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Depletion of CD4+CD25+ regulatory T cells enhances natural killer T cell-mediated anti-tumour immunity in a murine mammary breast cancer model

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Summary

Both invariant natural killer T (NK T) cells and CD4+CD25+ T regulatory cells (T_{regs}) regulate the immune system to maintain homeostasis. In a tumour setting, NK T cells activated by α-galactosylceramide (α-GalCer) execute anti-tumour activity by secreting cytokines. By contrast, T_{regs} intrinsically suppress antigen-specific immune responses and are often found to be elevated in tumour patients. In this study, we have shown that T_{regs} regulate NK T cell function negatively *in vitro*, suggesting a direct interaction between these cell types. In a murine mammary tumour model, we demonstrated that administration of either α-GalCer or anti-CD25 antibody alone markedly suppressed tumour formation and pulmonary metastasis, and resulted in an increase in the survival rate up to 44% (from a baseline of 0%). When treatments were combined, depletion of T_{regs} boosted the anti-tumour effect of α-GalCer, and the survival rate jumped to 85%. Our results imply a potential application of combining T_{reg} cell depletion with α-GalCer to stimulate NK T cells for cancer therapy.

Keywords: α-galactosylceramide, anti-tumour immunity, CD4+CD25+ regulatory T cells, innate, natural killer T cells

Introduction

Immunotherapy aimed at increasing anti-tumour immunity represents a powerful tool for cancer treatment. However, in order to achieve maximum effect, cancer immunotherapy should also focus upon removing negative factors that could inhibit the immune system. The immune system carries out its function by discriminating self from non-self. To keep the system balanced, there are at least two populations of T cells that regulate immune responses, natural killer T (NK T) cells and regulatory T cells (T_{regs}) [1,2].

There are at least two types of NK T cells restricted by the non-polymorphic major histocompatibility complex (MHC) class I-like CD1d molecule on the surface of antigen-presenting cells (APCs). Type I NK T cells express an invariant T cell receptor (TCR) rearrangement: Vα14–Jα18 in mice and Vα24–Jα18 in humans. They respond to stimulation with glycosphingolipid α-galactosylceramide (α-GalCer) presented by CD1d [3,4], and have been shown to be capable of inhibiting tumour growth [5]. The mechanism of type I NK T anti-tumour activity is not understood fully. However, it is believed to be due largely to the innate and adaptive immune responses activated by the T helper type 1 (Th1) cytokines released from α-GalCer-activated NK T cells [6,7]. Type II NK T cells, on the other hand, have not been studied as intensively as type I NK T cells. They do not express Vα14–Jα18 in mice and Vα24–Jα18 in humans consistently, nor do they respond specifically to α-GalCer stimulation, but they do respond to sulphatide [7]. Type II NK T cells have been shown to suppress immune responses and are important in controlling autoimmune diseases, possibly by enhancing the production of interleukin (IL)-13 [7].

T_{regs} represent another type of T cell which maintains immune system homeostasis and tolerance to self-antigens. They are characterized by expression of the CD4 molecule and the IL-2 receptor α (IL-2Rα) chain (CD25), as well as the intracellularly expressed transcription factor forkhead box (Fox)P3 [2]. T_{regs} play a key role in peripheral tolerance, preventing autoimmune diseases and controlling inflammatory diseases. The precise mechanism of T_{reg}-mediated inhibition is not clear, but it is generally believed that they may act in an antigen-specific manner [8] and carry out their function in a number of ways [9]: (i) releasing inhibitory cytokines such as IL-10 and transforming growth factor (TGF)-β; (ii) granzyme- and perforin-dependent killing of other T effector cells; (iii) absorbing local IL-2 by virtue of CD25 expression, although this mechanism remains controversial; and (iv) regulation of dendritic cells (DCs), for

example by inhibiting maturation or down-regulating the expression of co-stimulatory molecules on DCs, to suppress immune responses. T_{regs} are often observed to be elevated in various tumour forms [10–13], suggesting that they may prevent immune-mediated clearance of malignancies. Moreover, depletion of T_{regs} promotes anti-tumour immunity [14,15].

