Investigation of the Therapeutic Potential of Transgenic CD40 Ligand Expression

Michael Paul Brown

School of Medicine
Faculty of Health Sciences
University of Adelaide

Thesis by Prior Publication Submitted for the
Degree of Doctor of Philosophy
May 2007

TABLE OF CONTENTS

TABLE OF FIGURES AND TABLES

THESIS ABSTRACT

The CD40 ligand (CD40L) molecule is central to innate and adaptive immunity. CD40L expression is very tightly regulated whereas its CD40 receptor is constitutively expressed by many different cell types. CD40L is expressed transiently on helper T cells (Th) only after activation by specific immune recognition molecules carried by professional antigen presenting cells, in particular, dendritic cells (DC). CD40L subsequently binds to CD40 on DC to enable full Th activation. CD40 ligated DC produce interleukin-12 (IL-12) and contribute both to the development of IFNγ-secreting natural killer cells, a vital component of innate immunity, and of IFNγ-secreting type 1 Th (Th_1) cells. CD40 ligated DC also contribute to the development of IL-4- and IL-10-secreting Th_2 cells. CD40L on Th cells also binds CD40 on macrophages to enhance their cytotoxic functions. CD40L-expressing Th cells provide the 'help' pivotally required to activate other components of adaptive immunity responsible both for clearing invading pathogens and generating the memory cells required to prevent re-infection. Th-supplied CD40L binds (i) B cell CD40 to switch production of antibodies to more potent effector molecules that have higher avidity for antigen, and (ii) DC CD40 to prime then expand antigen-specific cytotoxic T lymphocytes (CTL). Activated NK cells and CTL are required both to eradicate malignant cells and cells infected with viruses or other intracellular pathogens.

Genetic CD40L deficiency causes the very rare HyperIgM Syndrome Type 1 (HIGM1), which is realistically modelled by genetically engineered CD40L-deficient mice. Neither CD40L-deficient patients nor mice make effective antibodies or mount cellular immune responses that would defend them against intracellular pathogens such as parasites. Consequently, the only potentially curative therapy is allogeneic stem cell transplantation or CD40L gene replacement. Here, we used a retroviral vector, which constitutively expressed CD40L, to genetically modify CD40L-deficient bone marrow cells, which were used to reconstitute partially the immunity of CD40L-deficient mice. The crucial importance of tight regulation of CD40L expression was revealed when these mice later developed lethal thymic T cell malignancy.

Growing tumours escape immune vigilance by genetic alterations that reduce their sensitivity to IFNγ. Using murine tumour models, we incorporated transgenic CD40L expression in therapeutic tumour vaccines to show that CD40L gene transfer augmented the immunogenicity of the host's tumour thus reducing its tumorigenicity. We translated this finding clinically to safety and immunogenicity testing of a transgenic CD40L- and IL-2-expressing leukaemia vaccine.

Finally, the common viral respiratory pathogen, respiratory syncytial virus (RSV) mainly infects young infants and the elderly to cause potentially lethal pneumonia. Both groups have reduced cellular and humoral immunity, which predisposes them to re-infection with RSV. Using a murine model, we showed first that simultaneous adenoviral expression of CD40L augmented primary RSV-specific Th1 responses that were associated with accelerated pulmonary viral clearance. Second, we showed that expression of CD40L in RSV-F and RSV-G subunit DNA vaccines elevated antibody and cellular immune responses to RSV challenge four and eight months after the initial immunisation.

These results demonstrate the potent ability of CD40L gene transfer to solve the absolute immune deficiency caused by genetic lesions of CD40L. However, physiological regulation of the transgene is required to prevent serious adverse consequences. In contrast, no adverse effects were observed after transgenic CD40L expression was used to overcome relative immune deficiencies imposed by malignancy and RSV infection.

