Anti-Tumour Therapeutic Efficacy Of OX40L in Murine Tumour Model

Selman A. Ali, [*] Murrium Ahmad,[*] June Lynam, Cornelia S. McLean, [†] Claire Entwisle, [†] Peter Loudon, [†] Esther Choolun, [*] Stephanie E. B. McArdle, [*] Geng Li, [*] Shahid Mian, [*] and Robert C. Rees [*]

*School of Science, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK; and the †Xenova Group plc, Cambridge Science Park, Cambridge CB4 4GN, UK.

Correspondence To:

Robert C Rees

Professor of Biomedical Science

Department of Life Sciences, The Nottingham Trent University

Clifton Lane, Nottingham NG11 8NS

Tel: + 44 0115 8486342

Fax: + 44 0115 8483384

Eml: robert.rees@ntu.ac.uk

Abstract

The OX40 ligand (OX40L), a member of TNF superfamily, is a costimulatory molecule involved in T cell activation. It is expressed on antigen presenting cells including dendritic cells (DC) and activated B cells. This molecule has been reported to provide potent costimulation in APC-T cell interactions upon binding to its cognate receptor, OX40 which is expressed by activated T cells. In this study systemic administration of OX40L fusion protein was used in the treatment of established murine subcutaneous colon and breast carcinomas. Intra-peritoneal injection of mOX40L fusion protein significantly inhibited the growth of subcutaneous 3 day established colon (CT26) and breast (4T1) carcinomas which was dose and route dependent. Effective therapy with OX40L required the the presence of tumour for 3 days prior to OX40L, concomitant therapy, given at the same time (day 0) as tumour cells was less effective. Furthermore, therapy with OX40L fusion protein was effective in significantly reducing CT26 experimental lung metastasis. In addition, inhibition of CT26 and 4T1 tumours in response to therapy with OX40L was significantly enhanced by combination treatment with intra-tumour injection of a DISC-HSV vector encoding mGM-CSF. Tumour rejection in response to OX40L therapy was correlated with splenocyte CTL activity against the gp70 CT26 tumour associated antigen. *In vivo* depletion studies demonstrated the requirement for both CD4+ and CD8+ T cells for effective OX40L therapy. Collectively these results demonstrate the potential role of the OX40L in cancer immunotherapy.

Introduction

T cells play an important role in immunological surveillance and eradication of cancer, and their activation requires at least two signals (Bretscher, et al 1992; Carreno and Collins 2002). The first signal is delivered through the T-cell receptor (TCR) via the antigen-specific recognition and binding to MHC/ peptide complex; this triggers the T cell activation cycle but is insufficient for the generation of effector function, which requires a second co-stimulatory signal to promote cytokine production and proliferation. This complex series of events is mediated through the interaction of surface molecules (ligands/receptors) on APC and their counterparts on the surface of T cells (Guinan et al, 1994; Hellstrom et al, 1996). There are a number of molecules normally expressed on the surface of antigen presenting cells (APC) that perform co-stimulatory functions by interacting with their counter ligand/receptor on T cells to provide the critical second signal for T-cell and/or APC activation including: CD80 (B7-1) / CD86 (B7-2) binding to CD28 and CTLA4 respectively; CD40L on activated CD4 helper cells binding to CD40 receptors on APCs; human CD58 and mouse CD48 (LFA-3) binding to CD2; and CD54 (ICAM-1) binding to LFA-1. Failure to provide secondary co-stimulation can cause T cell anergy, a form of non-responsiveness resulting from incomplete T cell activation (Croft et al, 1997). Furthermore generating CD8^{+} T-cell effector cells requires a CD4^{+} T-helper (Th) response, mediated principally by MHC restricted Th1 cells. The generation of these effector cells requires not only TCR-MHC peptide interaction, but also secondary signals that mediate cell survival (Oh etal, 2003).

Therefore the level of co-stimulation has a profound effect on the response to antigens, and strong co-stimulatory signal can convert a weak agonist into a full agonist and an agonist into a super-agonist (Murtaza etal, 1999). Co-stimulatory interactions can also play a crucial role

during the death phase of the T cell response and determine the number of effector T cells that survive to become memory T cells (Bansal-Paskala etal, 2001). Several members of the TNF R super-family including CD27, CD30, 4-1BB and OX40 have also been shown to transmit a co-stimulation signal for CD8$^+$ and CD4$^+$ T cell proliferation, cytokine production and cell survival in a manner similar to that of CD28 (Smith etal,1994, cell 76, 959; Gruss and Dower, 1995, blood, 85, 3378).

Improving cancer immunotherapy approaches requires an understanding of the molecular interactions responsible for inducing anti-cancer T cell responses. The availability of well-characterised tumour associated antigens "TAAs" combined with our knowledge of the requirements for T-cell activation allows us to propose new strategies for cancer vaccine based therapy. One important signal influencing T-helper cell activation is mediated via the OX40 pathway.