Because both NK T cells and T_{regs} possess regulatory functions in the immune system, it is of great importance to determine the nature of the relationship between these two populations. In particular, a key question is whether T_{regs} play any role in regulation of NK T-mediated anti-tumour immunity. In this study we studied the impact of T_{regs} on NK T cell functions *in vitro* and the effect of depletion of T_{regs} on α-GalCer-activated NK T cells *in vivo* using a murine breast cancer model.

Methods

Animals and tumour challenge

Six- to 8-week-old female BALB/c or C57BL/6 mice were used in this study. All animal protocols were approved by a local Ethical Committee (University of Oxford in the UK or Sun-Yat San University in China) and carried out following the guidelines of the British Home Office or the Chinese Sun-Yat San University.

BALB/c mice ($n = 55$) were divided into four groups according to anti-tumour treatments: group 1 mice ($n = 19$) were given control antibody [rat immunoglobulin (Ig)G, 1 mg/mouse] only; group 2 mice ($n = 14$) were given α-GalCer [Bingham, Nottingham, UK, 100 μg/kg, intraperitoneally (i.p.)] only; group 3 mice ($n = 9$) were given anti-CD25 antibodies (1 mg/mouse, i.p.) only; and group 4 mice ($n = 13$) were given both α-GalCer (AXXORA, 100 μg/kg, i.p.) and anti-CD25 antibodies (1 mg/mouse, i.p.). All groups were challenged with mammary tumour cell line 4T1. Briefly, BALB/c mice were challenged on day 0 by injecting 5×10^4 4T1 cells subcutaneously (s.c.) into the mammary gland area (Fig. 2a). Mice were monitored every 3 days for tumour growth. Mean tumour size was calculated from calliper measurements.

In vivo depletion of $CD25^+$ cells

The anti-CD25 monoclonal antibody PC61 was purified from the supernatant of hybridomas using HyTrap protein G columns (GE Healthcare, Buckinghamshire, UK). Depletion of $CD4^+CD25^+$ T cell subsets in naive BALB/c mice was achieved by i.p. injection of PC61 on day –3 (3 days before tumour cell challenge), at a dose of 1 mg PC61 per mouse. In a preliminary experiment, the total number of $CD4^+CD25^+$ lymphocytes in spleens remained decreased 7 days after the injection, as determined by flow cytometry.

Lung metastasis and histology

After mice were euthanized, lungs were collected and metastatic tumours were checked and calculated. Lung tissues were fixed with phosphate-buffered formalin and embedded in paraffin. Serial sections (5 μm) were stained consecutively in haematoxylin and eosin. Sections were analysed by microscopy.

Purification of liver lymphocytes and NK T cell fraction determination

Livers from C57BL/6 mice were collected and meshed through Falcon cell strainers (BD Labware, Oxford, UK). Cells were suspended in 80% Percoll/phosphate-buffered saline (PBS) and overlaid with an equal volume of 40% Percoll/PBS. After centrifugation at 863 ***g*** for 30 min, cells of the interface layer were harvested, followed by lysis of red blood cells. The NK T cell fraction was determined by flow cytometry after staining with anti-CD3-APC and α-GalCer/CD1d -PE dimers (BD, Oxford, UK).

Isolation of splenocytes and purification of splenic $CD4^+CD25^+$ T cells

Spleens of C57BL/6 mice were collected and meshed through Falcon cell strainers. Cells were resuspended in 10% fetal calf serum RPMI-1640 and overlaid onto an equal volume of Histoque (1·083) (Sigma-Aldrich, Dorset, UK). After centrifugation, the interface lymphocytes were collected. $CD4^+CD25^+$ cells were purified with the mouse $CD4^+CD25^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purification was determined by flow cytometry after staining cells with anti-CD3-APC, anti-CD4-FITC and anti-CD25-PE antibodies (eBiosciences, Hatfield, UK).

Purification of bone marrow-derived DCs (BM-DCs)

BM-DCs were generated from C57BL/6 mice. Cells were washed out from the bone marrows of femurs and tibiae, and plated in 10-cm culture dishes in complete RPMI-1640 medium with 50 μM 2-mercaptal ethanol (Sigma-Aldrich) and 20 ng/ml recombinant murine granulocyte–macrophage colony-stimulating factor (GM–CSF) (R&D Systems, Abingdon, UK). Cultures were fed with growth factor every 2–3 days and half the medium was changed. After 6–8 days' culture, non-adherent and loosely adherent cells were harvested, washed and ready for further experiments.

Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays

Assays were performed using ELISPOT kits (Mabtech, Stockholm, Sweden). Briefly, liver lymphocytes were stimulated

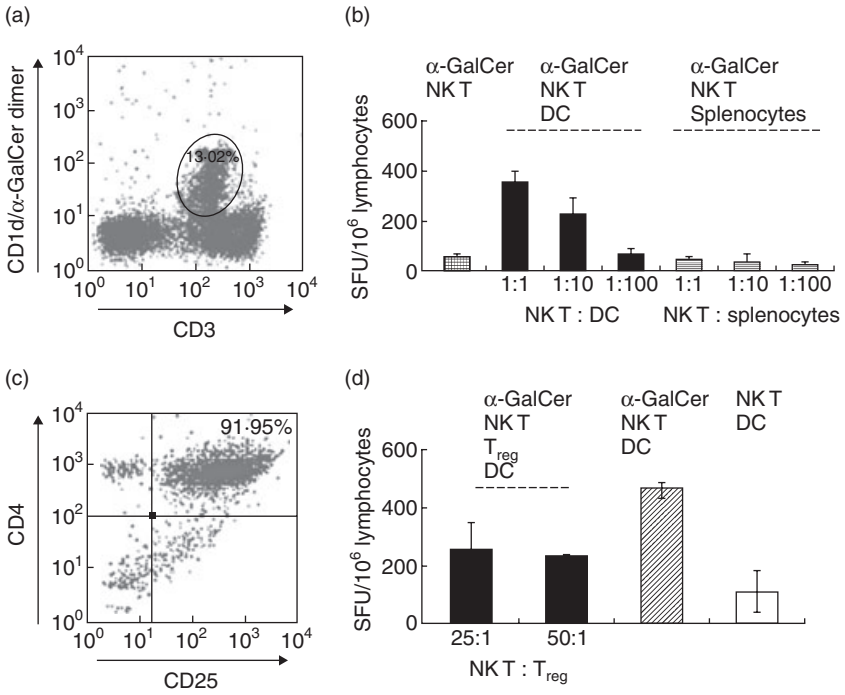

Fig. 1. *In vitro* interaction between natural killer T (NK T) cells and regulatory T cells (T_{regs}). (a) Mouse liver lymphocytes which contain 5–15% CD1d/α-galactosylceramide (α-GalCer) dimmer positive cells were used as NK T cells. (B) With the presence of α-GalCer and various ratios of dendritic cells but not splenocytes, NK T cells released interferon-γ, which can be detected by enzyme-linked immunospot (ELISPOT). (c) $CD4^+CD25^+$ cells with a purity of > 90% were separated from mouse spleens (see Materials and methods) and used as T_{regs}. (d) When culturing T_{regs} with NK T cells at different ratios in the presence of α-GalCer and dendritic cells, interferon-γ release was inhibited. The data represent three experiments with five mice per experiment.

with α-GalCer overnight, with or without the presence of varying ratios of BM-DCs or splenocytes, in anti-IFN-γ antibody precoated plates (Millipore, Bedford, MA, USA). For *in vitro* suppression experiments stimulation was performed as described above, in the presence or absence of varying ratios of splenic $CD4^+CD25^+$ T cells. Cells were discarded, and biotinylated anti-IFN-γ antibodies were added for 1 h at 37°C, followed by another 1 h of incubation at 37°C with anti-biotin antibody labelled with enzyme. After colour developed, the reaction was stopped by washing plates with tap water and plates were air-dried. Spots were counted with an ELISPOT reader (Autoimmun Diagnostike, Strasburg, Germany). Results are expressed as spot-forming units (SFU)/10^6 cells.

Statistical analysis

Significant differences in survival rate were determined by the log-rank (Mantel–Cox) test or Gehan–Breslow–Wilcoxon test using Prism 5 software.

