International Immunopharmacology 9 (2009) 1298–1303

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Oral administration of a soluble 1–3, 1–6 β-glucan during prophylactic survivin peptide vaccination diminishes growth of a B cell lymphoma in mice

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ARTICLE INFO

Article history:
Received 10 March 2009
Received in revised form 24 July 2009
Accepted 27 July 2009

Keywords:
A20 lymphoma
β-glucan
Survivin
Tumor peptide vaccination

ABSTRACT

β-glucans are biological response modifiers with activatory effects on macrophages, dendritic cells (DC), granulocytes and NK cells. In this study, we investigated the effect of a soluble yeast-derived β-(1–3), (1–6)-D-glucan on prophylactic peptide vaccination against the B cell lymphoma A20 in syngeneic Balb/c mice. We found that repeated immunizations with two MHC class-I restricted peptides derived from the tumor antigen survivin combined with oral co-administration of β-glucan could significantly diminish intradermal tumor growth, whereas peptide vaccination alone failed to control tumor growth. β-glucan as single agent induced only a weak but non-significant growth inhibitory effect. To determine whether the tumor inhibitory effect of the combined treatment was associated with the induction of a tumor-specific immune response we quantified splenic DC and macrophages, analyzed the maturation of DC and measured the frequency of peptide-specific $CD8^+$ and $CD4^+$ T cells. Treated mice showed significantly increased numbers of splenic macrophages and mature DC compared to untreated tumor-bearing mice. After restimulation with both peptides *in vitro* elevated levels of interferon (IFN)-γ-secreting $CD8^+$ T cells were found in two of four tested mice following treatment and one of four mice showed a strong increase of interleukin (IL)-4-secreting $CD4^+$ T cells. Our data reveal a beneficial effect of β-(1–3), (1–6)-D-glucan in tumor growth inhibition by tumor-specific peptide vaccination which may rely on a function of the polymeric sugar as immunological adjuvant.

1. Introduction

Prevention of cancer by vaccination using peptide or protein antigens is still hampered by weak induction of antitumor immune responses. To overcome the limited clinical responses observed so far vaccines are increasingly combined with immunological adjuvant. These agents such as Toll-like receptor (TLR) ligands, microbial extracts or cytokines like GM-CSF, IL-12 or IL-15 are able to intensify weak antigen-specific cellular or humoral immune responses evoked by the vaccine or to provoke additional stimulatory effects on the innate immune system not shown by the vaccine alone [1–4]. Alternatively, the immunogenicity of a peptide vaccine can be markedly increased by loading the peptides on professional antigen presenting cells (APC) such as dendritic cells (DC) termed "nature's adjuvant" [5].

β-(1–3), (1–6)-D-glucans, branched glucose polymers derived from the cell wall of a variety of plants and microorganisms such as barley, yeast and fungi, exert pleiotropic activation of the innate immunity in mice and humans. Activation of macrophages, neutrophil granulocytes and natural killer (NK) cells by β-glucans leads to elevated phagocytic and cytotoxic activities and production of reactive oxygen intermediates and proinflammatory cytokines *in vitro* and *in vivo* [6–12]. The immunostimulatory effects of β-glucans to leukocytes are mediated by binding to the C-type lectin receptor Dectin-1, to a lectin domain within the complement receptor type 3 (CR3; CD11b/CD18, MAC-1) or to Toll-like receptors (TLR) [8,13–16]. Recent studies revealed an adjuvant function of soluble or particulate β-glucans administered by different routes during protein vaccination due to their capability to induce the activation and maturation of DC and macrophages [17–20]. β-glucan activated DC and macrophages induced primarily the differentiation of Th1 and Th17 cells and primed antigen-specific cytotoxic T lymphocyte (CTL) responses as well as the production of vaccine specific antibodies [19–24]. Furthermore, the therapeutic efficacy of C-activating monoclonal antibodies (mAbs) is enhanced by the engagement of iC3b (inactivated complement 3b fragment)-coated tumor cells with β-glucan bound to CR3 on the surface of granulocytes or NK cells mediating CR3-dependent cellular cytotoxicity [25,26].

