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Generation of tumour-rejecting anti-carbohydrate monoclonal antibodies using melanoma modified with Fas ligand

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Abstract

Carbohydrate antigens such as glycolipids and glycoproteins are over-expressed in a variety of cancers and have therefore been identified as ideal candidates for tumour vaccines. Detection of anti-carbohydrate antibodies is associated with a good prognosis in cancer patients. However, generation of an efficient adaptive immune response has been hampered by the low immunogenicity of carbohydrates due to tolerance. Here, we describe a method by which tumour-rejecting antibodies directed against carbohydrates can be elicited in two different melanoma mouse models. Thus, using the murine melanoma B16F10 over-expressing Fas ligand (FasL), we have generated mAbs against cancer carbohydrate antigens expressed by the melanoma. Importantly, passive transfer of mAbs resulted in rejection of melanoma *in vivo*. Their protective effect *in vivo* was dependent on FcR and *in vitro* antibody-dependent cellular phagocytosis. They were also able to delay tumour growth when injected after the tumour was established. FasL-expressing tumours as an adjuvant are a novel way to generate anti-carbohydrate antibodies able to reject tumours *in vivo*.

Introduction

Melanoma is the main cause of death due to skin malignancy. Melanoma incidence has risen faster in the last 50 years than any other cancer, and so have mortality rates 1 in 82 women and 1 in 58 men in USA will develop melanoma [reviewed in (1)]. Although surgery can be curative for many patients, the 5-year survival period for patients with high-risk, thick melanoma is as low as 25% despite recent advances in melanoma therapy. Melanoma is quite resistant to traditional chemo- and radiation therapies and the poor prognosis has therefore made melanoma a target for testing new therapies. A considerable body of knowledge about cancer immunity comes from studies of melanoma (2).

One major form of immunotherapy aims to activate T cells to kill tumours. The characterization of T cell antigens has provided promise for the development of cancer immunotherapy. However, antibody-based approaches were the first form of immunotherapy to reach the clinic. mAbs such as anti-Her-2 (Herceptin) and anti-CD20 (rituximab) represent the fastest growing class of cancer therapeutics. Herceptin is directed against HER2/neu over-expressed on metastatic breast cancer, approved by Food and Drug Administration (FDA), improves response rates in combination with chemotherapy. Rituximab directed against the CD20 B cell differentiation antigen was approved by FDA in 1997 for treatment of low-grade or follicular B cell lymphoma (3). A third type of mAbs approved by FDA is avastin which has anti-angiogenic properties. Therefore, three types of antibodies are successful in cancer therapy, one targets surface molecules and recruits the immune system to kill the tumour, the second induces direct signalling for growth arrest or death of the tumour cell and the third inhibits angiogenesis.

Melanoma is known to over-express gangliosides, in particular GM2, GM3, GD2 and GD3, and therefore these have become the target of many immunotherapeutic attempts. Natural or anti-ganglioside antibodies induced by vaccination are indicative of a good prognosis for cancer patients (4). Several clinical trials were conducted in melanoma patients using gangliosides alone or in combination with

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adjuvants. Since carbohydrates are self-antigens, lymphocytes are tolerant to them. To overcome this lack of immunogenicity, gangliosides in combination with T cell carriers or non-self-derivatives were used and in some cases yielded clinical improvement [reviewed in (5, 6)]. Thus, carbohydrate antigens have proven to be an effective target for immune recognition and attack for humoral immunity in cancer vaccines.

In a previous study, we have shown that injecting Fas ligand (FasL)-expressing melanoma cells into mice induces tumour immunity by breaking tolerance to self-proteins (7). In this paper, we report that vaccination with B16FasL elicits tumour-rejecting antibodies recognizing carbohydrate structures, one of which is the self-antigen GM4.

Materials and methods

Mice and cells

Tumour cells were maintained in RPMI 1640 (Sigma) supplemented with 10% FCS, L-glutamine and penicillin–streptomycin (R10). Stable transfectant clones of B16FasL were generated as described previously (7) and were grown in R10 with 1.5 mg ml^{-1} G418 (Gibco). Tumour cells were lifted with PBS without Mg^{2+}/Ca^{2+} and washed twice before injection. C57BL/6 mice were bred in our animal facility; the $FcR\gamma^{-/-}$ mice on C57BL/6 background were obtained from Taconic (Germantown, NY, USA). All animal experiments were carried out under a valid animal licence and with home office approval.

Cell lines used were B16F10 (C57BL/6 melanoma), MC57 (C57BL/6 fibrosarcoma), Renca (BALB/c renal carcinoma), CT26 (BALB/c colon carcinoma), EL4 (C57BL/6 thymoma), A20 (murine B cell line), P815 (BALB/c mast tumour cell line), 293T (human embryonic kidney fibroblast), Jurkat (human $CD4^+$ lymphoma), K1735 (C3H melanoma) and B16F1 (subline of B16F10). GM95 cells (ganglioside-deficient derivative of B16F10) were a kind gift from Terry Butters (Glycobiology, Oxford, UK).

Generation and purification of mAbs

C57BL/6 mice were immunized with irradiated 1×10^7 B16FasL subcutaneous (s.c.) followed by at least three challenges with 5×10^5 live B16F10 s.c. The last challenge was 3 days before fusion. Splenocytes of tumour-free mice were fused to NS-1 myeloma cells using Polyethyleneglycol (PEG). Hybridomas were screened by staining B16F10 cells, followed by anti-mouse Ig PE (Dako) and analysis by FACS. Positive hybridomas were cloned three times. Isotypes were determined by using the DAKO isotyping kit. IgMs were purified from hybridoma supernatant over a protein L column (Pierce) according to the manufacturer's instructions followed by extensive dialysis. The IgG2a antibody was purified using a protein A (Sigma) column, followed by elution with 0.1 M glycine at pH 3 and extensive dialysis. The concentration of antibodies was determined by optical density and specificity was checked by staining B16F10 at 5–10 μg ml^{-1} and subsequent FACS analysis.

Immunhistochemistry

B16F10 cells were grown in six-well plates on cover slips, fixed with 3% formaldehyde then stained with mAbs (10–20 μg ml^{-1}) and secondary layer [anti-mouse IgG FITC (Sigma) or anti-mouse IgM Alexa (Molecular Probes)] at room temperature. TA99 (anti-TRP1) was obtained from Abcam (Cambridge, UK). Slides were analysed by fluorescent microscopy (Axiovert S100, Zeiss, Germany; software Openlab; digital camera Hamamatsu).

Glycosylation inhibitors

Tunicamycin (200 ng ml^{-1} final, Sigma) was added to 70% confluent B16F10 in R10 and incubated for 18 h. C4DNJ (1 mM final, *N*-butyl-deoxynojirimycin, Toronto Research Chemicals, Canada) was added to 50% confluent B16F10 in R10 and incubated for 72 h. After treatment, cells were harvested using PBS without Mg^{2+}/Ca^{2+}, stained and analysed by FACS.

FACS staining

B16F10 were stained with 10 μg ml^{-1} polyclonal anti-GM4 antibody (Matreya, Pleasant Gap, PA, USA) followed by anti-rabbit-FITC (Sigma). Inhibition experiments were performed incubating B16F10 with 20 μg ml^{-1} M5.2 or M3.4 (negative control) for 30 min followed without washing by staining with anti-GM4 polyclonal antibody (as above). Vice versa, B16F10 were incubated with 20 μg ml^{-1} GM4 or rabbit polyclonal serum (negative control) for 30 min followed without washing by staining with M5.2 and anti-IgM–FITC (Sigma).