The crucial significance of the ligand for CD40

The ligand for CD40 is a molecule critical in human biology and medicine (Chapters 1 and 2). CD40 ligand (CD40L) acts at the point of specific immune recognition of an antigen to activate the immune system and so produce immunity. If immune activation results then a chain of events is set in motion that result in elimination of antigen from the body. An integral feature of immunity is immunological memory, which is the ability to mount a similar or augmented immune response in the event that the antigen is again encountered. CD40L plays a vital role in coordinating, amplifying and recalling the immune response, which necessarily includes CD40L-mediated cooperation between the innate and adaptive arms of the immune system. If CD40L does not act and immune activation does not occur then the immune system sleeps in a state of immunological tolerance for the antigen. Although there are other causes of immunological tolerance, tolerance is the functional outcome as tumours grow in the body.

After antigen recognition, the decision within the immune system to activate, or not, may be considered a binary 'on' or off' response that may produce outcomes deleterious to the organism's survival if the decision is made out of its proper context. Hence, an evolutionary imperative will have dictated that appropriate mechanisms be built into the organism's immune system to control appropriately 'on' or 'off' decisions and so maximise the organism's ability to survive and reproduce. The term antigen is nominally an experimental construct. In the real world, antigens are part of infectious pathogens, the altered body components of cancer, and normal body components subject to autoimmune attack. Consequently, failure of the organism's immune system to make correct 'on' or 'off' decisions in response to antigen recognition may impair the organism's chances of survival.

How is this key 'on' or 'off' decision made and controlled?

The antigen recognition event initiates the decision making. A peptide derived from the antigen is bound by the major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC), which is required to be a dendritic cell (DC) in order to prime rather than recall the T cell responses of immunity or tolerance. If the 'key fits the lock' then the peptide-MHC (pMHC) complex interacts with the T cell receptor (TCR) on the surface of a CD4-expressing T helper lymphocyte. The duration and strength of this cognate interaction determines the probability that T cell activation will occur. The first essential signal after T cell activation is the induction of CD40L expression. By ligating its CD40 receptor on the DC surface, CD40L then conditions the DC to engage further in crosstalk with the T cell so that a more durable immunological synapse between the two cells is assembled. Subsequently, the DC is licensed to prime the killer CD8-expressing T lymphocyte, which kills virally infected cells and tumour cells. Thus, CD40L expression promotes the development of type 1 immune responses, which are cytolytic. The CD4- and CD40L-expressing T lymphocyte can also provide 'help' to B lymphocytes in the form of ligation of its CD40 receptor so that it can produce more effective antibodies able to neutralise pathogens. Finally, antigen-experienced and CD40L-expressing T lymphocytes traffic to peripheral tissues at the site of antigen challenge where, *via* the ligation of CD40 on accessory immune cells, inflammation is promoted. In the permissive inflammatory microenvironment, CD40L is also expressed by other accessory immune cells and, *via* CD40 ligation, inflammation is reinforced and the ongoing development of immunity is enhanced.

To control this cascade of possibly injurious events, it will be noted that CD40L expression is for the most part only inducible in the most temporally and spatially restricted circumstances following lymphocyte activation whereas its CD40 receptor is constitutively expressed by both immune and non-immune cells such as endothelial cells, fibroblasts and epithelial cells

throughout the body. This compartmentation of the CD40L activating signal ensures that the responding cells receive it only in the context of immune recognition.

What are the implications if the CD40L-dependent activation signal is not made and it should be?