Results

$CD4^+CD25^+$ T_{regs} inhibit α-GalCer-stimulated NK T cell function *in vitro*

First we investigated whether $CD4^+$ $CD25^+$ T_{reg} were able to inhibit α-GalCer–stimulated NK T cells *in vitro*. We took mouse liver cells as the source of NK T cells because, in the mouse, the liver is known to contain the greatest concentration of NK T cells compared to other organs [16]. In our system, we found that 5–15% of lymphocytes in the liver were α-GalCer–CD1d dimmer positive cells (Fig. 1a). Hereafter, we refer to the liver-derived lymphocytes as NK T cells.

We measured IFN-γ secreted by NK T cells because IFN-γ has been reported to play an important role in α-GalCer-induced anti-tumour activity [17].

We tested whether the NK T cells could be stimulated by the CD1d ligand α-GalCer with or without the presence of irradiated DCs or splenocytes. As Fig. 1b shows, only DC-presented α-GalCer was able to stimulate the NK T cells. Furthermore, this was observed only at NK T : DC ratios of 1:1 or 1:10 (Fig. 1b). We chose an NK T : DC ratio of 1:10 for subsequent experiments.

To investigate whether T_{regs} inhibit NK T cell activation by α-GalCer, T_{reg} cells, which were purified from mouse splenocytes (Fig. 1c), were co-cultured with NK T and α-GalCer presented by irradiated DCs (Fig. 1d). At a T_{reg} : NK T ratio of 1:25 or 1:50, T_{regs} inhibited α-GalCer–induced IFN-γ secretion (Fig. 1d). These experiments indicate that T_{reg} cells are able to down-regulate NK T cell function *in vitro*.

Depletion of T_{regs} *in vivo* enhances α-GalCer-induced anti-tumour activity

Given that NK T cells stimulate anti-tumour activity [6,7], T_{regs} are elevated in tumours [10–13] and T_{regs} regulate NK T cells negatively (Fig. 1d), we wondered whether depletion of T_{regs} *in vivo* would enhance α-GalCer-induced anti-tumour activity significantly.

First, a mouse mammary tumour model was established by subcutaneous inoculation of breast cancer cells (4T1) into the mammary glands of BALB/c mice. The transplanted 4T1

