almost all cell types. CD40 also associates with TRAF2, a molecule first identified as an addaptor molecule bound to TNF-R2 (42). Finally, both TRAF5 and TRAF6 were cloned by yeast two-hybrid using CD40 as bait (43, 44).

Early mutagenesis studies already suggested a critical role for the Thr residue at position 254 (complete coding sequence)(45), and this residue was implicated in TRAF3 binding (39). 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)(44). Such a separation of the TRAF members in two groups is supported by the more distant amino acid homology of TRAF4 and TRAF6 compared to TRAF1,2,3,5 which are more homologous (11). A comparable difference in binding domain of TRAF6 has been demonstrated for RANK (46). In accordance, pepscan analysis of CD40 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 (47). In this study, TRAF5 binding could only be demonstrated indirectly via TRAF3, which was confirmed by others (48). Binding and signaling of TRAF6 could even be optimized by generating a N237D mutant (49). Using wild-type CD40 and a mutant only containing the binding site for TRAF6, it was demonstrated that CD40 activates ERK both by a ras-dependent pathway and a ras-independent pathway involving TRAF6 (50).

3.2.3. Genetic inactivation of TRAF molecules

To unravel this complex network and to understand the in vivo role of the different TRAF molecules, several members have been genetically inactivated in mice. TRAF3 knock out mice appear normal at birth, but die around the age of 10 days (51). B cells from these mice show an apparantly normal proliferation and signaling in B cells, suggesting that the action of TRAF3 might be compensated by other TRAF molecules. In contrast, it was found that T cell activation is impaired, but signaling via which receptor is responsible for this defect is not clear (51).

Inactivation of TRAF2 showed that mice were born normal, although at lower frequency, but they also died early in life (52). Initial experiments had suggested that TRAF2 was both critical for JNK and NF-kB activation, but analysis of embryonic fibroblasts of these TRAF2-KO mainly showed hampered JNK activation, but almost normal NF-kB. A similar observation was made in transgenic mice, overexpressing a dominant negative form of the TRAF2 molecule (53).

Inactivation of TRAF5 resulted in a milder phenotype and no gros abnormalities of the mice (54). Stimulation of TRAF5 -/- B cells via several receptors (CD40, CD27, TNF) resulted in normal NF-kB and JNK activation. Nevertheless, the same cells showed defects in CD40-mediated proliferation and expression of CD23, CD54, CD80, CD86 and Fas/CD95 (54), suggesting a critical role for TRAF5 in CD40 signaling.

After it's initial cloning as a CD40 associated molecule, the role of TRAF6 has become more complex. It was found that TRAF6, via two additional adaptor molecules (IRAK and MyD88), is also critical in the signal transduction induced by the Toll / IL1-R family (55-58). Within the TNF-R family, association with TRAF6 is restricted to CD40, RANK and the p75 NGF-R (11). Generation of TRAF6 KO mice showed low birth rates of -/- mice and early death (59, 60). Importantly, these mice exhibited severe osteopetrosis, confirming the critical role of RANK-OPGL in bone remoddeling (61). In line with it's role in different signaling pathways, absence of TRAF6 not only interfered with CD40-mediated B cell proliferation, but also B cell proliferation induced by LPS or induction of iNOS in macrophages by IL-1 or LPS (59, 60). These recent observations of TRAF6, which link immune regulation (CD40) and inflammation (IL-1/LPS), might be helpfull in understanding the role of CD40 expression on cells outside the immune system.

3.2.4. CD40 signaling and EBV

TRAF3 was also cloned by screening for proteins which can interact with the EBV transforming gene product LMP1 (latent infection membrane protein-1)(40). It is thought that 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 signalling pathway to activate and immortalize B lymphocytes. Continuous expression of LMP1 is essential for the proliferation of the EBV-immortalized B cells (62, 63). Generation of chimeric proteins suggested that CD40-induced and LMP1-induced pathways are overlapping (64), although it has been shown that TRAF6 can not associate with the LMP-1 molecule

(44). In vivo experiments showed that transgenic overexpression of LMP-1 in a CD40 deficient background resulted in extrafollicular B cell proliferation and Ig production, but not in the generation of germinal centers (65). Although this points to differences in signal transduction, an alternative explanation might be the absence of CD40-CD40L interaction and subsequent "CD40L triggering" which has been suggested to be important for GC formation (66).