Absolute loss of the CD40L-dependent activation signal results in profound cellular and humoral immunodeficiency, which may be complicated by lethal opportunistic infections and, less comprehensibly, by malignancy of the hepatobiliary tree. The most straightforward although rare cause of absolute signal loss is inherited mutations in the genes for CD40L or its receptor. *Thus, a clear rationale exists for the replacement of the CD40L gene in the case of its genetic deficiency (Chapters 2 and 3).*

Relative loss of the CD40L-dependent activation signal occurs in the neonatal period and early infancy when CD40L expression is less responsive to T cell activation. A teleological explanation for the relative deficiency of CD40L expression would be that the immature immune system is less responsive because it has the important tasks of positively selecting lymphocytes that recognise foreign antigens and negatively selecting autoreactive lymphocytes. 'Hair-trigger' induction of CD40L expression may compromise the elimination of autoreactive lymphocytes and predispose to autoimmune disease. To continue the teleological argument, while thymic T cell education is underway, transfer of mature maternal antibodies *via* the placenta and breast milk compensates for the relative lack of CD40L-dependent antibody maturation and so reduces the infant's susceptibility to infection. However, the most common respiratory pathogen of early infancy is respiratory syncytial virus (RSV), which does not evoke effective immunity and predisposes to re-infection even in adults, which includes the mothers of susceptible infants. RSV infection tends toward the induction of type 2 rather than type 1 immune responses and so deviates the host immune response toward a less effective anti-viral response. Given that peak exposure to RSV occurs in early infancy, relative CD40L deficiency may also contribute to the development of ineffective RSV-specific immune responses. *In the presence of ineffective RSV-specific immunity and immune deviation, it is reasonable to test the hypothesis that CD40L gene augmentation will correct the immune deviation and provoke effective RSV-specific immunity (Chapter 4).*

A plethora of defects in immune function accompany malignancy. One of the most outstanding is the state of immunological tolerance or even frank immune suppression that progresses as the malignancy itself progresses. Here, it is important to note that abundant evidence now exists to indicate that immune recognition of tumour associated antigens does occur. Therefore, the host's immunological tolerance of the malignancy belies the threat that it poses to life.

A distinction will be made between the malignancies that express CD40 and those that do not. CD40-expressing malignancies more commonly originate from the lympho-haemopoietic system although a significant proportion arise from the epithelium, which is the most important source of malignancy overall. In many instances, CD40 ligation of CD40-expressing malignancies induces at least one of two effects. First, CD40 ligation induces upregulation of MHC, costimulatory and cell adhesion molecules, which together directly enhance the antigen processing and presentation functions of the tumour cell itself. Second, CD40 ligation induces tumour cell death, which indirectly promotes antigen processing and presentation by bystander APC. In this second event, it is both the greater efficiency in APC uptake of tumour antigens and CD40L-mediated enhancement of bystander APC function that promote anti-tumour immunity. On the other hand, in CD40-negative malignancies, CD40 ligation of bystander APC is most important, and anti-tumour immune effects may be reinforced if another modality of anti-tumour treatment such as cytotoxic chemotherapy induces tumour cell death. Of course, in any one malignancy, tumours may or may not express CD40 and any of the abovementioned mechanisms may operate to increase anti-tumour immunity. *Hence, it is reasonable to test the hypothesis that genetic augmentation*

with the gene for CD40L, which is a critical positive regulator of immunity, will induce effective anti-tumour immune responses (Chapter 5).

What are the implications if the CD40L-dependent activation signal is made and it should* not *be?

Several studies show that transgenic unregulated overexpression of CD40L induces autoimmunity. However, although retroviral gene transfer partially corrected CD40L deficiency in the mouse model, unregulated transgenic expression of CD40L by bone-marrow derived cells, which had repopulated the CD40L-deficient mice, produced thymic lymphoproliferations and lymphoblastic lymphoma after a latent period (Brown MP *et al*., 1998). In contrast, a later study of regulated transgenic CD40L expression also corrected the immunodeficiency but did not cause malignancy and, therefore, vindicated the physiological relevance of regulated CD40L expression *(Chapter 6)*.