Tumour rejection in vivo

Five intra-peritoneal (i.p.) injections of 150 μg of mAbs in PBS were performed into C57BL/6 (five mice per group unless indicated otherwise) on alternate days. On the first day of antibody injection, mice were also challenged with 2.5×10^5 B16F10 s.c. Tumours were measured twice a week. Statistics on survival graphs were performed with Prism using the chi-square test. For therapy experiments, mice were injected with 1.5×10^5 B16F10 and left to develop a palpable tumour (1–2 mm^2), and then antibody injections were performed on alternate days. Isotype controls (IgG2a and IgM) were either grown or purchased as ascites from Sigma and purified as the relevant antibodies. To analyse the statistical significance (days elapsed for tumour size to reach 200 mm^2), we used the two-tailed *t*-test.

K1735 melanoma was stably transfected with FasL as described previously (7); 5×10^6 irradiated K1735 or K1735FasL were injected s.c. into C3H mice; 8 weeks later mice were challenged with 1×10^6 K1735 s.c. Serum, taken at time of sacrifice, was used at 1:50 dilution to stain K1735 cells followed by an anti-mouse Allophycocyanine antibody.

Metastatic lung model of B16F10

Mice were injected with 1×10^5 B16F10 intravenously (i.v.). Mice were monitored daily for distress during the experimental period. After 18 days, animals were sacrificed and lungs were removed and fixed in 10% formaldehyde. The number of metastatic pulmonary colonies was counted. *P*-values were calculated using the Tukey's multiple comparison test (Figs 1 and 5).

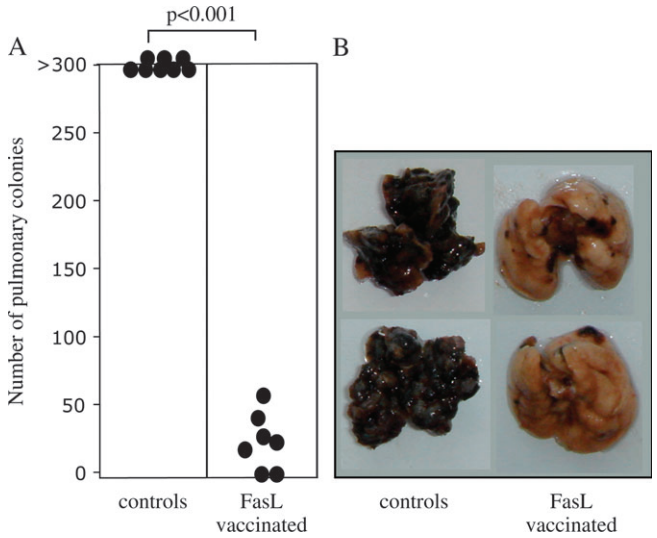

Fig. 1. Mice vaccinated with FasL are protected from lung metastasis. C57BL/6 mice were vaccinated with irradiated 1×10^7 B16FasL or B16F10, and then challenged 6 weeks later with 10^5 B16F10 i.v. (A) Eighteen days later mice were sacrificed and black colonies were counted on the lungs. (B) Representative lungs of mice sacrificed on day 18. Statistics see Fig. 5.

Antibody-dependent cellular phagocytosis assay

Peritoneal macrophages were elicited by injecting 1 ml of 3% thioglycollate i.p. Five days later, macrophages were retrieved from peritoneum with syringe and labelled with PKH26 (orange, Sigma) according to the manufacturer's instructions. B16F10 cells (2×10^6) were labelled with PKH67 (green, Sigma). mAbs (50 μg ml^{-1}) were added to a mixture of labelled effectors and targets cells (ratio of 10:1) for 2 h. Then, cells were analysed by flow cytometry.

Results

FasL protects mice using the lung metastasis and a second melanoma model

In a previous study, we showed that FasL expressed on the melanoma cell line B16F10 induces tumour immunity when both the immunizing B16FasL and the challenge wild-type B16F10 were injected s.c. (7). We therefore attempted to extend this finding to the B16F10 lung metastasis model. C57BL/6 mice were injected s.c. with 1×10^7 irradiated B16F10 stably transfected with FasL or irradiated B16F10 and subsequently challenged with wild-type B16F10. All control mice developed >300 black colonies on their lungs, whereas B16FasL vaccination controls metastatic tumour growth with all mice developing fewer than 60 colonies (Fig. 1).

Generation of mAbs

In our previous study, we demonstrated that the FasL-mediated tumour immunity could be transferred by serum from B16FasL but not from wild-type B16-treated mice (7). The protective serum from B16FasL but not from B16-treated mice stained B16F10 by FACS (7). We therefore set out to make mAbs from the B16FasL-vaccinated mice using FACS analysis of B16 as the screening method. Mice were vaccinated with irradiated B16FasL followed by at least three challenges with live B16F10. As described previously, 60% of mice remained tumour free after the first challenge (7). All tumour-free mice were challenged at least twice more, all remaining tumour free for 12 months or longer. Three days after the last challenge, spleens were dissected and fused to the myeloma line NS-1. Of 190 hybridomas, 4 hybridomas tested positive by FACS and were cloned three times, isotyped and purified for subsequent analysis. We obtained four mAbs which were then characterized. Three IgMs (M2.7, M5.2 and M3.4) gave a strong signal by FACS, whereas the IgG2a (G4.2) staining of B16F10 was somewhat weaker (Fig. 2). The same staining was observed with the stable transfectant B16FasL implying that the antibodies are not directed against human FasL. Immunohistology of adhered formaldehyde-fixed B16F10 revealed a punctate staining pattern for the M2.7 and M5.2 (Fig. 2). The G4.2 also stained B16F10 in a punctate manner but different from the IgMs. In contrast, the M3.4 showed membrane staining and the morphology was reminiscent of the mAb TA99 specific for the melanocyte differentiation antigen TRP1 and used as a control mAb (Fig. 2).

mAbs are directed against carbohydrates on melanoma

In order to further identify the specificity of the novel mAb, we stained 11 transformed and non-transformed syngeneic, allogeneic and xenogeneic cells for flow cytometry. All murine and human cell lines tested, MC57, Renca, CT26, EL4, A20, P815, 293T, Jurkat, C57BL/6 thymocytes, were found negative upon staining with either of the four mAbs. Positive are only the two murine melanoma cell lines K1735 and B16F1 with the IgMs giving the strongest staining and the IgG2a the weakest (data not shown), suggesting that the mAbs might be specific for melanoma. However, four human melanoma cell lines (AFJ, Trombelli, mm25 and mm9) were found negative using either of the four antibodies (data not shown). A fuller survey of antibody reactivity against normal mouse tissues may be required to conclude whether this antibody is truly tumour specific. The increased avidity of the IgM pentamer for repetitive epitopes may compensate for a lack of affinity maturation when compared with the IgG dimer. This result rules out that antibodies recognize FCS or MHC antigens.