The OX40 receptor (CD134) is a membrane bound glycoprotein belonging to the tumour necrosis factor (TNF) receptor superfamily and recent evidence suggests that this ligand - receptor pathway is important for promoting the full effector function of CD4$^+$ T - helper cells (Weinberg et al, 2000). OX40 Ligand (OX40L) is a type II transmembrane protein and is expressed on B cells, dendritic cells (DCs) and endothelial cells (Stuber etal, 1995; Imura et al, 1996). Signals transmitted via CD28 and OX40 are highly synergistic for CD4$^+$ T cell proliferation and survival (Gramaglia et al, 1998), and interaction of OX40L with its receptor (CD134) has been shown to increase the expansion and survival of naïve CD4$^+$ T cells, and to enhance memory Th cell survival by inhibiting peripheral deletion (Maxwell et al, 2000 J; Gramaglia et al, 2000). Modulating the OX40-OX40L pathway can influence the in vivo pathogenesis of infection and cancer. Altering the Th1/ Th2 balance by administering anti-OX40 L mAb abrogates progressive Leishmaniasis in susceptible Balb/c mice, which was associated with reduced production of Th2 cytokines (Akiba et al, 2000). In animal tumour

models, *in vivo* ligation of OX40R by administration of OX40L:immunoglobulin fusion protein or OX40R mAb resulted in a significant prolongation of survival of tumour-bearing mice in four histologically distinct tumour models (Weinburg et al, 2000). The therapeutic efficacy of OX40 mAb was influenced by the tumour burden, the site of tumour growth and the intrinsic immunogenecity of the tumour (Kjaergaard et al, 2000). Furthermore, OX40$^+$ Th cells have been detected in human breast, melanoma and head and neck cancer tissue (Vetto etal, 1997).

In the present study, the role of OX40L as a co-stimulatory molecule for cancer therapy was further analysed. Intraperitoneal administration of OX40L-immunoglobulin conjugates to tumour-bearer animals inhibited tumour growth in a dose and time dependent manner. The inhibition was shown to correlate with splenocytes CTL activity and *in vivo* depletion of CD4$^+$ and CD8$^+$ T cells abrogated antitumour activity. This study also demonstrated that therapy with OX40L fusion protein combined with direct intra-tumour injection with a disabled single cycle herpes simplex virus encoding the gene for murine granulocyte - mecrophage colony stimulating factor (DISC-mGM-CSF) enhanced the therapeutic response and induced maximum tumour growth inhibition.

Materials and Methods

Animals

Female Balb/c mice were purchased from Harlan (UK) Ltd, and maintained in accordance with the Home Office Codes of Practice for housing and care of animals.

Peptides

A known murine leukemia virus (MuLV) gp-70 derived H2L^d restricted peptide AH-1 (SPSYVYHQF, 138-147) and a control β-galactosidase H2L^d restricted peptide (TPHPARIGL, 877-88) were synthesised (Company) and used for *in vitro* assays.

Cell lines and Tumour Therapy Protocols

  The CT26 cell line is a N-nitroso-N-methylurethane induced Balb/c murine colon carcinoma, maintained by serial *in vitro* passage in DMEM tissue culture media supplemented with 2mM L-glutamine and 10% FCS. This cell line was provided by Prof Ian Hart, (ICRF, London, UK). The A20 Balb/c murine B cell lymphoma, RENCA (a balb/c renal cell carcinoma line) and the NK sensitive YAC-1 lymphoma were maintained by serial *in vitro* passage in RPMI 1640 tissue culture media supplemented with 2mM L-glutamine, 10% FCS and 0.05mM 2-Mercaptoethanol. Subcutaneous (s.c) CT26 and 4T1 tumours were induced by the injection of $1x10^4$ cells on the right flank. Ten animals per group were injected intra-peritoneally or as indicated for individual experiment at times / intervals after tumour cell implantation with OX40L conjugated protein or media in a volume of up to 300μl. For combination therapy experiments, animals were injected s.c with $8-10^4$ to $1x10^5$ tumour cells; mice with tumors of a size of 0.09-0.36 cm^2 diameter were selected at 8-10 days. Intra-tumour therapy was performed by directly injecting DISC/-HSV/mGM-CSF virus (50μl volume) into the tumour followed by intra-peritoneal OX40L fusion protein injection; the dose of vector and time of administration is shown for individual experiments. Statistical analysis was performed using Graph Pad Software (Student *t* test and).

DISC/mGMCSF viral vector

DISC/mGM-CSF-HSV (dH2B) was constructed by recombination as previously described (Ali, etal, 2000; Todryk, et al, 1999). The DISC/mGM-CSF virus (dH2B) is thymidine kinase

negative (TK⁻), gH negative (gH⁻) and expresses murine granulocyte-macrophage-colony stimulating factor (mGM-CSF) following infection of normal or complementing cells.

OX40L Preparation

The MM1 (murine OX40L-murine IgG1) and MH1 (murine OX40L-human IgG1) are hybrid proteins constructed by linking the appropriate truncated murine or human immunoglobulin gamma 1 gene (hinge-CH2-CH3) to the extracellular domain (aa50-198) of the murine OX40 ligand gene (Genbank U12763). The linkage is achieved via a mutated heterologous hinge region, which is identical to the native sequence except that the cysteine residues have been changed to alanine in order to increase the flexibility of the linkage. The fused polypeptides are designed as secreted products which are achieved by use of a signal sequence from the murine B72.3 antibody (Whittle etal, 1987) ligated at the NH_2 terminal.