Besides the usage of an appropriate adjuvant a potent antitumor vaccine requires the application of immunogenic tumor-specific peptides. The survivin protein is a member of the inhibitor-of-apoptosis (IAP) gene family and is expressed in almost all malignant tissues, but is

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doi:10.1016/j.intimp.2009.07.013

not present in normal differentiated adult tissues [27]. In syngeneic tumor models immunization with DC loaded with survivin-derived peptides induced specific CTL and significantly prolonged survival or induced protective immunity against tumor challenge [28,29]. Furthermore, the presence of survivin-specific CTL has been detected in patients with various cancers, and vaccination with a HLA-A2 restricted survivin peptide was successful in a patient suffering from pancreatic cancer leading to complete remission of liver metastasis [30,31].

In the present work, we evaluated the effect of a soluble β-(1–3), (1–6)-D-glucan, derived from *Saccharomyces cerevisiae*, on the growth of the intradermally (i.d.) implanted syngeneic A20 B cell lymphoma when orally co-administered during prophylactic vaccination of mice with tumor-specific peptides. We further analyzed the cellular immune response elicited by the combined treatment in tumor-bearing mice.

2. Materials and methods

2.1. Mice and cell line

Female, 8-week-old Balb/c mice were purchased from Charles River (Sulzendorf, Germany) and were maintained in the pathogen-free animal facility of epo GmbH (Berlin-Buch, Germany) following institutional guidelines and with approval from the responsible authorities. A20, a Balb/c-derived B lymphoma cell line, was kindly provided by Genethor GmbH (Berlin, Germany) and was cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 2 mM glutamine and supplemented with 10% FBS and 50 μg/ml gentamicin.

2.2. Reagents and antibodies

FITC- or PE-labelled anti-mouse CD4 (L3T4), CD8a (53-6.7), CD11c (N418), F4/80 (BM8), CD40 (1C10), CD86 (GL1) antibodies and appropriate isotype control antibodies were purchased from eBioscience (Frankfurt, Germany). Anti-mouse MHC class-II ($I\text{-}A^d$, CL8713) antibody was purchased from Cedarlane (Canada). The peptides $survivin_{66–74}$ (GWEPDDNPI) and $survivin_{85–93}$ (AFLTVKKQM) were obtained from Biosyntan (Berlin-Buch, Germany). Incomplete Freund's adjuvant (IFA) was from Sigma-Aldrich (Taufkirchen, Germany). Aqueous preparation of underivatized soluble β-(1–3), (1–6)-D-glucan (20 mg/ml) derived from the cell wall of *S. cerevisiae* was provided by Biotec Pharmacon (Tromso, Norway). Endotoxin level was <0.05 EU/ml.

2.3. Vaccination and tumor challenge

Equal amounts of the survivin peptides were dissolved in phosphate buffered saline (PBS) to a final concentration of 2 mg/ml. The vaccine consisted of 100 μg peptides in 50 μl PBS emulsified with an equal volume of IFA and was injected subcutaneously (s.c.) into the left flank of Balb/c mice. Viscous β-glucan solution (400 μg) was dissolved in 100 μl PBS and administered intragastrically. To determine an effect of the β-glucan on peptide vaccination four groups of mice (all $n=8$) were inoculated i.d. with 1×10^6 tumor cells one week after initiation of the treatment with either peptides and β-glucan, the single agents or PBS as control. Injection of peptides was repeated twice in weekly intervals and β-glucan was administered on the first three days of each immunization. The tumor size was determined by measuring the width and length twice weekly using an electronic calliper. Mice were sacrificed when the tumor size reached 500 mm^2. To evaluate the cellular immune response induced by prophylactic peptide vaccination and co-administration of β-glucan a lower dose of 1×10^4 intraperitoneally (i.p.) passaged lymphoma cells was inoculated i.d. into the left flank of mice six days after the first immunization ($n=8$). The initial immunization was followed by two booster vaccinations in weekly intervals and β-glucan was co-administered once daily over three weeks. Untreated tumor-bearing mice ($n=8$) served as control. Four representative mice of both groups were sacrificed one week after the treatment had finished to analyze the splenocytes. The remaining animals were sacrificed when the tumor reached 250 mm^2. All animal experiments were performed according to the German Animal protection law.