Using standard protein-based techniques, we found no evidence that the four mAbs bind to proteins directly. However, addition of the N-linked glycosylation inhibitor tunicamycin followed by FACS staining indicated that the epitopes for all mAbs were carbohydrate structures (Fig. 3A); tunicamycin also reduced Con A–FITC binding used as a control as it preferentially binds to mannose. Furthermore, we incubated B16F10 for 72 h with diverse imino sugars that are non-toxic inhibitors of *N*-glycosylation or glycosphingolipid biosynthesis or both [*N*-nonyl-DNJ, *N*-butyl-DGJ and *N*-butyl-DNJ, respectively (8)]. All showed reduced staining with the mAbs including the control lectin from *Sambucus nigra* (SNA–FITC) that binds preferentially to sialic acid (Fig. 3B). However, both glycosylation inhibitors tunicamycin and imino sugars may have an inhibitory effect on the surface

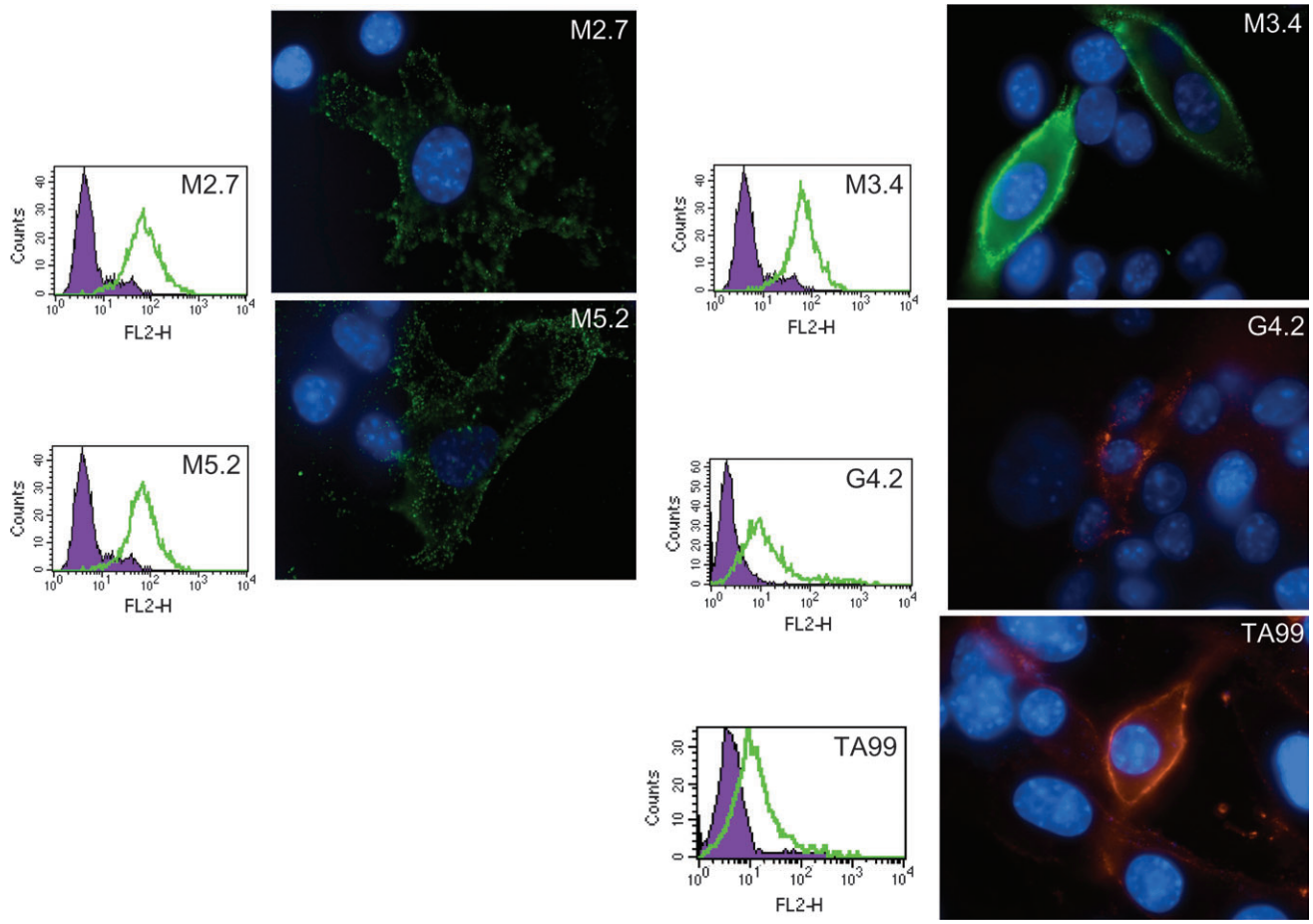

Fig. 2. mAbs were generated from FasL-vaccinated mice. B16F10 was stained with the mAb G4.2, M2.7, M3.4, M5.2 and TA99 (TRP-1) and analysed by FACS (histograms) or immunofluorescent microscopy (objective ×100, photographs). In the FACS plots, filled histograms are isotype controls, open histograms are staining with indicated mAbs.

expression of glycosylated proteins. We therefore stained GM 95 with the mAbs. GM 95 is a B16F10-derived cell line that has reduced levels of ceramide glucosyl transferase (9) and therefore reduced levels of glycosphingolipids (some are detectable by sensitive HPLC; T. Butters, personal communication). Again staining was reduced or sometimes totally absent for all mAbs and the control lectin SNA (Fig. 3C). An overlay of thin layer chromatography including the following common brain gangliosides (GM1, 2, 3, GD1a and b, GT1b, GQ1b, GA1, GD3, sulphatide and globoside) was found negative although the anti-GD1 antibody yielded a strong band (data not shown). This indicates that the mAbs are not directed against common brain gangliosides, a finding that was confirmed by ELISA. We concluded that the carbohydrates recognized by the mAbs are found on not so common glycolipids and to some extenton proteins expressed at the cell surface. Glycoproteins and glycolipids often share glycosylated residues. Cross-reactive mAbs to glycoproteins and glycolipids have been described elsewhere (10).

Indeed, in a collaboration with the National Institutes of Health-funded glycan consortium (http://www.functionalglycomics.org/static/index.shtml), when an array of >200 carbohydrate structures were screened, one of the epitopes for the M5.2 antibody was found to be the ganglioside GM4 (Fig. 4A). The M5.2 binds weakly but specifically to GM4. There is no signal detected with the other gangliosides in the array: GM1, GM2, GM2 (NAc), GM3, GM3 (Gc), GD2, GD3 and GT3. We next determined whether B16F10 expresses GM4. We found that B16F10 expresses GM4 by staining with a commercially available polyclonal anti-GM4 antibody (no staining with polyclonal rabbit serum) whereas the GM95, the ganglioside-lacking derivative of B16F10, is negative when stained with anti-GM4 antibody (Fig. 4B).

Expression of this unusual minor ganglioside of the gala-series glycoplipids has never been described in melanoma before. Moreover, this staining was weakly but specifically inhibited by an addition of excess of M5.2 but not of an irrelevant IgM. Similarly, M5.2 staining of B16F10 was inhibited by polyclonal anti-GM4 but not with rabbit polyclonal serum (Fig. 4C). Furthermore, this finding correlated with the punctate staining pattern found using immunohistology with M5.2 (and M2.7) (Fig. 2) that is typical for ganglioside staining. However, staining was not inhibited completely and the fact that M5.2 stains GM95 despite its total apparent lack of GM4 indicates that this antibody is not specific for GM4