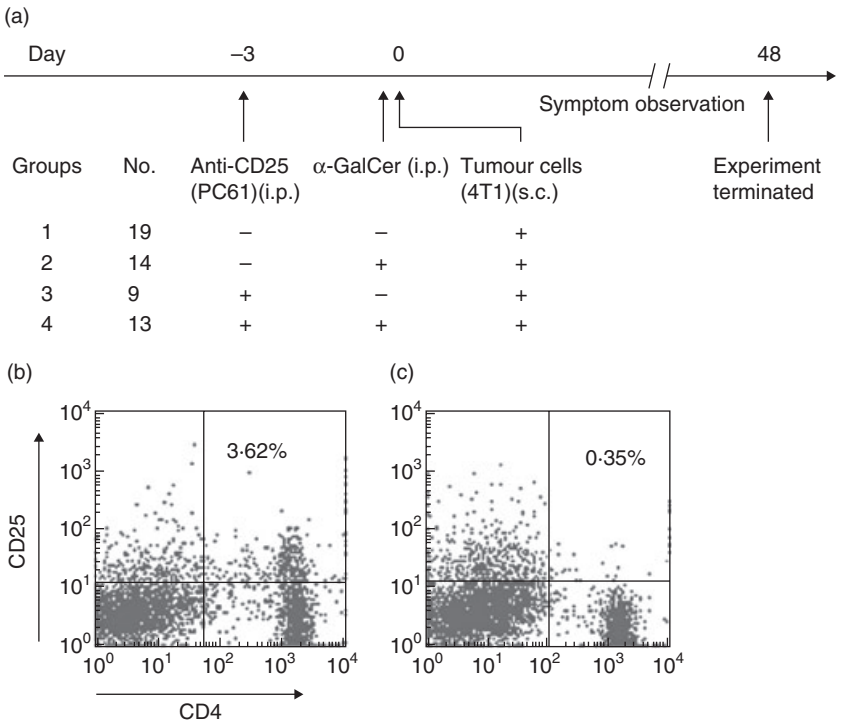

Fig. 2. (a) Groups and schedule of the *in vivo* study. Group 1 mice ($n = 19$) were given control antibody [rat immunoglobulin (IgG), 1 mg/mouse] only; group 2 mice ($n = 14$) were given α-galactosylceramide (α-GalCer) [AXXORA, 100 μg/kg, intraperitoneally (i.p.)] only; group 3 mice ($n = 9$) were given anti-CD25 antibodies (1 mg/mouse, i.p.) only; and group 4 mice ($n = 13$) were given both α-GalCer (AXXORA, 100 μg/kg, i.p.) and anti-CD25 antibodies (1 mg/mouse, i.p.). Symptom observation was terminated at day 48. (b,c) $CD4^+CD25^+$ population in splenocytes. The $CD4^+CD25^+$ population in splenocytes is shown by fluorescence activated cell sorter before (b) and after (c) *in vivo* injection of anti-CD25 monoclonal antibody.

tumour cells grow locally, and metastasize to the lung and liver [18]. Visible nodules are formed in these organs [19].

The schedule of the *in vivo* experiment and groups of BALB/c mice divided according to different treatments are described in Methods and shown in Fig. 2a. The effectiveness of *in vivo* depletion of $CD4^+CD25^+$ cells by anti-CD25 antibody was predetermined in a pilot experiment: a single dose (1 mg) of the anti-CD25 antibody injection *in vivo* resulted in at least 10-fold decreases in $CD4^+CD25^+$ T cells (Fig. 2b,c).

All mice in the control group were dead by 48 days post-challenge with tumour cells. More than 42% of mice receiving α-GalCer survived; more than 44% of mice given anti-CD25 antibody survived. The survival rate of the group which received a combination of α-GalCer and anti-CD25 antibody was increased further to more than 85%. This survival rate is significantly higher than that of the control group ($P = 0{\cdot}0001$) (Fig. 3); it is also significantly higher than the survival rates of the groups given α-GalCer or anti-CD25 antibody alone ($P = 0{\cdot}0013$ and $0{\cdot}0024$, respectively). This result not only confirmed previous observations that α-GalCer increases the survival rate after tumour cell challenge [5], but also demonstrated that the depletion of T_{regs} enhanced α-GalCer-mediated anti-tumour activity significantly.

We analysed tumour volume among these groups during the days after challenge with 4T1 cells. As shown in Fig. 4, mice given α-GalCer or anti-CD25 antibody alone reduced the tumour volume at the site of injection, but the reduction was not significant; in contrast, treating mice with both α-GalCer and depletion of $CD4^+CD25^+$ cells reduced the tumour volume significantly (Fig. 4).

Tumour regression was also manifested by disappearance of tumour nodules at the site of injection. Tumour nodules started to appear on day 5 and they began to disappear on day 20 in the dual treatments group. As shown by Table 1, tumour nodules did not disappear in control, α-GalCer-stimulated or anti-CD25 antibody alone groups, although we observed that the numbers of nodules in the latter two groups were reduced. In the group in which mice received a combination of α-GalCer and anti-CD25 antibodies, tumour nodules disappeared in about 15% of mice (Table 1).

Another parameter of tumour regression is lung metastasis. Without any treatment, all mice had lung

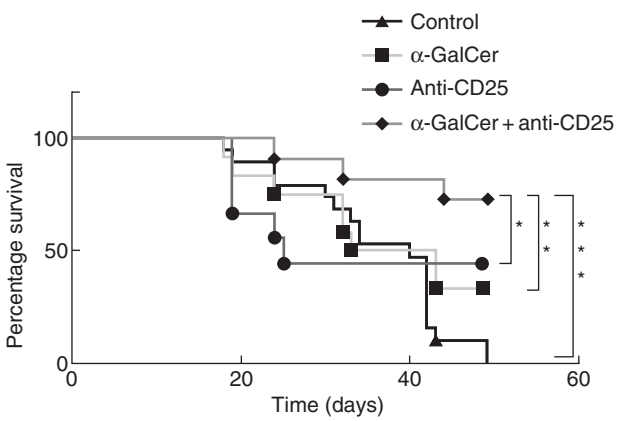

Fig. 3. Challenge with tumour (4T1) cells. The survival rates of four groups of mice with different treatments were recorded. Statistical significance was calculated as described in Materials and methods. $^*P = 0{\cdot}0024$; $^{**}P = 0{\cdot}0013$; $^{***}P = 0{\cdot}0001$.