The MM1 and MH1 proteins were produced in stable transfected Chinese Hamster Ovary (CHO) cells (Lonza Biologicals, UK) grown in hollow fibre or roller bottle culture in serum containing media. The growth media was purified on a protein G sepharose column and the final product was dialysed against PBS (pH7.4).

In Vitro Generation of CTL and chromium release cytotoxicity assay

Spleens were harvested from tumour bearer mice receiving therapy (test) and non-treated normal mice (control). Cells were flushed from the spleen (lymphocyte fraction) with serum free RPMI media using a 25g needle and syringe. The remaining spleen tissue was cut into four pieces, digested with 2ml of enzyme cocktail (1.6 mg/ml collagenase and 0.1% DNase in serum free RPM1 media, Sigma, UK) at 37°C in a 5% CO_2 in air humidified atmosphere, for 60 minutes. The spleen tissue was then dissociated by gentle pipetting. The cells

(parenchymal fraction) were collected, washed twice in serum free medium, mixed with the lymphocyte fraction and suspended in CTL media and cultured at a concentration of 5 x 10^6 cells/2ml (RPMI 1640 supplemented with 1% glutamine, 10% FCS, 20mM HEPES, 50 μM 2ME, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.25 μg/ml fungizone) with 10μM of the relevant peptide in to 24 well plates at 2ml / well for 5 days.

On day 5 of *in vitro* stimulation, splenocytes were harvested, washed twice in serum free media, re-suspended in CTL media, counted and used as effector cells. Target cells were harvested by trypsinisation, washed, and labeled with chromium-51. A standard 4 hours Cr - release assay was performed and the percentage specific cytotoxicity was determined using the following equation: -

% Specific Cytotoxicity = (Experimental Release - Spontaneous Release) / (Maximum Release - Spontaneous Release) x 100

CD4$^+$ and CD8$^+$ T-cell depletion

The *in vivo* depletion of CD4$^+$ and CD8$^+$ was carried out as previously described (Ali et al, 2000). Briefly, Groups of 10 mice were given three i.p injections of 400 μg of anti-CD4$^+$ (YTS191.1.2), anti-CD8$^+$ (YTS169.4.2.1), control isotype antibody (YTH24), or a combination of anti-CD4$^+$ and anti-CD8$^+$ antibodies over a period of one week.

Results

In vitro T Cell Proliferation in Response to OX40L and OX86

The effect of murine OX40L and anti-murine OX40 antibody (OX86) on T cell proliferation was tested by bioassay *in vitro*. Balb/c mice were immunised with Staphylococcal enterotoxin B (SEB) i.p. and twenty four hours later lymph nodes were removed and

processed and their lymphocytes were cultured *in vitro* with 0.5 μg /ml SEB and varying concentrations of murine OX40L (mOX40L), OX86, murine IgG1 or human OX40L (Hox40L) for 72 hours. Cells were pulsed with ^{3}H thymidine for the final 6 hours of culture. The results (Fig 1) clearly demonstrates that both m OX40L and OX86 but not control human OX40L and murine IgG1, enhanced T cell proliferation in a dose dependent manner. Murine OX40L stimulated T cell proliferation at 0.001 μg /ml whereas OX86 required 1000 fold more protein to induce similar levels of proliferation.

OX40L Therapy

It has previously been shown that engagement of OX40 receptors with antibody agonist or the OX40L delivers a potent co-stimulatory signal to effector T cells (Pan etal, 2002; Morris etal, 2001). In the present study, the therapeutic effect of systemic administration of mOX40L fusion protein into CT26 tumour bearer animals was investigated. Intraperitoneal injection of mOX40L protein 3 days after the subcutaneous injection of $1x10^4$ CT26 cells (X10 TD$_{50}$) induced a significant (p=<0.05) inhibition of tumour growth (Fig 2). Similarly, significant tumour inhibition (p=<0.01) was obtained using this protocol to treat 4T1 tumour -bearer mice. Three and five out of the10 treated animals injected with CT26 and 4T1 tumours cells respectively remained tumour free throughout the period of the experiment, and these mice remained immune to subsequent subcutaneous challenge with parental tumour cells (data not shown). The therapeutic effect of mOX40L was species specific since the human hOX40L conjugated to human IgG1 failed to inhibit tumour growth (Fig 3).

The tumour growth inhibitory effect of mOX40L therapy was shown to be dose and route dependent (Fig 4 A&B). Using the 4T1 tumour model, increasing the dose of mOX40L administered i.p from 5x100 to 5x250 μg per mouse increased the degree of tumour growth inhibition and increased the overall survival (from 3/10 to 6/10 for mice receiving 5x100 and

5x250 μg per mouse respectively). Similarly the i.p therapeutic efficacy of mOX40L have been increased when the dose was increased from 2x250 μg to 2x500 μg per mouse despite the administration frequency decreased from 5 to 2 (Fig 4 A). Results shown in Fig 4 B clearly demonstrates a significant difference (P = <..........) in efficacy between the i.m versus the i.p route of administering of mOX40L; five doses of 100μg and 250μg mOX40L given i.m reduced tumour growth and tumour incidence in this model compared with mice receiving i.p. injections of OX40L. [Selman - needs clarification]. We also observed that the murine mOX40L fusion protein was comparable to the anti-murine OX40 (OX86) at reducing 4T1tumour growth when administered at equivalent concentrations (data not shown).