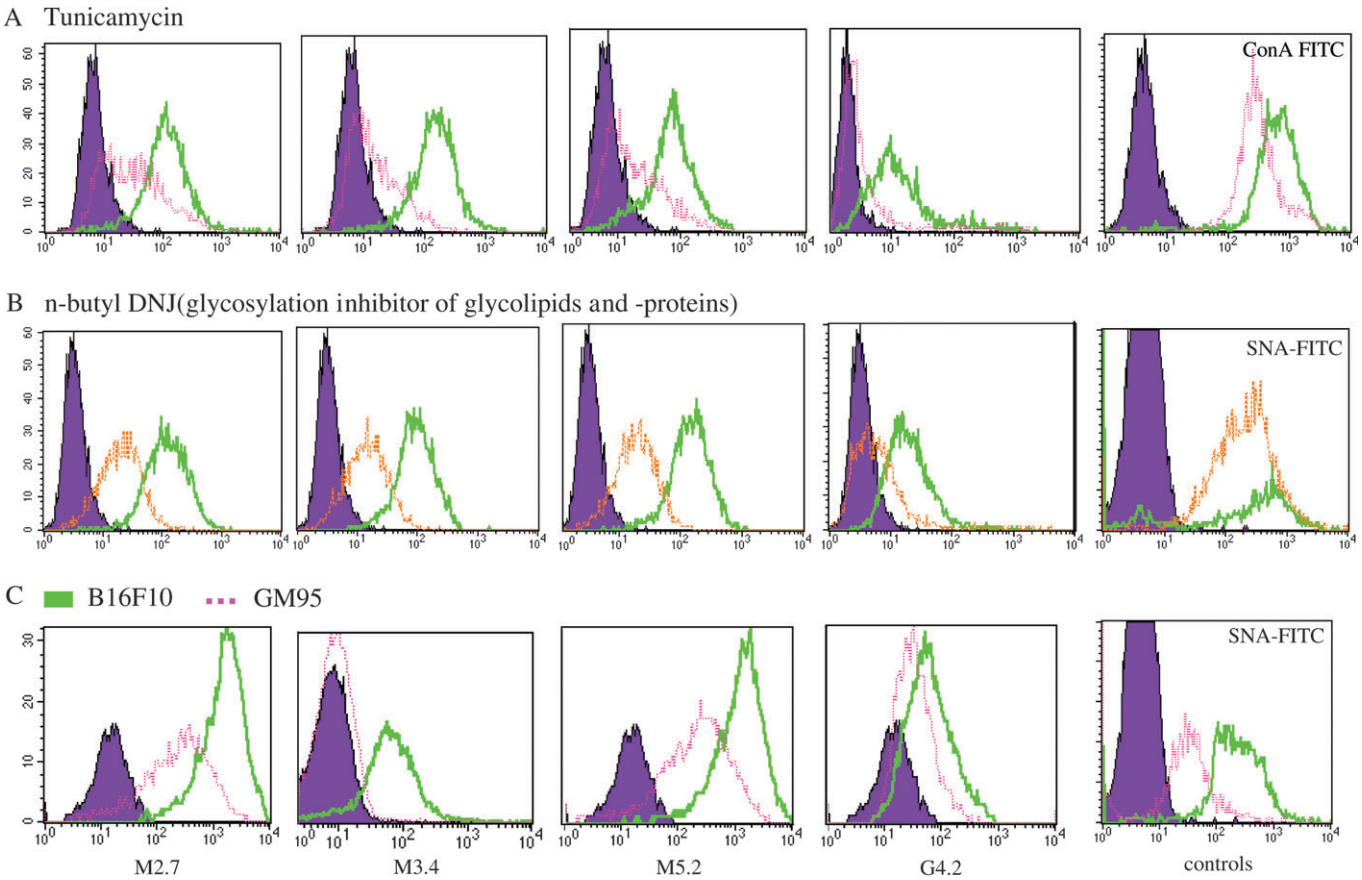

Fig. 3. mAbs recognize carbohydrate structures. (A) B16F10 were grown in the presence of tunicamycin, (N-linked glycosylation inhibitor) for 18 h and then stained with mAbs and analysed by FACS. (B) The glycolipid and glycoprotein glycosylation inhibitor C4DNJ was added to B16F10 for 72 h [For (A and B), filled histograms = no mAb, light grey line = mAbs, cells grown in R10, dark grey line = mAbs, cells grown with inhibitor]. (C) B16F10 (light grey line) and GM95 (cell line with reduced levels of glycosphingolipids, dark grey line) were stained with mAbs (filled histograms, isotype control).

alone. We cannot rule out that the other generated antibodies are similarly cross-reactive.

To extrapolate our findings to other tumours, we tested whether in a second melanoma model K1735 (C3H strain), FasL treatment protects mice from a subsequent challenge mediated by antibodies. Although less efficient than in the B16F10-C57BL/6 system, 50% of C3H mice are protected from a subsequent K1735 challenge after vaccination with irradiated K1735FasL. All mice receiving the irradiated K1735 challenge developed tumours (Fig. 5A). Anti-tumour antibodies were elevated significantly in mice that were treated with K1735FasL (Fig. 5B) particularly in those mice that were protected from tumour growth and in which tumour growth was delayed (open symbols). The staining of K1735 was reduced when K1735 cells were treated with tunicamycin *in vitro*, indicating that polyclonal antibodies generated in this melanoma model are also directed against carbohydrate residues (Fig. 5C).

Mice are protected from tumour growth by anti-tumour antibodies

We then postulated that the newly generated mAbs are able to reject tumours *in vivo*. Using the pulmonary model, we injected mice with either 150 μg isotype control or G4.2 or M5.2 on days 2, 4, 5, 7 and 9. On day 0, C57BL/6 mice were challenged with 1.5×10^5 B16F10 i.v. Whereas the M5.2 had some but not significant effect on the formation of pulmonary metastases (sacrificed on day 17), the G4.2 protected mice significantly from tumour growth (sacrificed on day 20) (Fig. 6A and B). We then followed the same protocol using the s.c. tumour model. Again mice were injected five times with antibody as well as receiving a challenge of 2.5×10^5 B16F10 on day 0. Seventy-five per cent of mice that received the G4.2 remained tumour free for a period of at least 8 weeks (Fig. 7A). Overall in all experiments performed, 38 of 42 mice tumours grew with five injections of irrelevant antibody, whereas 10 of 23 mice grew tumours with five injections of 150 μg ml^{-1} of G4.2 (Fisher's exact $P < 0.0001$). Despite identical kinetics, tumour and antibody doses used in >10 experiments, we observed a variability between experiments that can be explained by the inherent variability of B16F10 *in vivo* growth in general. The dose per body weight (μg g^{-1}) used was equivalent to the dose of Herceptin given to breast cancer patients. No hypopigmentation (vitiligo) or other signs of autoimmunity was observed in treated mice.