2.4. Preparation of splenocyte suspensions and staining of cellular surface marker

Single cell suspensions were prepared from the spleens of two healthy mice and from four mice of each of the treated and untreated tumor-bearing groups. In brief, the splenic tissue was pressed gently through a 70-μm-cell strainer (BD Falcon, Heidelberg, Germany) using a syringe plunger to remove connective tissue and debris. Subsequently, splenocytes were washed twice with RPMI 1640 medium and were used for *in vitro* experiments or evaluated for the percentage of (mature) DC and macrophages using anti-CD11c, anti-CD86, anti-CD40, anti-MHC class-II, anti-F4/80 and appropriate isotype control antibodies. Splenocytes were resuspended in PBS buffer supplemented with 2% FCS and 0.1% sodium azide and incubated with CD16/CD32 FcγIII/II receptor block (BD) before staining with the respective antibodies over 1 h at 4 °C. Thereafter, the cells were washed and 1×10^4 cells were measured by flow cytometry. Data were analyzed using the CellQuest software (BD).

2.5. Quantification of IFN-γ and IL-4-secreting T cells

IFN-γ-secreting $CD8^+$ T cells and IL-4-secreting $CD4^+$ T cells were quantified in splenocyte suspensions one week after the last treatment by flow cytometry using cytokine-specific secretion assays (Miltenyi Biotec GmbH, Germany). In brief, splenocytes (1×10^6/well) were seeded in a 24-well plate in 1 ml RPMI-1640 medium containing 10% FBS and 50 μg/ml gentamicin and were stimulated with both peptides (5 μg/ml of each) over four days. Subsequently, the cells were re-stimulated with the same amount of both peptides as before over 4 h. Thereafter, the cells were stained for CD4 or CD8 and for secreted IL-4 or IFN-γ, respectively, following the manufacturer's instructions. 1×10^5 cells were measured followed by data analysis using the CellQuest software and based upon gating on $CD4^+$ or $CD8^+$ T cells, respectively.

2.6. Statistical analysis

Data are presented as mean ± the standard error of the mean (SEM). Statistical significance of the data was calculated by two-sided, paired Student's *t*-test. Significance levels of $P<0.05$ (*), and $P<0.005$ (**) were chosen.

3. Results

3.1. Inhibitory effect of β-glucan co-administered to prophylactic peptide vaccination on A20 tumor challenge

We first examined the effect of orally administered β-glucan alone or in combination with prophylactic peptide vaccination on growth of the lymphoma. As shown in Fig. 1, repeated peptide immunization and co-administration of β-glucan significantly diminished the growth of 1×10^6 i.d. implanted tumor cells resulting in a 35% decrease of the mean tumor size ten days after end of the treatment (day 24) compared to untreated mice. β-glucan as single agent slightly suppressed tumor growth, leading to a reduction of the mean tumor size of 21% at day 24, without being significant for that effect. In contrast, at this time point the mean tumor size of mice only immunized with peptides increased by 25% compared to control mice.

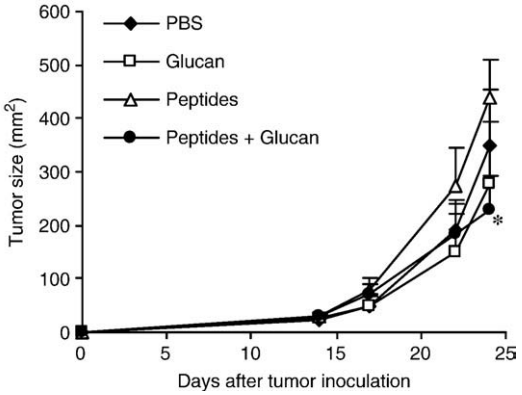

Fig. 1. Inhibitory effect of prophylactic vaccination with survivin peptides and co-treatment with β-glucan on i.d. growth of A20 lymphoma. Mice were immunized by three s.c. injections of the peptides survivin$_{66-74}$ and survivin$_{85-93}$ at weekly intervals or were orally administered with β-glucan (400 μg) during the first three days of each vaccination or received both treatments. Control mice were injected with PBS. One week after the first immunization mice were challenged with 1×10^6 tumor cells. Data are presented as means ± SEM ($n=8$). *, $P<0.05$ versus value for untreated control mice.

In a second experiment we determined whether the combined treatment shows a higher efficacy when the mice are challenged with a smaller number of implanted tumor cells (1×10^4) and β-glucan is continuously administered once per day. Treatment with the single agents was not included due to their lack of a significant growth inhibitory effect against larger tumors. The combined treatment induced significant growth inhibition due to in average 61% and 55% lower tumor sizes in treated versus untreated mice at days 22 and 24 after tumor inoculation, respectively (Fig. 2). However, growth of the tumors increased afterwards.