Both OX40 and its ligand are upregulated during $CD4^+$ T-cell priming and it was reported that expression of OX40 on T cells in the T cell zone in lymph nodes was maximal 3 days after antigen administration (Stuber & Strober 1996, j exp med 183, 979). Based on these observations we undertook experiments to establish whether the timing of mOX40L fusion protein administration after tumour implantation affected the therapeutic response. The results in Fig 5 clearly show that a priming period of 3 days with CT26 tumour cells was required to obtain the maximal anti-tumour inhibitory effect with mOX40L (p<0.01). Seven out of 10 animals remained tumour free when 100μg of mOX40L fusion protein was injected on day 3 compared with 0/10, 1/10, 0/10 and 1/10 for control mice injected with PBS or OX40L on day 0, 6 and 10 respectively.

Effect of mOX40L fusion protein Therapy on Experimental Metastasis

Experimental lung metastasis was induced by injecting $1x10^4$ CT26 cells intravenously and based on the results shown in Fig 5. Treatment with OX40L was started on day 3: control mice were given 5 injections of 200μl PBS i.p at 3 days intervals, the remaining 3 groups

were given 5 injections of either 50, 100 or 250 μg mOX40L fusion protein i.p, respectively.

Tumour inhibition was shown to be mOX40L dose dependent; 50% of animals treated with 5

injections of 250 μg OX40L fusion protein remained tumour free for the duration of the

experiment in comparison to 33% for groups treated with 50 or 100μg of mOX40L fusion

protein (Fig 6). Similar results were obtained when the OX40L fusion protein was injected

intramuscularly (results not shown).

Combination Therapy of OX40L and DISC/mGM-CSF virus.

We have previously shown that direct intra-tumour injection of subcutaneous CT26 tumours

with DISC/mGM-CSF induces the complete regression of tumours in a proportion of treated

animals (Todryk et al, 1999; Ali et al, 2002). In the present investigation the effect of

combination therapy of mOX40L fusion protein and DISC/mGM-CSF therapy on established

subcutaneous 4T1 and CT26 tumours was determined. 4T1 and CT-26 tumours were

established by s.c. injection of 1-2 x 10^5 tumour cells, and therapy was administered on day 8

when tumours were 0.09-0.36 cm^2 diameter. The results for 4T1 tumours is given in Fig. 7A.

Two direct injections of 1.7x10^7 pfu of DISC-HSV-mGM-CSF at 2 day intervals or intra-

peritoneal injection of 2x250μg of mOX40L fusion protein resulted in tumour growth

inhibition but no complete tumour regressions were detected. However, a significant increase

in tumour growth inhibition was observed in mice receiving direct intra-tumour injection of

DISC/mGM-CSF followed by i.p injection of the mOX40L fusion protein (see Materials and

Methods for details). Six out of the ten animals treated with combined therapy survived and

remained tumour free (p=<0.01), and rejected a further challenge with parental 4T1 cells

(results not shown). Similar results were observed when mice with subcutaneous CT26

tumours were treated with combination DISC-HSV-mGM-CSF and mOX40L fusion protein

(Fig 7B); 3/10, 7/10 and 10/10 mice rejected their tumour following therapy with DISC, OX40L and DISC+OX40L, respectively compared with control mice

CTL Activity in Response to Therapy with OX40L

To elucidate the potential role of cytotoxic T-lymphocytes (CTL) in tumour rejection induced by mOX40L infusion protein therapy, a series of experiments were undertaken to assess the presence of CTL activity in CT26 tumour bearer mice treated with mOX40L. The H2-L^d - restricted AH-1 peptide of the gp70 tumour antigen is endogenously expressed by CT26 tumour cells and represents an appropriate target peptide for assessing the MHC class I restricted CTL response. Splenocytes were harvested from mice that had rejected subcutaneous CT26 tumours following i.p injections of the mOX40L fusion protein and mice that failed therapy. Splenocytes were cultured for 5 days in the presence of the AH-1 peptide (SPSYVYHQF) and assayed for cytolytic activity against CT26 cells, A20 pulsed with the AH-1 peptide and YAC-1 cells; the immunogenic TPHPARIGL peptide derived from the bacterial protein β-galactosidase was used as an irrelevant control peptide. Spleen cells from all test mice (five out of five) that had rejected CT26 tumours in response to therapy with mOX40L fusion protein exhibited significant (P<0.001) CTL activity against CT26 and A20 cells pulsed with the AH-1 peptide (Fig 8 A&B). The average cytolytic activity (at 50:1, E:T ratio) of splenocytes responding to the SPSYVYHQF peptide was 60% and 72% against CT26 and A20 AH-1 peptide pulsed cells, respectively. The CTL response was peptide specific since lymphocytes from the same mice cultured for 5 days with the irrelevant peptide (β-gal: TPHPARIGL) were not cytotoxic for CT26 or A20 peptide pulsed target cells (Fig 8 A&B). However, only one in four mice with progressor tumours exhibited a low but significant CTL activity (data not shown).