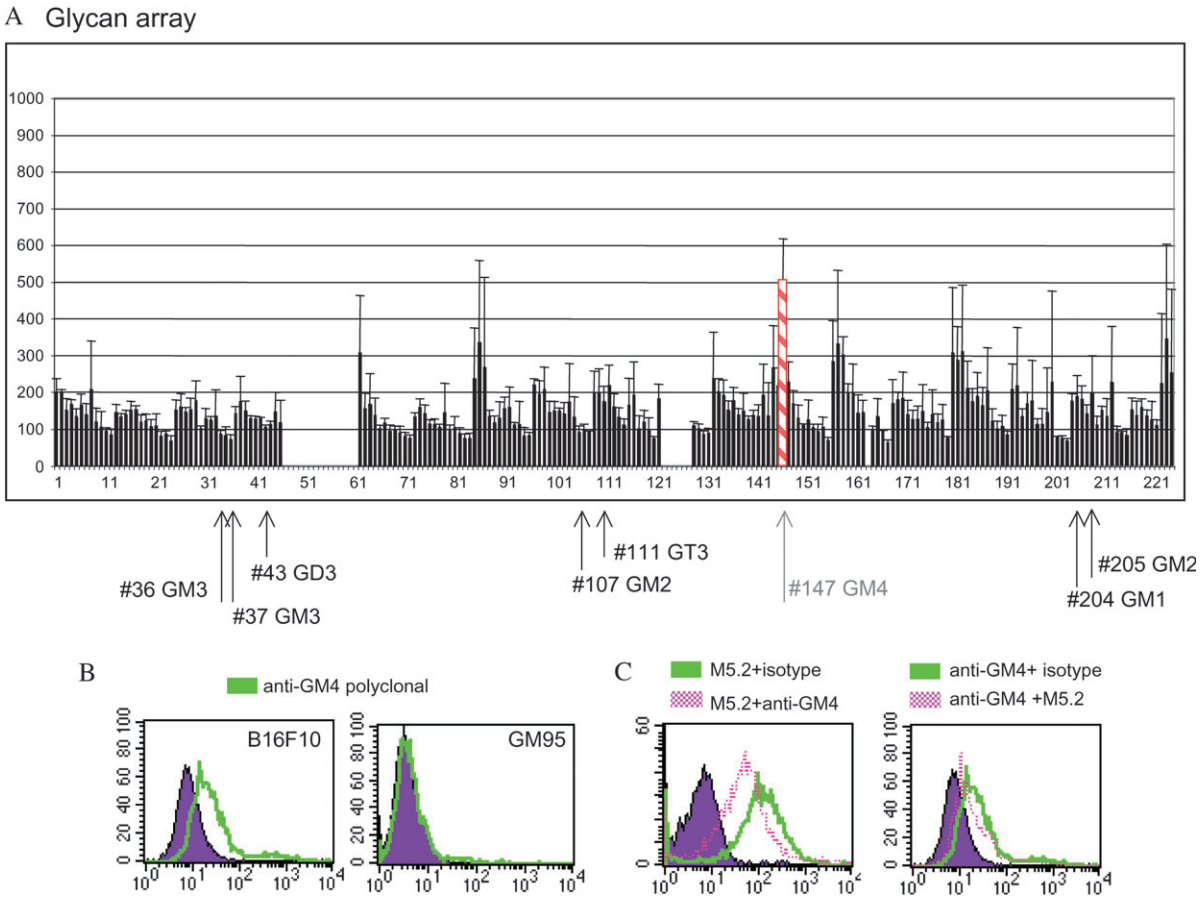

Fig. 4. The mAb M5.2 recognizes the glycolipid GM4. (A) By screening an array of >200 carbohydrate structures, the glycan consortium found that the M5.2 recognizes GM4 specifically (red stripes). Indicated are other selected gangliosides (GM1, GM2, GM3, GT3 and GD3) that were not found to be positive with this antibody. (B) GM4 is expressed on B16F10 as shown by staining with polyclonal anti-GM4 antibody (green). (C) Staining with M5.2 is inhibited with polyclonal anti-GM4 but not normal rabbit serum and (D) staining with polyclonal anti-GM4 is inhibited with M5.2 but not an irrelevant monoclonal IgM.

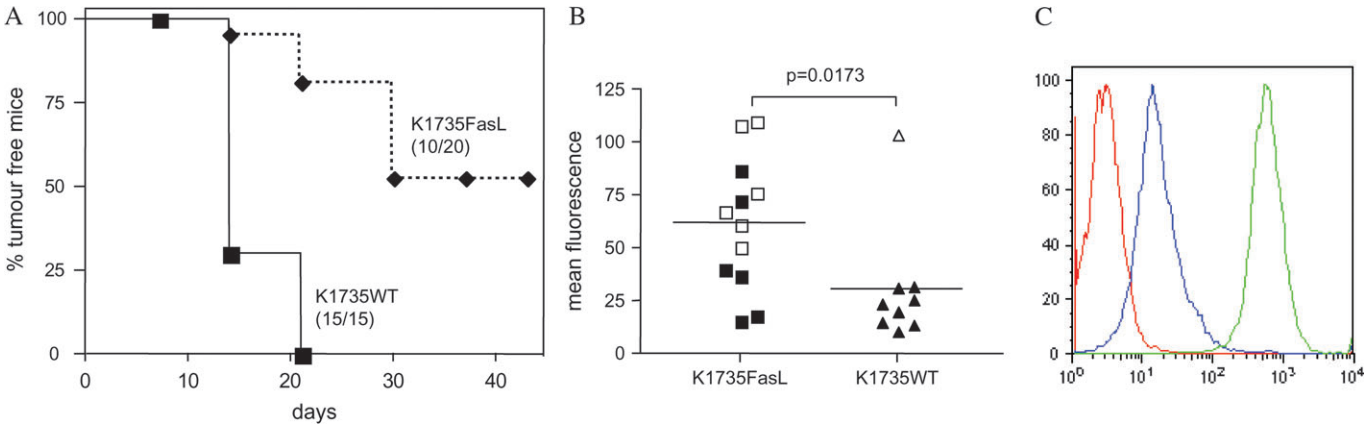

Fig. 5. FasL vaccination elicits anti-tumour antibodies in second tumour model. Eight weeks after vaccination with 5×10^6 irradiated K1735 or K135FasL cells, C3H mice were challenged with 1×10^6 K1735 cells. (A) Tumour growth was measured over a period of 7 weeks once a week. (B) At time of sacrifice, serum was retrieved from some mice and used to stain K1735 cells. Open symbols are mice with slow-growing tumours or tumour-negative mice. (C) K1735 cells were treated with tunicamycin (blue) or not (green and red) and stained with mixed serum obtained from five C3H mice inoculated with K1735FasL (blue and green) or normal mouse serum (red).

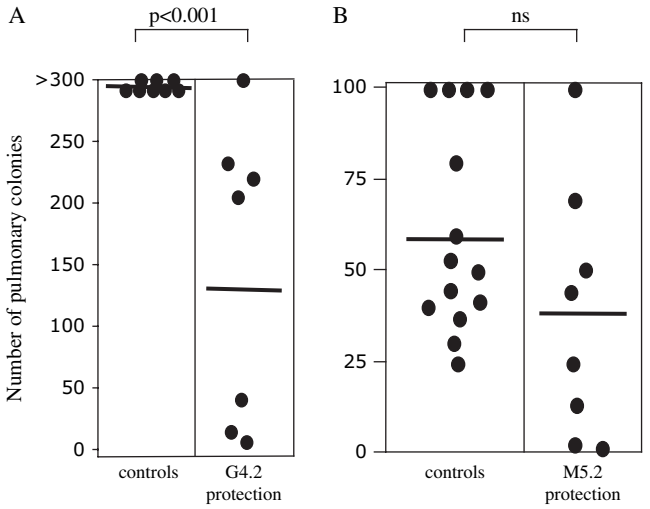

Fig. 6. Both IgG and IgM antibodies protect mice from pulmonary metastases. C57BL/6 mice were challenged with 2×10^5 B16F10 tumour cells i.v. at the same time as the first antibody injection of 150 μg, four more antibody injections were performed on alternate days. (A) Mice were injected with tumour and G4.2 antibody; 20 days later mice were sacrificed and black colonies were counted on lungs. (B) Seventeen days after tumour challenge and five injections of M5.2, black colonies were counted on lungs. For Figs 1 and 5, *P*-values were calculated using Tukey's multiple comparison test. This experiment was repeated three times with similar results.