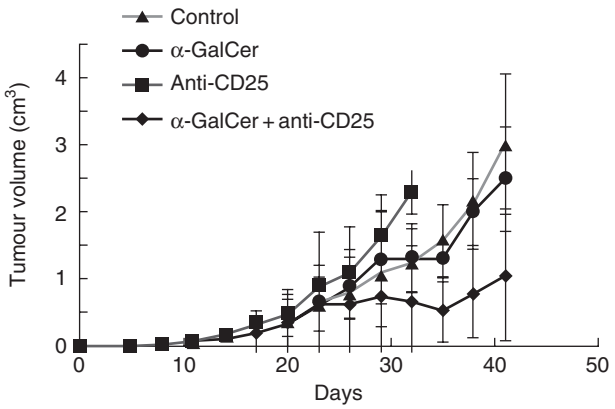

Fig. 4. Tumour volumes (size). The sizes of tumour nodules in the four groups of mice were measured in the days after the challenge with tumour cells.

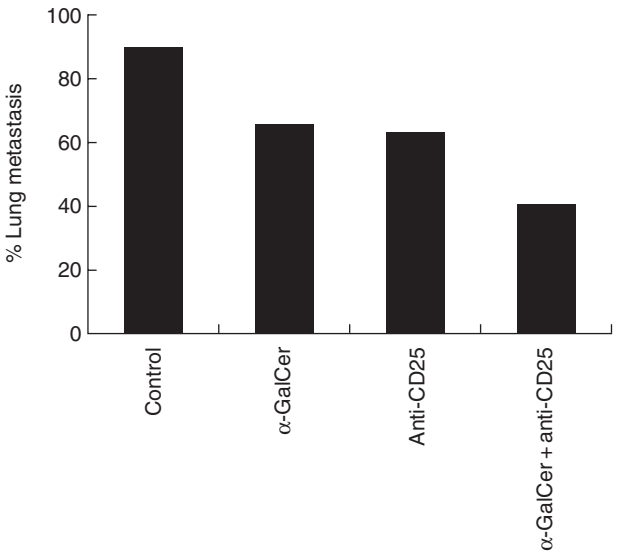

Fig. 5. Lung metastasis. At the end of the *in vivo* experiment, mouse lungs were examined for metastasized tumour nodules.

metastasis. With either α-GalCer alone or anti-CD25 antibody treatment alone, about 60% of mice had tumours which had metastasized to the lungs. This number was reduced to around 38% if mice were given a combination of α-GalCer and anti-CD25 antibody (Fig. 5).

We further examined whether different treatments would affect lung pathological changes. There were many tumour nodules in the lungs of control mice (group 1). Under light microscopy, histological examination of the lung tissue with haematoxylin and eosin (H&E) staining showed large tumour nodules and infiltrating lymph cells around pulmonary vesicles, bronchia and parenchyma (Fig. 6). When mice were given α-GalCer, the number of tumour nodules and the degree of lymph cell infiltration around the bronchia were both reduced; however, there were still many tumour nodules on the lung surface and lymph cell infiltration in the parenchyma. The tumour nodules and infiltration in the bronchia were reduced further when mice received a combination of α-GalCer and anti-CD25 antibody. Furthermore, in mice receiving dual treatments, the tumour nodules were seen only on the edge of the lung, and there was only minor infiltration of lymph cells in the parenchyma of the lungs (Fig. 6). These data suggest that the combination of α-GalCer and CD4⁺CD25⁺ depletion enhanced significantly the effect of α-GalCer-mediated resistance to tumour metastasis.