To confirm the involvement of $CD4^+$ and $CD8^+$ T lymphocytes in the tumour rejection resulting from mOX40L fusion protein therapy, mice were depleted of the respective T cell population by administration of monoclonal antibodies against either CD4 or CD8 antigens (see materials and methods). Four days after the initiation of the antibody treatment, mice were injected s.c with $1x10^4$ CT26 cells and 100 μg of mOX40L fusion protein was given on days 3, 6 and 9 following tumour cell injection. The results given in Fig 9 show that mice depleted of CD4 or CD8 or both failed to respond to treatment with OX40L fusion protein, whereas mice receiving either the isotype control serum or media demonstrated a therapeutic response to mOX40 therapy. These results confirm the requirement for $CD8^+$ T-cells in mediating an anti-tumour effect and infer that both $CD4^+$ and $CD8^+$ T-cells are required for effective therapy using mOX40L fusion protein.

Discussion

OX40-OX40L interactions control primary T cell expansion, regulate the numbers of antigen-specific T cells, promote the survival of T cells and the size of the memory T cell pool (Gramaglia et al, 2000, Bansal-Pakala et al, 2001; Rogers et al, 2001). The implication of OX40 in auto-reactive disorders is very well documented in human and animal studies (Weinberg et al, 1996), and antibodies directed against the up-regulated OX40 receptors (CD134) on activated auto-antigen-specific T cells selectively delete them and reduce auto-reactive inflammation (Weinberg et.al, 1998). Signaling via OX40 can reverse an already established state of tolerance even after the onset of antigen-specific hypo-responsiveness suggesting that OX40 represents an appropriate target for therapeutic intervention in a variety of diseases (Bansal-Pakala et al 2001), however the role of OX40R/OX40L interaction in the immune response to malignancies has only recently been recognised. OX40R over-

expression was reported in tumour infiltrating lymphocytes and tumour-draining lymph node cells from melanoma, breast cancer, colon cancer and head and neck cancer patients, and their presence suggested a correlation with overall survival rate (Vetto etal, 1997; Weinberg et al, 2000, Petty et al, 2002). Unlike its involvement in autoimmunity where reduced clinical signs induced by blocking the OX40R-OX40L interaction or depletion of CD4^{+} cells, the anti-tumour adjuvant properties of the OX40 is based on it's engagement with its ligand during the initiation process of active immunisation, which leads to enhanced cytokine production and increased numbers of antigen-specific memory cells (Weinberg, 2002). It was proposed that expansion of tumour reactive OX40R+ T cells localising in cancer tissues could lead to an increase in tumour-specific immunity (Weinberg et.al, 2000). Furthermore, NK cells, for example are known to express OX40L (Kashii et al, 1999), which may on ligation enhanced their apoptotic anti-tumour killing.

In the present study, injecting mOX40L infusion protein into mice bearing moderately or poor immunogenic CT26 and 4T1 tumours respectively induced a dose and route dependant inhibition of tumour growth. Unlike the results reported by Kjaergaad et al (2000) where anti-OX40 administration had no impact on the growth of the poorly immunogenic B16/D5 melanoma, we have shown that both mOX40L and anti OX40R antibody induced significant tumour growth inhibition of 4T1 in spite of its poor immunogenecity. Murine OX40L fusion protein was more potent than the anti-OX40 antibody (OX86) in inducing the proliferation of T-cells from *Staphylococcal enterotoxin* B primed Balb/c mice, when used at equivalent protein concentrations, however they induced comparable tumour growth inhibition when injected i.p into 4T1 tumour bearer mice (data not shown). Why OX86 antibody appears to be more effective *in vivo* is unclear although one possibility is its *in vivo* functional half-life or OX40L/OX40R may have selective affinity towards cells expressing OX40R.

Engagement of OX40 during an ongoing immune response has proved to be a potent adjuvant that enhances antigen-specific immunity (Weinberg, 2002). CT26 tumour rejection in response to therapy with mOX40L was correlated with CTL activity; spleens from all tumour regressor, but not tumour progressor mice when cultured with the AH-1 peptide showed a potent *in vitro* CTL activity against CT26 or AH-1 peptide pulsed A20 cells. It has been shown that OX40 expression and activation of intestinal intra-epithelial lymphocytes was positively correlated with cell-mediated cytotoxicity and IFNγ synthesis (Wang and Klein, 2001). In the present study, mice that rejected their tumours (regressor) following therapy with the mOX40L, remained immune to further challenge with parental tumour cells indicating the presence of immunological memory. Furthermore, the *in vivo* depletion experiments clearly demonstrated the requirement for both CD4^{+} and CD8^{+} cells in tumour rejection in response to therapy with mOX40L fusion protein. Similarly, using OX40R monoclonal Ab, the induced GL261 glioma regression in B6 mice was also found to be dependent on both CD4^{+} and CD8^{+} (Kaergaard et al, 2000). These results are consistent with our previous observation that both T-cell populations are involved in tumour rejection following therapy of established tumours with DISC/mGM-CSF (Ali et al, 2000, 2002).