FcR mediate the tumour rejection in vivo

As the G4.2 had tumour-rejecting abilities, we focused our attention on this mAb. There are two major ways in which antibodies can have an effect on tumour growth. First, binding of the mAb to the tumour cell can directly affect the proliferative capacities or even induce cell death of the tumour. Alternatively, the antibody-coated tumour cells are being recognized and eliminated by the immune system via FcR and/or complement. We investigated both of these possibilities. B16F10 were seeded at different concentrations and grown in the presence of either plate-coated or cross-linked IgGB16 or isotype control for 1 or 3 days. Then live/dead cells were counted by the trypan blue exclusion and also stained with Annexin V as a readout for apoptosis. There was no difference observed between the isotype control or G4.2-treated B16F10 (data not shown). To investigate the anti-proliferative capacities of the mAb, we also labelled B16F10 with CarboxyFluoresceinSuccinimidylEster (CFSE) and grew them in the presence of IgG or isotype control, cross-linked for 1, 2, 4 or 7 days followed by FACS analysis. Again we observed no difference between the isotype control mAb and the G4.2 (data not shown), confirming that this antibody has no anti-proliferative or apoptosis-inducing effect on the tumour cell line *in vitro*.

Alternatively, the anti-tumour effects of the mAb may be mediated by the immune system. B16F10 tumour cells were readily killed in culture by adding any of the four mAbs and complement (data not shown). However, *in vivo* experiments using mice deficient in complement 3 (C3-/-) on the C57BL/6 background (C3-/-) were not possible as surprisingly the control untreated C3-/- mice failed to develop tumours (data not shown). To investigate the contribution of antibody-dependent cytotoxicity, we injected the G4.2 into $Fc\gamma R^{-/-}$ mice. The IgG antibody protected wild-type C57BL/6 mice from a B16F10 challenge, whereas this protection was completely lost in $Fc\gamma R^{-/-}$ mice (Fig. 7B). This strongly suggests that FcR are essential in tumour rejection in this IgG2a-mediated tumour cytotoxicity.

Only the IgG2a antibody mediates ADCP in vitro

Previous studies and our own data show that anti-B16F10 antibody of the IgG2a subtype such as TA99 or GM4.2 do not produce efficient ADCC (11, 12). As this suggests a non-cytolytic mechanism, we performed an *in vitro* assay of ADCP (antibody-dependent cytotoxicity phagocytosis) to test whether the mAbs mediate tumour cytotoxicity. As described in (13), tumour target cells were labelled with a lipophylic dye in green (PKH67), coated with antibody, then mixed with peritoneal macrophages labelled with another membrane dye in orange (PKH26). Phagocytosis was measured by the appearance of double labelled events analysed by flow cytometry. Whereas phagocytosis in the presence of irrelevant IgG2a antibody yielded very little phagocytosis, there was a 2-fold increase when targets were coated with G4.2 but not when coated with any of the IgM antibodies Fig. 8). Engulfment of target cells by macrophages was confirmed by fluorescence microscopy (Fig. 8 inlet). However, all antibodies mediated complement lysis *in vitro* (data not shown).

The mAbs shows some therapeutic anti-tumour effects

Anti-tumour antibodies are of major clinical value if they can eliminate or reduce an existing tumour. We therefore injected the G4.2 mAb into mice bearing tumours. All mice were injected with 2×10^5 B16F10 and had developed a palpable tumour before they received 200 μg of mAb every 2–3 days throughout the duration of the experiment. The mAb therapy delayed the tumour growth significantly in all mice and in 2 of 5 mice by 1 week (Fig. 9). In order to test the hypothesis whether targeting several antigens with different mAbs would improve tumour regression, we injected mice with a low dose of tumour (10^5 B16F10) followed on day 12 with either G4.2 (6 mice in each group) or a cocktail of antibodies containing G4.2 + M5.2 + M3.4 (10 mice) or the equivalent dose of isotype control antibodies on day 12. Most mice had developed a palpable tumour at that time point. Twenty-five per cent of mice in the control group tumour growth regressed; however, 60% of mice regressed when treated with a cocktail of tumour-specific antibodies (data not shown). We observed an improvement over the G4.2 treatment alone in three experiments; however this improvement was not significant (data not shown).

Discussion

As described in a previous study, FasL expressed on the murine melanoma B16F10 protects mice from a s.c. challenge with the wild-type melanoma. FasL behaves as an adjuvant and induces antibody-mediated tumour immunity (7). Here, we demonstrate that FasL-vaccinated mice are also protected from lung metastases when this melanoma is

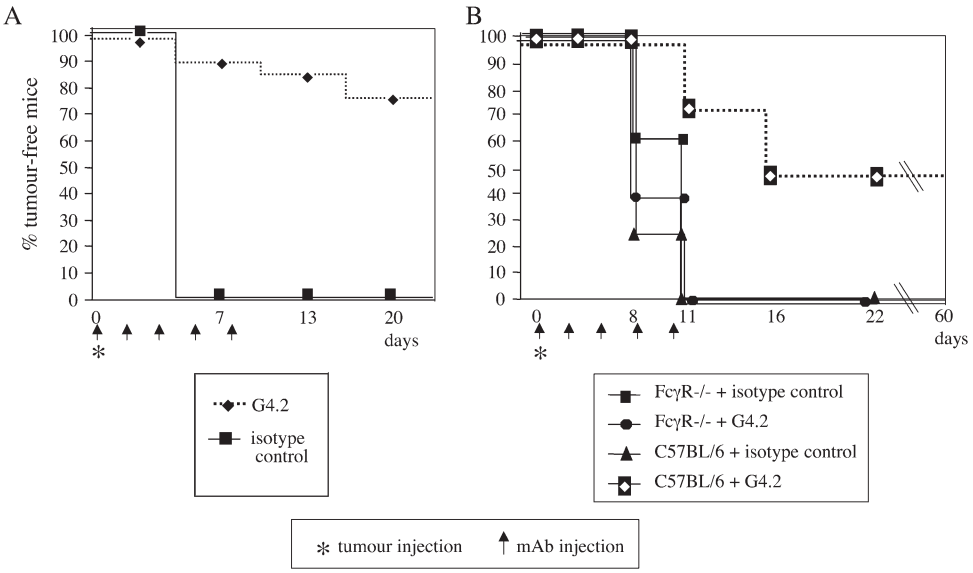

Fig. 7. The G4.2 protects mice from s.c. tumour growth *in vivo* via FcR. Mice were challenged with 2×10^5 B16F10 s.c. On the same day, the first dose (150 μg mouse) of G4.2 mAb was administered i.p. followed by four more mAb injections on alternate days. (A) Mice used in this experiment were C57BL/6. Shown is one representative experiment out of three performed. (B) C57BL/6 or FcR$\gamma^{-/-}$ mice were treated as in (A). Shown is one out of two identical experiments.