3.2. Combined treatment increases the number of splenic macrophages and DC and their maturation

To determine the underlying mechanism of tumor inhibition by prophylactic peptide vaccination and co-administration of β-glucan we quantified splenic macrophages and DC, representing key mediators of cellular immune responses, and analyzed the phenotype of DC by flow cytometry one week after the last treatment. Splenocytes from healthy or untreated tumor-bearing mice were 4.9 ± 0.8% CD11c$^+$ or 6.1 ± 1.4% CD11c$^+$, respectively. The combined treatment increased the number of CD11c$^+$ cells in the spleen twofold to 12.0 ± 0.8% and strongly enhanced the maturation of DC (Fig. 3A). Similarly, the number of macrophages

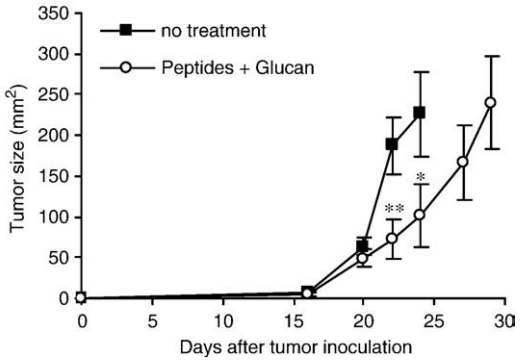

Fig. 2. Inhibitory effect of prophylactic vaccination with survivin peptides and oral administration of β-glucan on i.d. growth of 1×10^4 A20 lymphoma cells. ○, mice immunized by three consecutive s.c. injections of the peptides survivin$_{66-74}$ and survivin$_{85-93}$ at weekly intervals accompanied by continuous daily administration of β-glucan (400 μg). Treatment started six days before tumor inoculation; ■, untreated mice. Data are presented as means ± SEM ($n=8$). *, $P<0.05$; ** $P<0.005$.