The time of administration of mOX40L following tumour cell implantation clearly has an impact on tumour growth; results demonstrate that therapy initiated on day 3 after tumour cell injection gave the best anti-tumour effect. Therapy given on days 0, 6 or 10 did not affect tumour growth, and correlates with the known *in-vitro* effects of OX40 signalling on T-cell survival and immune response. Three days may be required to activate T cells following exposure to antigen, to express OX 40R, which is only expressed by naïve CD4^{+} T cells 2-3 days after activation by antigen; OX40R expression then decreases by 4-5 days (Bansal-Pakala et al, 2001). Flow cytometry analysis of blood CD4^{+} and CD3^{+} T-cells performed 17, 24 or 72 hours following CT26 tumour implantation did not show a significant increase in

OX40R expression (data not shown), although it has been reported that unlike naïve T cells, the majority of effector T cells up-regulate their expression of OX40R by 4 hours after antigen stimulation (Gramaglia et al, 1998).

Primary tumours are frequently cured by conventional therapies, and metastatic lesions often do not respond to these treatments or they progress after a period of growth cessation. Therefore, the development of vaccine-based therapies for established metastatic disease or prophylactic vaccination to prevent the development of latent metastasis would be of great value in clinical therapy. In the present study it was shown that i.p injection of mOX40L fusion protein 3 days after i.v inoculation of CT26 tumour cells inhibited experimental lung metastasis, in a dose dependent manner. This provides further direct evidence that mOX40L mediated inhibition of experimental metastasis correlates with reports that engagement of OX40 during the tumour-specific adoptive immunotherapy improves the ability of T cells to eradicate lung and brain metastasis (Kjaergaard et al, 2001). We further tested the efficacy of the mOX40L administration in combination with the intra-tumour injection of DISC/mGM-CSF virus vector, and have previously reported that intra-tumour injection of established s.c tumours with DISC/mGM-CSF induced tumour growth inhibition and complete tumour regression in a proportion of treated mice (Ali et al, 2002). The combination therapy of OX40L fusion protein and DISC/mGM-CSF virus reported in this study was more effective in inhibiting both CT26 and 4T1 s.c tumours than either of the single therapies. This approach may circumvent the limited therapeutic "window" for use of mOX40L as a single therapeutic agent, and that combined with DISC/mGM-CSF an enhanced T-cell response can be generated. Thus, DISC/mGM-CSF (given on day 0) might activate naive T-cells that are then able to respond to mOX40L (given on day 3).

Legends

Fig 1 In vitro T cell proliferation Enhancement by OX40L and OX86 Antibody

Balb/c mice were immunised i.p with *Staphylococcal enterotoxin B* (SEB). Twenty

four hour later lymph nodes were removed and cultured *in vitro* with 0.5 μg/ml SEB

and varying concentrations of murine OX40L, OX86 or human OX40L for 72 hours.

Murine IgG1 was also included as control (Data not given). Cells were pulsed with

3H thymidine for the final 6 hours of culture.

Fig 2. Effect of therapy with OX40L on tumour growth *in vivo*

A&B. 1×10^4 of CT 26 and 4T1 tumour cells were respectively implanted s.c in to two groups

of 10 Balb/c mice. One group was injected i.p on day 3 and 7 with 250μg of OX40L fusion

protein. The other group was injected i.p with PBS and used as a control. Animals were

inspected regularly and their tumour progression was recorded.

Fig 3. Species specificity of OX40 L

1×10^4 4T1 cells were implanted s.c in 4 groups of 10 Balb/c mice. On day 3 and 7, mice were

injected i.p with 250 μg of hOX40L-h IgG or mOX40L-h IgG or mOX40L-m IgG fusion

protein or PBS. Tumour growth was monitored regularly.

Fig 4. Effect of Dose and Route of OX40L Administration on Tumour Growth *In Vivo*

A. Five groups of 10 Balb/c mice were implanted s.c with $1x10^4$ 4T1 cells. Two groups were given 5 doses of 100 and 250 μg per mouse i. p respectively, while the other 2 groups were given 2 doses of 250 and 500 μg respectively. The fifth group was injected i.p with PBS and used as control.

B. Five groups of 10 Balb/c mice were implanted s.c with $1x10^4$ 4T1 cells. Four groups were given 5 doses of 100 or 250μg per mouse either i.p or i.m, respectively. The fifth group was injected i.p with PBS and used as control.

Fig 5. Treatment Initiation time Influences Responses to OX40L Therapy

Five groups of 10 Balb/c mice were implanted s.c with $1x10^4$ CT26 cells. Therapy with 100μg OX40L was started i.p on day 0, 3, 6 or 10 for each group respectively. The fifth group was given PBS i.p as control. S: number of survivals per group.

Fig 6 Inhibition of Experimental Lung Metastasis by OX40L therapy

Four groups of 10 Balb/c mice were implanted i.v with $2x10^4$ CT26 cells. On day 3 therapy with OX40L fusion protein was initiated. Five doses of 50 or 100 or 250 μg/mouse at 3-4 days intervals were given for each group respectively.