3.2.5. Mechanisms of transducing signals

Despite the wealth of new information on molecules implicated in CD40 signaling, the exact mechanism how signals are transmitted is still not clear. For a long time it has been assumed that the trimeric configuration of the different ligands, as proven by chrystallography of TNF, LT and CD40L (16, 67-69), will trimerize the receptor and thereby launch the signal machinery. This resembles the concept of signaling by other cytokines and growth factors. However, crystallography of the TRAF-C domain of TRAF2 which showed spontaneous trimerization already complicated this picture (35, 36). Moreover, recent studies identified a domain (pre-ligand assambly domain; PLAD) in the extracellular parts of TNF-R members, including CD40, which was responsible for the assembly of a trimeric receptor complex in the absence of ligand (70). Whether there is a signaling function for ligand-independent trimers and what is the consequence of ligand binding remains to be determined. For instance different levels of crosslinking might have different biological consequences as shown by functional differences in IL-6 production after anti-CD40 antibody or membrane bound CD40L exposure (71).

A constitutive association between CD40 and TRAF2 has been described (72). Others described that CD40 activation in B cells results in translocation of TRAF2 from cytoplasm to the membrane (73). More importantly, it seems that upon activation, CD40 together with associated TRAF2 and TRAF3 move to lipid microdomains which brings them in close vicinity of different src kinases (73). Interaction between TRAF2 and these kinases can be prevented by TRAF mutants or zinc chelators. Similar results on the importance of membrane rafts for CD40 signaling has been demonstrated in dendritic cells (74). These exciting new developments might provide clues to the exact mechanisms of signal transduction, explaining the array of different activities and differences between cell types.

3.3. Functions of CD40 *in vitro*

3.3.1. B Iymphocytes

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 (2, 5, 6). CD40 ligation activates resting B cells as shown by increase in size and expression of new surface molecules involved in homotypic and heterotypic aggregation (CD23, VLA-4), T cell costimulation (CD80/CD86) and increased expression of MHC class I, MHC class II and TAP transporter (75). Furthermore, CD40-activated B cells secrete a panel of cytokines which may act as autocrine and paracrine growth and differentiation factors (76, 77). 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. In human, IL-4/IL-13 induce switch to IgE and IgG4, IL-10 induces switch to IgG1 and IgG3, whereas combinations of IL-10 and TGF-beta promote switch to IgA. More recently, it has become clear that also other molecular interactions, like interaction with dendritic cells, might enhance the level of Ig production (78), or even directly promote the switch to a specific isotype (IgA)(79).

For selection of high affinity memory B cells, a complex network of interactions exists, involving CD40, Fas, B cell Antigen-receptor (BCR) and cytokines. CD40 crosslinking induces Fas expression and sensitivity for Fas-mediated apoptosis (80-82). However, the simultaneous triggering of the BCR increases the resistance to Fas-induced apoptosis (83). In contrast, apoptosis of germinal center B cells can be induced by prolonged crosslinking of the BCR, an effect which can be prevented by the addition of IL-4 (84, 85). The interplay of these various signalling pathways has been confirmed *in vivo* using HEL transgenic mice (86).

Dual triggering of resting B Iymphocytes through their CD40 and antigen receptor induces a phenotype characteristic of cells from germinal centers (87, 88), the anatomical site where B cells undergo somatic mutation, selection, isotype switching and become either plasma or memory cells. Prolonged triggering of CD40 skews the maturation of B cells into memory cells while interruption of CD40 signalling allows plasma cell differentiation (89, 90).

Although most normal and malignant B cells proliferate in response to CD40 engagement, plasma cells appear unresponsive (91). Furthermore, CD40 ligation appears to inhibit the proliferation of diffuse B cell Iymphomas, thus illustrating a possible negative role of CD40 on cell proliferation (92, 93)

3.3.2. Monocytes and dendritic cells

The expression of CD40 on antigen presenting cells like monocytes and dendritic cells is well established now (94, 95). Activation of the CD40 receptors is one of the critical signals which allow the full maturation of dendritic cells into the most powerfull antigen presenting cells (96). CD40 ligation on monocytes and dendritic cells results in: i) increased expression of costimulatory molecules such as CD54/ICAM-1, CD58/LFA-3, CD80/B7-1, CD86/B7-2; ii) the secretion of cytokines (such as IL1, IL6, IL8, IL10, IL12, TNFa, MIP1a) and enzymes such as matrix metalloproteinase (MMP); iii) an enhanced survival of these cells; iv) enhanced monocyte tumoricidal activity; v) NO synthesis; vi) promote

differentiation of DC from CD34^{+} hematopoietic progenitors. Therefore the interaction between CD40 and CD40L has important consequences for both APC function and T cell function. The full impact of CD40 for DC biology is outside the scope of this paper and is reviewed elsewhere (96-98).