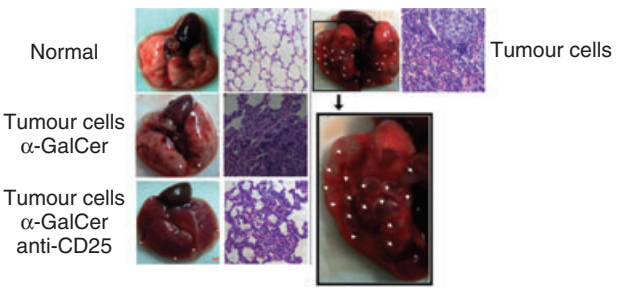

Fig. 6. Pathogenesis of lungs. Representative pictures of lungs and microscopy of haematoxylin and eosin staining of lungs in the three groups of mice are shown. Tumour nodules are indicated with white arrows as shown by the enlarged inset picture on the right panel.

Discussion

For many years, successful cancer immunotherapies have been the Holy Grail for cancer researchers and yet little success has been achieved, with few passing phase III clinical trials. Among the many reasons for failure (e.g. lack of specific tumour antigens, etc.), inhibitory factors in the tumour microenvironment such as myeloid-derived suppressor cells

Table 1. Tumour regression.

Groups	Treatment	No.	% Mice with tumour regression (disappearance of tumour nodules between days 20 and 47)
1	Control (rat IgG)	19	0
2	a-GalCer	14	0
3	Anti-CD25 antibody	9	0
4	a-GalCer anti-CD25 antibody	13	15·38%

Ig: immunoglobulin; α-GalCer: α-galactosylceramide.

(MDSC), cytotoxic T lymphocyte antigen-4 (CTLA-4) and $CD4^+CD25^+$ T_{regs} may also account for the poor results of current cancer immunotherapies [20].

Here we have shown that $CD4^+CD25^+$ T cells inhibit α-GalCer-stimulated NK T cell activity *in vitro*; in the animal model, injection of either α-GalCer alone or anti-CD25 antibody alone inhibits tumour formation significantly; depletion of $CD4^+CD25^+$ cells together with α-GalCer injection renders even higher tumour regression.

α-GalCer bound to CD1d molecules on APCs activates NK T cells which co-ordinate the innate and adaptive immune responses through release of cytokines [6]. Therefore, α-GalCer is regarded as a potential agent for anti-tumour and vaccine adjuvant purposes. On the other hand, $CD4^+CD25^+$ T_{regs} play an important role in maintaining immune homeostasis and protecting the host from autoimmune diseases. Activation of T_{regs}, however, suppresses immune responses and may facilitate tumour growth. The mechanism of immune suppression by T_{regs} may involve inhibition of IFN-γ and IL-2 secretion, as well as secretion of immunological suppressing substances such as TGF-β and IL-10.

The issue we sought to address in this study is whether the anti-tumour effect of α-GalCer could be dampened by suppressive $CD4^+CD25^+$ T_{regs}. Some evidence shows that depletion of $CD4^+CD25^+$ T_{regs} promotes immune responses against tumours such as pancreatic cancer [15,21]; however, there is little direct evidence of cross-regulation between these two regulatory populations in the immune system in a tumour setting; indeed, little is known of the action of T_{regs} on NK T cell function *in vivo*.

Our *in vitro* study has shown clearly that T_{regs} are able to suppress directly NK T cell functions such as the release of IFN-γ (Fig. 1). This provided a rationale to study whether *in vivo* depletion of T_{regs} would enhance NK T cell activity against tumour formation.

In the mammary tumour model, injection of α-GalCer inhibited tumour formation markedly, as was shown by an increase in the survival rate of mice challenged with mammary tumour cells (Fig. 3). It also reduced, to a certain extent, lung metastasis (Figs 5 and 6). Depletion of $CD4^+CD25^+$ T_{regs}, as expected, contributed greatly to the anti-tumour activity of α-GalCer (Figs 3–6). This may be due to the removal of a negative regulation factor for NK T cells, as we have shown *in vitro* that T_{regs} inhibit NK T cell activity. The double act of α-GalCer and depletion of $CD4^+CD25^+$ T_{regs} seems to have a synergistic effect on tumour regression. Our results have demonstrated that this dual treatment approach has potential applications in cancer immunotherapy.

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Disclosure

No conflict of interest to declare.

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