Fig 7. Combination Therapy of OX40L and DISC/mGM-CSF virus

A. Four groups of 10 Balb/c mice were implanted s.c with $1x10^4$ 4T1 cells. Therapy started when tumours reached the size of 0.09-0.36 cm^2, which is usually on days 8-10. *DISC*: mice were injected twice at 2 days intervals with $2.5X10^7$ pfu DISC/mGM-CSF virus. *OX40L*: mice were injected i.p with 250 μg/mouse on days 8 and 14. *DISC+OX40L*: mice were first injected intra-tumourally with DISC virus and concomitantly injected i.p with OX40L fusion protein as above. PBS: mice were injected intra-tumorally and i.p with PBS as control.

B. Four groups of 10 Balb/c mice were implanted with 1×10^4 CT26 cells and treated with DISC virus and OX40L as with 4T1 tumours.

Fig 8. CTL Activity of Splenocytes from Mice Rejected CT26 Tumours Following Therapy with OX40L

Splenocytes from mice with complete regressed tumours following therapy with OX40L fusion protein were cultured for 5 days in the presence or absence of the gp70 AH-1 peptide (SPS..), or the non relevant β-gal peptide (TPH..) and tested against CT26 and A20 peptide pulsed targets. *Reg SPS+CT26*: splenocytes cultured with SPS peptide and tested against CT26 targets. *Reg SPS+A20:* splenocytes cultured with SPS peptide and tested against A20 cells. *Reg TPH+CT26*: splenocytes cultured with TPH and tested against CT26 cells. *Reg none+CT26*: splenocytes cultured with no peptide and tested against CT26 cells.

B. Splenocytes were cultured as above but tested against A20 targets pulsed with SPS or TPH peptides.

Fig 9. OX40L Therapy in Mice Depleted of CD4$^+$ and CD8$^+$ T cells.

Treatment of mice with anti-CD4$^+$ or anti-CD8$^+$ monoclonal antibody, given 3 time i.p over a period of 1 week, reduced the circulating CD4$^+$ and CD8$^+$ T cells by 95% and 50%, respectively as determined by antibody staining and flow cytometery (data not shown; Ali et al, 2000). Tumour cells injection and OX40L therapy started on days 4 and 7 after the initiation of Ab treatment, respectively. *CD4&CD8 Dep*: mice depleted of lymphocytes receiving OX40L therapy. *CD4 Dep:* mice depleted of CD4$^+$lymphocytes receiving OX40L therapy. *CD8 Dep:* mice depleted of CD8$^+$lymphocytes receiving OX40L therapy. *Isotype Dep*: mice were given isotype control Ab and receiving OX40L therapy. *Media* : Control mice receiving media and OX40L therapy. *Control*: neither depletion nor therapy.

Carreno BM and Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol; 20:29-53. 2002.

Bretscher P. The two-signal model of lymphocyte activation twenty-one years later. Immunology Today. 13(2):74-6, 1992 Feb.

Stuber E, Neurath M, Calderhead D, Fell HP, Strober W. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity. 2: 507-21. 1995

Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, Imamura S, Uchiyama T. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J Exp Med. 183:2185-95. 1996

Hellstrom KE. Chen L. **Hellstrom** I. Costimulation of T-cell-mediated tumor immunity. [Review] [30 refs] *Cancer Chemotherapy & Pharmacology. 38 Suppl: S40-1, 1996*

Guinan EC. Gribben JG. Boussiotis VA. Freeman GJ. Nadler LM. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. [Review] [244 refs] *Blood. 84: 3261-82, 1994*

Croft M. Joseph SB. Miner KT. Partial activation of naive CD4 T cells and tolerance induction in response to peptide presented by resting B cells. Journal of Immunology. 159: 3257-65, 1997.

Oh S, Hodge JW, Ahlers JD, Burke DS, Schlom J, Berzofsky JA. Selective induction of high avidity CTL by altering the balance of signals from APC. J Immunol 2003, 170: 2523-30.

Murtaza A, Kuchroo VK, Freeman GJ. Changes in the strength of co-stimulation through the B7/CD28 pathway alter functional T cell responses to altered peptide ligands. Int Immunol 1999;11:407-16.

Bansal-Pakala P. Jember AG. Croft M. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nature Medicine. 7):907-12, 2001*

Gruss HJ, Dower SK. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood 1995 Jun 15;85(12):3378-404

Gramaglia I. Weinberg AD. Lemon M. Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *Journal of Immunology. 161: 6510-7, 1998.*

Gramaglia I. Jember A. Pippig SD. Weinberg AD. Killeen N. Croft M. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *Journal of Immunology. 165:3043-50, 2000*

Maxwell JR, Andrew Weinberg, Rodney A. Prell, and Anthony T. Vella Danger and OX40 Receptor Signaling Synergize to Enhance Memory T Cell Survival by Inhibiting Peripheral Deletion. J Immunol 2000 164: 107-112

Akiba H, Miyahira Y, Atsuta M, Takeda K, Nohara C, Futagawa T, Matsuda H, Aoki T, Yagita H, Okumura K. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. J Exp Med. 2000;191:375-80

Weinberg AD, Rivera MM, Prell R, Morris A, Ramstad T, Vetto JT, Urba WJ, Alvord G, Bunce C, Shields J. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. J Immunol 2000;164:2160-9

Kjaergaard J. Tanaka J. Kim JA. Rothchild K. Weinberg A. Shu S. Therapeutic efficacy of OX-40 receptor antibody depends on tumor immunogenicity and anatomic site of tumor growth. Cancer Research. 60: 5514-21, 2000

Vetto JT, Lum S, Morris A, Sicotte M, Davis J, Lemon M, Weinberg A. Presence of the T-cell activation marker OX-40 on tumor infiltrating lymphocytes and draining lymph node cells from patients with melanoma and head and neck cancers. Am J Surg. 1997;174:258-65.