3.3.3. Endothelial cells, epithelial cells and fibroblasts

Apart from hematopoietic cells, the expression of CD40 has now been observed on many other cell lineages. Although the expression is generally low in normal tissues, the molecule is clearly upregulated under various pathological conditions. In accordance, 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, IL-17 or IFN-gamma. The functional relevance of the CD40 molecule on these cells is relatively less investigated. Most likely, CD40 expression on non-hematopoietic cells might be relevant for inflammatory responses. Initial *in vitro* studies, using various cell types, have demonstrated prominent effects of CD40 crosslinking on the production of cytokines and chemokines. Moreover, also other inflammatory mediators are increased, including expression of adhesion molecules, production of matrix metalloproteinases (MMP) and procoaggulant activity.

Alternatively, on some cell types, including carcinomas, it has been demonstrated that CD40 activation results in an induction of cell death (99-101).

3.4. *In vivo* functions of CD40/CD40-L interactions

3.4.1. Hyper IgM Syndrome

The first 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 (102). This disease is characterized by a severe impairment of T cell dependent antibody responses with no B cell memory and no circulating IgG, IgA and IgE (103, 104). The demonstration of preformed CD40L in platelets has been instrumental in the development of an easy diagnostic tool (105). Next 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 signalling (106).

Patients with hyper-IgM syndrome have an enhanced susceptibility to opportunistic infections, such as *Pneumocystis carinii* pneumonia and *Cryptosporidium* diarrhea. This indicates a role for CD40-CD40-L interactions in cell-mediated immune responses. Indeed, CD40-L knockout mice display a considerable impairment of antigen specific T cell priming (107) and appear particularly susceptible to Leishmania infection (108-110). This most likely results from a defective Th1 response which is related to an impaired production of IL12 by antigen presenting cells.

3.4..2 CD40-CD40L: humoral immune responses

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 (111). The generation of CD40 and CD40-L knockout mice revealed a phenotype comparable to that of the patients suffering from the hyper IgM syndrome (112-114).

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, SLE nephritis and experimental autoimmune encephalomyelitis (EAE). On the other hand, administration of agonistic anti-CD40 antibodies can result in strong, isotype-switched, antibody responses against pneumococcal polysaccharides (115). Similarly, an expression plasmid containing the CD40L-trimer has been administered together with *Leishmania* Ag, and was successfull in controling infection with *Leishmania major* (116). 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 immunecompromized individuals.

3.4.3. CD40-CD40L: priming of T cell responses

The contribution of CD40-CD40L interactions to the process of T cell priming, differentiation and effector functions has been extensively reviewed (95, 117-119). The notion that CD40 is expressed on (professional) antigen presenting cells has been a break through in this field. There has been some controversy whether antigen presentation in the absence of CD40-CD40L interaction leads to tolerance (120). In some models, like the cellular immunity against LCMV or VSV virus, T cell priming seems to be independent of CD40L (121), although differences might exist between the induction of the CTL response and maintenance of CTL memory (122, 123). 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'. It has become clear that CD40-CD40L interaction between the CD4+ T cell and the professional APC (dendritic cell), stimulates the DC in such a way that it aquires the capacity to subsequently stimulate CD8+ cytotoxic T cells (124-126). The molecular mechanism underlying this capacity of the APC is at present unknown. However, it seems that other signals, like TNF, LPS or viral infection may substitute for the CD40L-mediated signals, explaining the existence of CD4-independent CD8 T cell responses (127). Stimulating CD40 antibodies have been used to convert a tolerogenic peptide vaccine into strong CTL priming, and to show regression of established tumors (128-130).

3.4.4. CD40-CD40L: role in autoimmunity

Administration of blocking anti-CD40L has been demonstrated to be benificial 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 experimental allergic encephalomyelitis (EAE)(131). In patients with SLE, an increased expression of CD40L was observed on circulating lymphocytes (132, 133), and CD40L positive cells could also be demonstrated in kidney sections of SLE patients (134). Moreover, serumlevels of soluble CD40L seem to be increased in patients with SLE (135). Similarly, activated helper T cells expressing CD40-L surface protein are detected in multiple sclerosis patient brain sections where CD40 bearing antigen presenting cells can be found (136).