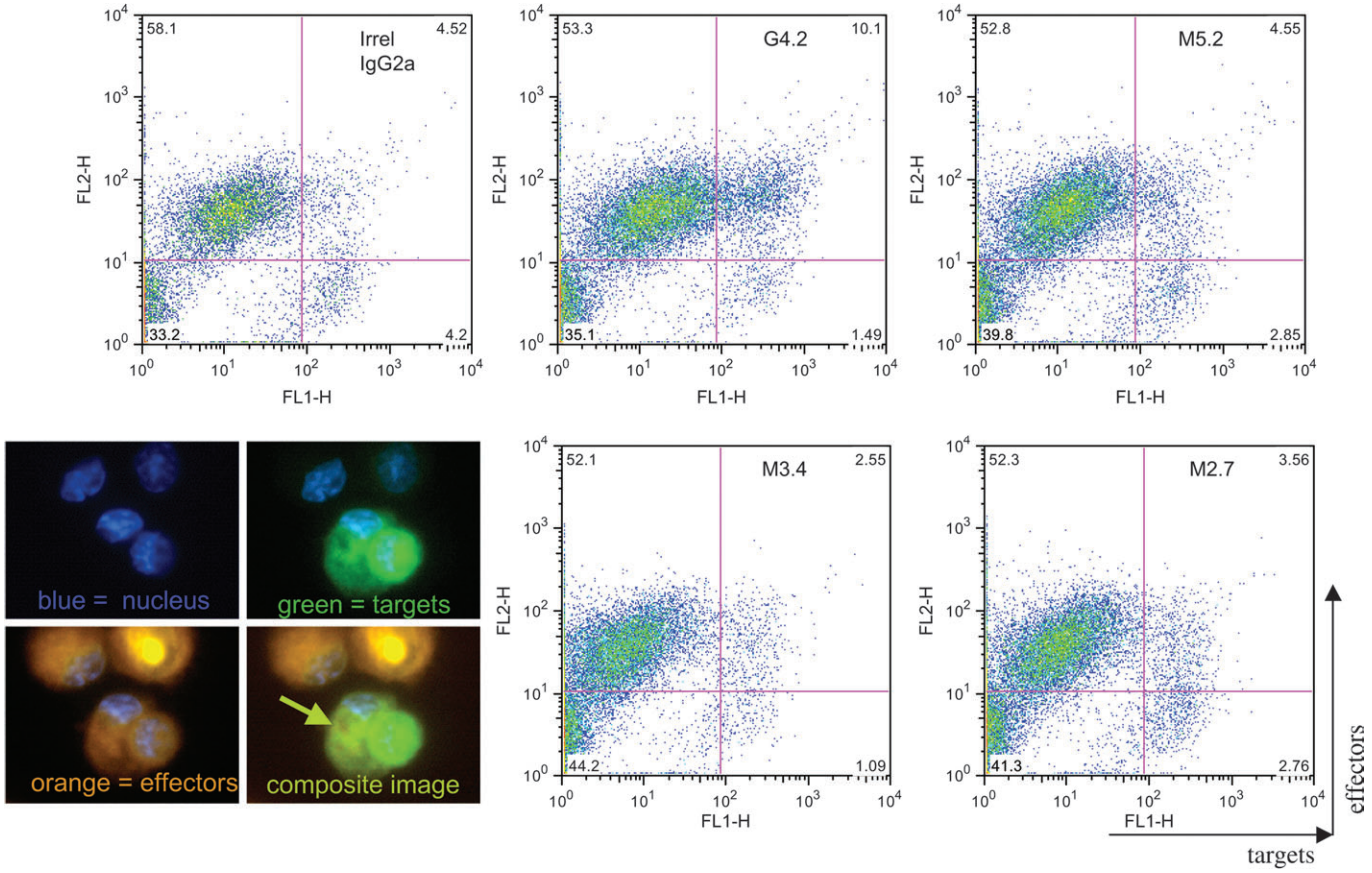

Fig. 8. Only the mAb G4.2 mediates ADCP labelled B16F10 target cells (green) were mixed with labelled thioglycollate elicited macrophages (orange) at 10:1. Only G4.2 but none of the IgMs mediates ADCP in this system as detected by double positive cells in FACS. Inlet shows pictures taken by Ikoniscope® imaging system of an orange macrophage (arrow) that has engulfed a green tumour cell and therefore contains two nuclei. Other macrophages containing only one nucleus have not engulfed any targets.

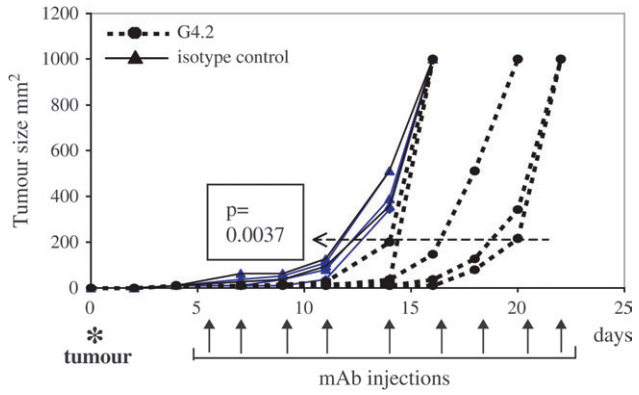

Fig. 9. The G4.2 shows some therapeutic anti-tumour effects and protects from lung metastases. (A) C57BL/6 mice were challenged s.c. with B16F10, when tumours were palpable in all mice, 200 μg of G4.2 was injected every other day. The unpaired *t*-test was used to calculate the delayed tumour growth at tumour size 200 mm^3.

injected i.v., suggesting that once the antibody response is established, the site of tumour growth and rejection is irrelevant. To further understand the mechanism of rejection, four mAbs were generated from FasL-vaccinated tumour immune mice, three of the IgM and one IgG2a isotype. As predicted from the literature, whole cell immunization yields mostly antibodies of the IgM and IgG3 isotypes, recognizing repetitive carbohydrate structures at the cell surface. Whether they are glycoprotein- or glycolipid-specific antibodies, the carbohydrate moiety is usually the epitope. However, we also generated an IgG2a antibody which using other types of adjuvants is only rarely achieved (5).

Interestingly, the antibody with best tumour-rejecting abilities was the IgG2a (G4.2). As shown in other studies, IgG2a is better than IgMs for *in vivo* tumour protection (14). In our study, the *in vivo* rejection was mediated by FcR. The protective effect therefore depended on the ability of IgG2a to mediate ADCP, a function that was not shared by IgMs. Indeed, recently it was shown that the subclasses of IgG have different affinities for the activating and inhibitory FcR which results in significant differences in their *in vivo* activity (15). The IgG2a subclass shows the best value when the activatory to inhibitory ratio was calculated of all IgG subclasses. As complement lysis was shown to be similarly effective for all isotypes in this study, our results indicate that elimination of tumours, either through cytotoxicity or phagocytosis, play a major role. In addition, it has been shown that mAbs can also work by binding to cell surface molecules that are involved in life, death and growth decisions of cancer cells. Indeed, Herceptin that is used in the clinic has been shown to cause cell cycle arrest (16). Similarly, the anti-CD20 mAb B1 that has potent therapeutic activity induces apoptosis in lymphoma cell lines (17). Furthermore, anti-ganglioside antibodies have been shown to signal through microdomains (18, 19). However, we found no evidence of growth arrest or induction of apoptosis in the case of the G4.2 antibody.

Furthermore, the G4.2 shows a statistically significant improvement when given to mice with established tumours; tumour growth is delayed by 5–7 days in three mice out of five. Unfortunately, in none of the mice we observe a total tumour regression. Further improved tumour regression was observed when a cocktail of IgG and IgM antibodies was administered indicating that escape can be minimized by targeting several tumour antigens. However, for immunotherapy the best primary targets are thought to be early micrometastases that may persist after apparent resection of all residual tumours. It is also of note here that the administration of an adjuvant, such as β-glucan, improves the effector mechanisms of, for example, the anti-tumour antibody rituximab.

The identification of new tumour antigens recognized by the cellular or the humoral arm of the immune system is important for cancer immunotherapy. These include carbohydrate antigens in particular gangliosides that are up-regulated upon malignant transformation. Consequently, the carbohydrates are perceived as self and B cells expressing high affinity antibodies for these structures would have been deleted during the development. In an attempt to generate a carbohydrate-based tumour vaccine, copies of synthetic tumour-associated glycans were linked to the immunogenic keyhole limpet haemocyanin (20, 21). Thus, not all glycan-specific B cells are deleted, and given T cell help, anti-glycan antibodies can be generated. Like protein tolerance, therefore, carbohydrate tolerance can be maintained at the T cell level. We found that only the IgG2a antibody rejected melanoma *in vivo* efficiently. The need of T cell help for the isotype switch was most likely provided by T cells recognizing peptide antigens expressed by the tumour. However, it is also possible that the carbohydrate as part of a glycoprotein was presented by MHC class II as it was demonstrated using the tumour antigen mucin MUC1 (22). Similarly, the glycan on a viral glycopeptide presented by MHC class I is available for direct recognition by the T cell receptor as shown in a crystallographic study (23).