Weinberg AD … 2000, i.immunol 164, 2160.

Todryk, S., C. S. McLean, S. A. Ali, C. Entwisle, M. Boursnell, R. C. Rees, and R. Vile. 1999. Disabled infectious single cycle herpes simplex virus vector for immunotherapy of colorectal cancer. Hum Gene Ther. 1999;10:2757-68.

Ali, S. A., C. S. McLean, E. G. Boursnell, G. Martin, C. L. Holmes, S. Reeder, C. Entwisle, D. M. Blakeley, S. Todryk, R. Vile, and R. C. Rees. The pre-clinical evaluation of whole cell vaccines for prophylaxis and therapy: the use of DISC HSV vector to transduce cytokine genes. *Cancer Research* 2000, 60:1663 .

Ali SA, Lynam J, McLean CS, Entwisle C, Loudon P, Rojas JM, McArdle SE, Li G, Mian S, Rees RC.Tumor regression induced by intratumor therapy with a disabled infectious single cycle (DISC) herpes simplex virus (HSV) vector, DISC/HSV/murine

granulocyte-macrophage colony-stimulating factor, correlates with antigen-specific adaptive immunity. J Immunol. 2002 Apr 1;168(7):3512-9.

Stuber E, Strober W. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. J Exp Med. 1996 Mar 1;183(3):979-89. J Exp Med. 1996; 183: 979-89.

Rogers PR. Song J. Gramaglia I. Killeen N. Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity. 2001, 15: 445-55.*

Weinberg AD. Bourdette DN. Sullivan TJ. Lemon M. Wallin JJ. Maziarz R. Davey M. Palida F. Godfrey W. Engleman E. Fulton RJ. Offner H. Vandenbark AA. Selective depletion of myelin-reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis. *Nature Medicine. 2:183-9, 1996*

Weinberg AD, Antibodies to OX-40 (CD134) can identify and eliminate autoreactive T cells: implications for human autoimmune disease. Mol Med Today. 1998; 4:76-83.

Petty JK, He K, Corless CL, Vetto JT, Weinberg AD. Survival in human colorectal cancer correlates with expression of the T-cell costimulatory molecule OX-40 (CD134). Am J Surg. 2002; 183: 512-8.

Kashii Y. Giorda R. Herberman RB. Whiteside TL. Vujanovic NL. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. [Journal Article] *Journal of Immunology. 163:5358-66, 1999.*

Weinberg AD. OX40: targeted immunotherapy--implications for tempering autoimmunity and enhancing vaccines. Trends Immunol 2002 Feb;23(2):102-9

Wang HC. Klein JR. Multiple levels of activation of murine CD8(+) intraepithelial lymphocytes defined by OX40 (CD134) expression: effects on cell-mediated cytotoxicity, IFN-gamma, and IL-10 regulation. *Journal of Immunology. 167:6717-23, 2001.* **Gramaglia** I. Weinberg AD. Lemon M. Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. Journal of Immunology. 161(12):6510-7, **1998**

Kjaergaard J, Liaomin Peng[*], Peter A. Cohen[*], Judith A. Drazba[†], Andrew D. Weinberg[‡] and Suyu Shu Augmentation Versus Inhibition: Effects of Conjunctional OX-40 Receptor Monoclonal Antibody and IL-2 Treatment on Adoptive Immunotherapy of Advanced Tumor[1] *The Journal of Immunology*, 2001, 167: 6669-6677.

Whittle N, Adair J, Lloyd C, Jenkins L, Devine J, Schlom J, Raubitschek A, Colcher D, Bodmer M. Expression in COS cells of a mouse-human chimaeric B72.3 antibody. Protein Eng. 1987 ;1: 499-505.

Pan PY, Zang Y, Weber K, Meseck ML, Chen SH. OX40 ligation enhances primary and memory cytotoxic T lymphocyte responses in an immunotherapy for hepatic colon metastases Mol Ther 2002; 6:528-36

Morris A, Vetto JT, Ramstad T, Funatake CJ, Choolun E, Entwisle C, Weinberg AD. Induction of anti-mammary cancer immunity by engaging the OX-40 receptor in vivo. Breast Cancer Res Treat 2001; 67:71-80

Kashii Y. Giorda R. Herberman RB. Whiteside TL. Vujanovic NL. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. [Journal Article] *Journal of Immunology. 163(10):5358-66, 1999 Nov 15.*

Annu. Rev. Immunol. 2001. 19:225-252.