Since 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). Interestingly, 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 (136, 137). Part of the mechanism has been suggested to relate to a block in Th1 development and diminished IFN-gamma production (138).

3.4.5. CD40-CD40L: role in transplantation

Interference with CD40 activation prolongs 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, as well as the respective xenografts (139-142). Although treatment with anti-CD40L alone prolongs graft 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 (143). However, it has been shown that the results are strongly dependent on the strain combinations used. In several cases, rejection seems to occur indendent of CD40-CD40L and are mediated by CD8 positive T cells (144, 145). Moreover, it has been shown that the Th1 or Th2 background of the mice affects the result of anti-CD40L blockade (146).

The treatment regimen has been different in the various studies, ranging from once at the time of transplantation until continuous administration every 72 hours to prevent chronic rejection in aortic allografts (147). In the latter case, also CD40L effector functions operating within the transplanted graft might be of importance (see 3.4.4). Local expression of CD40L has been found in human renal and heart allografts, and expression seems to correlate with rejection (148, 149).

Studies with a humanized anti-CD40L antibody have been performed in renal allotransplantation in rhesus monkeys. An extended course of anti-CD40L as the only treatment over a 6 months period resulted in long-term survival (150, 151). Importantly, the organs were not rejected when treatment was stopped, and graft survival reached up to 10 months post-treatment. Combinations of current immunosuppressive drugs, including steroids, MMF, and tracolimus, seems to counteract the benificial effect of anti-CD40L. The same humanized Ab has been used in a primate model of cardiac transplantation, showing improved survival and less vascular pathology (152).

Although the present data do not provide sufficient evidence to show permanent transplant tolerance, these data are promising for the development and use of blocking CD40L reagents. These studies have resulted in a phase-II human clinical trial in renal allograft recipients. However, this trial has been discontinued, due to adverse effects, specifically thrombo-embolic complications (153). It is expected that these trials will be resumed, but it underlines the need for further detailed analysis of the mechanism of action of CD40L blockade in basal studies.

3.4.6. 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 fibrosis, are characterized by the local presence of infiltrating mononuclear cells, including T cells, B cells and monocytes (154, 155).

Administration of soluble CD40L in the lung was demonstrated to induce pulmonary inflammation (156), whereas the use of CD40L KO mice prevented the development of lung inflammation and fibrosis (157). Treatment with anti-CD40L was shown to prevent pulmonary inflammation and fibrosis as a consequence of oxygen-induced respiratory distress syndrom (158). In a chimeric model, it was shown that CD40 positive bone marrow derived cells paly a critical role in this process (159).

Development of atherosclerosis is thought to be dependent on local communication within the vessel wall, possibly orchestrated by activated T cells. In vitro studies already indicated that vascular cells, like endothelial cells and smooth muscle cells (SMC) are responsive to CD40 signaling. CD40 activation of both cell types results in an increased expression of adhesion molecules, cytokines, matrix metalloproteinases and tissue factor (160-163). More importantly it was shown that CD40-CD40L is also relevant for the process of atherosclerosis in vivo. Treatment with anti-CD40L in hyperlipidemic mice (lacking LDL receptor) reduced the size of atherosclerotic lesions (162, 164). Evenmore, in models with progressive atherosclerosis, delayed treatment with anti-CD40L was still able to change the composition of the plaque and reduced further disease development (165, 166). These data were confirmed in a model of ApoE and CD40L double knock-out mice (167). Finally, transplant arteriosclerosis, occurring during chronic rejection, was prevented by anti-CD40L treatment and was associated with an increased expression of the protective, anti-apoptotic genes heme oxygenase-1, Bcl-xL and A20 (168).

4. CONCLUDING REMARKS

In recent years, the focus of CD40 research has been shifted from the study of B cell regulation (humoral immunity), to the study of a general regulator of immune

and inflammatory processes. Multiple disease states appear to be improved by interruption of CD40/CD40-L interactions. This is presently accomplished by preventing the association of the receptor with its ligand using specific antibodies and has resulted in phase II clinical trials. However, for long term clinical applications, such reagents may not be optimal. The identification of small synthetic chemical agents preventing the interaction of CD40 with its ligand would be of interest. Recently important progress has been made in unravelling the signal transduction pathways of CD40 in different target cells. This might allow the development of pharmacologic agents which will specifically block the intracellular pathways turned on after ligation of either CD40 or CD40-L.

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