Summary of the laboratory research program

The complementary DNA (cDNA) for murine CD40L (mCD40L) was cloned (Dilloo D *et al*., 1997) and the cDNA for human CD40L (hCD40L) was obtained. The mCD40L cDNA was subcloned into the retroviral shuttle plasmid, pG1a, which was subsequently used to make and clonally select a high-titre retroviral producer cell line (Grossmann ME *et al*., 1997). The supernatants obtained from this mCD40L retroviral producer clone were frozen in aliquots and stored for later use. The pG1a.mCD40L was provided to make a fibroblast cell line, which stably expressed mCD40L (Dilloo D *et al*., 1997). The mCD40L retroviral producer supernatants were provided to make a neuro2a cell line, which stably expressed mCD40L (Grossmann ME *et al*., 1997). The mCD40L retroviral producer supernatants were used to transduce CD40L-deficient murine bone marrow cells in readiness for transplantation (Brown MP *et al*., 1998). Bone marrow transplantation, measurement of gene transfer efficiencies, harvesting and phenotypic analysis of thymic tumours, and analysis of dinitrophenol-specific IgE responses were done as described (Brown MP *et al*., 1998). The mCD40L cDNA was subcloned into an adenoviral transfer plasmid, which was used to make a mCD40L-expressing adenoviral vector (Ad-mCD40L) after triple-plaque purification. An empty vector control (Ad-VC) was made in a similar way. Scaled-up stocks of these vectors were provided for co-infection experiments with RSV in BALB/c mice (Tripp RA *et al*., 2000) and a scaled-up stock of Ad-mCD40L was also supplied to perform transductions of MB49 murine bladder cancer cells (Loskog A *et al*., 2001). The hCD40L cDNA was subcloned into an adenoviral transfer plasmid and used to make a hCD40L-expressing adenoviral vector (Ad-hCD40L), which after triple-plaque purification was used subsequently to perform adenoviral transductions of human myeloma cell lines *in vitro* (Dotti G *et al*., 2001). The same vector was used to create the clinical leukaemia vaccines reported by Rousseau RF *et al*. (2006).

Significance of the major research findings

A recent survey conducted under the auspices of the U.S. National Cancer Institute ranked CD40L and anti-CD40 monoclonal antibodies fourth out of 20 molecules with high potential for use in treating cancer (http://web.ncifcrf.gov/research/brb/site/home.asp.). The publications presented herein were the first to show that:

(i) Genetic correction of CD40L deficiency was possible by gene replacement (Brown MP *et al*., 1998)

(ii) Deleterious effects resulted from constitutive CD40L transgene expression (Brown MP *et al*., 1998)

(iii) Transgenic CD40L gene expression conferred anti-tumour properties using *in vivo* models of CD40-positive and CD-negative malignancies (Dilloo D *et al*., 1997; Grossmann ME *et al*., 1997)

(iv) CD40 was not detected on primary human prostate cancer cells (Moghaddami M *et al*., 2001)

(v) Transgenic CD40L gene expression enhanced anti-RSV immunity in a model of RSV infection (Tripp RA *et al*., 2000; Harcourt JL *et al*., 2003).

Consequently, the nine publications included in this thesis have been cited 251 times in total.

STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University Library.

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Signed: Date: 23 May 2007

DEDICATION

This work is dedicated to the memories of my late father, Reginald Frederick Brown, my late aunt, Heather Gabrielle Brown, and my late father-in-law, Peter William Gage, who all at various stages of my life provided the inspiration or impetus for this work.

ACKNOWLEDGMENTS

I am greatly indebted for the support, encouragement and mentorship of my supervisors, Professors Malcolm Brenner and Ian Olver. My wife, Michelle Anne Gage Brown, has provided the encouragement and sustenance that I needed to complete this task during which I depended also on the forbearance of our children, Liam, Alice, and Daniel.

STATEMENTS OF THE CONTRIBUTIONS OF JOINTLY AUTHORED PAPERS

Each publication included in this thesis was jointly authored. As permitted by Professor Richard Russell, Dean of Graduate Studies, University of Adelaide, appended to this thesis (Appendix A) is a statement for each publication, which gives written and signed permission by each author for the paper to be included in the thesis and which provides a detailed description of the contribution made by the PhD candidate as an author on each paper.