From our previous study, we know that FasL can break tolerance to self-proteins of the melanocyte differentiation antigen family (7). We also showed that CD4 T cells were essential in the generation of an effective anti-tumour antibody response (but not in the effector phase), suggesting that the presence of melanoma-specific T cells helped to break B cell tolerance to self-antigens of carbohydrate structure. Breaking tolerance be it at the B cell or T cell level leads to autoimmunity. However, we have never observed any overt signs of autoimmunity such as vitiligo in mice treated with B16FasL or mAbs. This excludes the possibility that melanocytes are being attacked by antibodies as it has been demonstrated for the anti-TRP1 antibody (24). However, we cannot exclude that treatment with anti-ganglioside antibodies could lead to autoimmune signs in the nervous system where gangliosides and in particular galactoshingolipids such as GM4 are primarily expressed. Interestingly, GM4 has not yet been found to be over-expressed in melanoma, possibly due to the commonly employed detection method using ceramide glycanase that does not hydrolyse gal–ceramide linkages which are present in galactosphingolipids.

Other ganglioside-specific antibodies have been tested in murine melanoma models including a vaccine based on GM3 inserted in proteoliposomes derived from *Neisseria meningitidis*, which was capable of inducing GM3-specific antibodies. This promising therapeutic has led to a phase I

clinical trial in patients with melanoma which has shown attractive results (25).

Several spontaneous murine melanoma models have become available in recent years. It would be interesting to know whether such tumours express the antigenic determinants recognized by these antibodies in view of clinically relevant tumour settings in which to test the therapeutic potential of such antibodies (26).

Making hybridomas using FasL as an adjuvant may be a suitable tool in the search for novel mAbs endowed with anti-tumour activity *in vivo*. Immunization of humanized mice with FasL tumours should identify new tolerant antigens on human tumours.

Human tumours frequently display a loss of MHC class I molecules, thus escaping from CD8+ T cell control. The stimulation of an efficient antibody response against MHC class I-negative tumours will be therefore of major interest in the development of a cancer vaccine in humans. To avoid escape one would ideally attack the tumour with several antibodies with different specificity as well as T cells.

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Abbreviations

FasL	Fas ligand
FDA	Food and Drug Administration
i.p.	intra-peritoneal
i.v.	intravenous
s.c	subcutaneous
SNA	Sambucus nigra

References

1 Houghton, A. N. and Polsky, D. 2002. Focus on melanoma. *Cancer Cell* 2:275.
2 Houghton, A. N., Gold, J. S. and Blachere, N. E. 2001. Immunity against cancer: lessons learned from melanoma. *Curr. Opin. Immunol.* 13:134.
3 McLaughlin, P., White, C. A., Grillo-Lopez, A. J. and Maloney, D. G. 1998. Clinical status and optimal use of rituximab for B-cell lymphomas. *Oncology (Williston Park)* 12:1763; discussion 1769–1770, 1775.
4 Livingston, P. O., Wong, G. Y., Adluri, S. *et al.* 1994. Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. *J. Clin. Oncol.* 12:1036.
5 Ragupathi, G. 1996. Carbohydrate antigens as targets for active specific immunotherapy. *Cancer Immunol. Immunother.* 43:152.
6 Bitton, R. J., Guthmann, M. D., Gabri, M. R. *et al.* 2002. Cancer vaccines: an update with special focus on ganglioside antigens. *Oncol. Rep.* 9:267.
7 Simon, A. K., Gallimore, A., Jones, E., Sawitzki, B., Cerundolo, V. and Screaton, G. R. 2002. Fas ligand breaks tolerance to self-antigens and induces tumor immunity mediated by antibodies. *Cancer Cell* 2:315.
8 Butters, T. D., Dwek, R. A. and Platt, F. M. 2005. Imino sugars for treating the lysosomal glycosphingolipidoses. *Glycobiology.* 15:43R.
9 Nores, G. A., Dohi, T., Taniguchi, M. and Hakomori, S. 1987. Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: requirements for tumor-associated antigen and immunogen. *J. Immunol.* 139:3171.
10 Fox, D. A., He, X., Abe, A. *et al.* 2001. The T lymphocyte structure CD60 contains a sialylated carbohydrate epitope that is expressed on both gangliosides and glycoproteins. *Immunol. Invest.* 30:67.
11 Takechi, Y., Hara, I., Naftzger, C., Xu, Y. and Houghton, A. N. 1996. A melanosomal membrane protein is a cell surface target for melanoma therapy. *Clin. Cancer Res.* 2:1837.
12 Clynes, R., Takechi, Y., Moroi, Y., Houghton, A. and Ravetch, J. V. 1998. Fc receptors are required in passive and active immunity to melanoma. *Proc. Natl Acad. Sci. USA* 95:652.
13 McEarchern, J. A., Oflazoglu, E., Francisco, L. *et al.* 2007. Engineered anti-CD70 antibody with multiple effector functions exhibits *in vitro* and *in vivo* antitumor activities. *Blood* 109:1185.
14 Orengo, A. M., Di Carlo, E., Comes, A. *et al.* 2003. Tumor cells engineered with IL-12 and IL-15 genes induce protective antibody responses in nude mice. *J. Immunol.* 171:569.
15 Nimmerjahn, F. and Ravetch, J. V. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310:1510.
16 Le, X. F., Claret, F. X., Lammayot, A. *et al.* 2003. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. *J. Biol. Chem.* 278:23441.
17 Cragg, M. S. and Glennie, M. J. 2004. Antibody specificity controls *in vivo* effector mechanisms of anti-CD20 reagents. *Blood* 103:2738.
18 Kasahara, K., Watanabe, Y., Yamamoto, T. and Sanai, Y. 1997. Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. *J. Biol. Chem.* 272:29947.
19 Kasahara, K. and Sanai, Y. 2000. Functional roles of glycosphingolipids in signal transduction via lipid rafts. *Glycoconj J.* 17:153.
20 Slovin, S. F., Keding, S. J. and Ragupathi, G. 2005. Carbohydrate vaccines as immunotherapy for cancer. *Immunol. Cell Biol.* 83:418.
21 Livingston, P. O. 1995. Approaches to augmenting the immunogenicity of melanoma gangliosides: from whole melanoma cells to ganglioside-KLH conjugate vaccines. *Immunol. Rev.* 145:147.
22 Vlad, A. M., Muller, S., Cudic, M. *et al.* 2002. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. *J. Exp. Med.* 196:1435.
23 Glithero, A., Tormo, J., Haurum, J. S. *et al.* 1999. Crystal structures of two H-2Db/glycopeptide complexes suggest a molecular basis for CTL cross-reactivity. *Immunity.* 10:63.
24 Trcka, J., Moroi, Y., Clynes, R. A. *et al.* 2002. Redundant and alternative roles for activating Fc receptors and complement in an antibody-dependent model of autoimmune vitiligo. *Immunity.* 16:861.
25 Guthmann, M. D., Bitton, R. J., Carnero, A. J. *et al.* 1997. 2004. Active specific immunotherapy of melanoma with a GM3 ganglioside-based vaccine: a report on safety and immunogenicity. *J. Immunother.* 27:442.
26 Chin, L. 2003. The genetics of malignant melanoma: lessons from mouse and man. *Nat Rev. Cancer* 3:559.