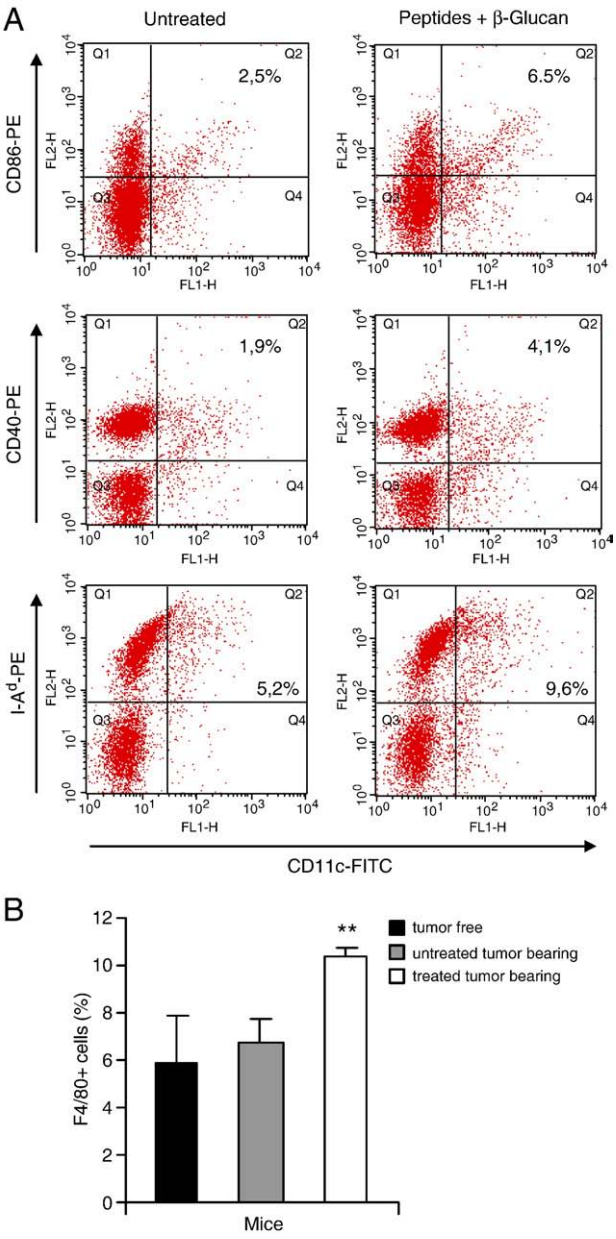

Fig. 3. Impact of repeated peptide immunization and co-administration of β-glucan on DC maturation and macrophage number in the spleen. Cells were analyzed one week after end of treatment by flow cytometry. (A) Representative dot plots of CD11c$^+$ versus CD86$^+$, CD40$^+$ or MHC class-II$^+$ cells from treated and untreated mice. Numbers indicate the mean percentages of fluorescence-positive cells from four mice in the corresponding squares. (B) Percentage of F4/80$^+$ cells in the spleen of healthy mice ($n=2$), and untreated or treated tumor-bearing mice ($n=4$). Data are presented as mean ± SEM. **, $P<0.005$ versus value for untreated tumor control mice.

was significantly elevated in treated mice compared to untreated healthy or tumor-bearing mice (Fig. 3B).

3.3. Combined treatment increases IFN-γ-secreting CD8$^+$ T cells and sporadic expansion of IL-4-secreting CD4$^+$ T cells

In order to test the induction of a tumor peptide-specific T cell response we further examined the activation of CD8$^+$ T and CD4$^+$ T cells one week after end of the combined treatment by flow cytometry. Following restimulation with peptides over four days *in vitro* strongly elevated levels of IFN-γ-secreting CD8$^+$ T cells were found in splenocytes from two of four tested mice of the treated group

and in one of four tested control mice compared to the tested healthy mouse and two mice of the control group representing the baseline level (Fig. 4). The frequency of IL-4 secreting $CD4^+$ T cells was moderately augmented in three of four investigated mice and was strongly induced in one mouse of the treated group compared to healthy and tumor-bearing control mice.

4. Discussion

The efficacy of tumor peptide vaccination depends on the usage of immunogenic peptides, potent adjuvants which increase the immune response to a vaccine but lack any specific antigenic effect by itself, and from the stage of the disease [1–4]. In the present study, we investigated the efficacy of combined prophylactic peptide immunization and oral administration of the soluble β-(1–3), (1–6)-D-glucan to induce a strong tumor-specific cellular immune response inhibiting A20 tumor growth.

The therapeutic effect of a tumor peptide vaccine is thought to rely primarily on the induction of tumor-specific $CD8^+$ CTL by antigen presenting mature DC or macrophages. β-glucans are well-described enhancers of DC maturation and activation of macrophages heightening their release of IL-12 to prime T helper cell differentiation into IFN-γ-secreting Th1 cells [18–22]. Full activation of naïve $CD8^+$ T cells to effector CTL requires IFN-γ secreted by Th1 cells [32]. Furthermore, vaccination with both peptides loaded on syngeneic mature DC was recently shown to induce a strong CTL response and to prevent A20 lymphoma growth in mice [28]. Nevertheless, in our study preventive treatment with both peptides and β-glucan failed to induce a detectable antigen-specific $CD8^+$ T cell response in half of the investigated mice (Fig. 4). Despite enhanced maturation of DC and an increase of the number of splenic macrophages even mice challenged with a low tumor dose (1×10^4 cells) developed tumors (Figs. 2–4).

Since the chosen dose of orally given β-glucan was proven to be effective and well tolerated when administered repetitively or continuously to immunotherapeutic antitumor approaches or as single agent in mice the dosage was not varied [20,33–35]. Accordingly, short-term administration of β-glucan significantly enhanced peptide vaccine efficacy and both schedules of treatment with β-glucan lacked any toxicity. Whether the prolonged co-administration of β-glucan in this study increased the vaccine efficacy compared to the shorter administration of the drug remains open due to the challenge with different tumor cell doses. An extended treatment of invasive micropapillary carcinoma (IMC) of the breast with β-glucan reportedly increased the growth inhibitory effect of the drug [36]. In the short-term schedule of β-glucan administration only a moderate activation of specific T cells occurred despite enhanced maturation of DC. Thus, we assign the greater tumor inhibitory effect in the second experiment rather to the challenge with a smaller tumor dose than to a further enhanced maturation of DC by the prolonged administration of the β-glucan.

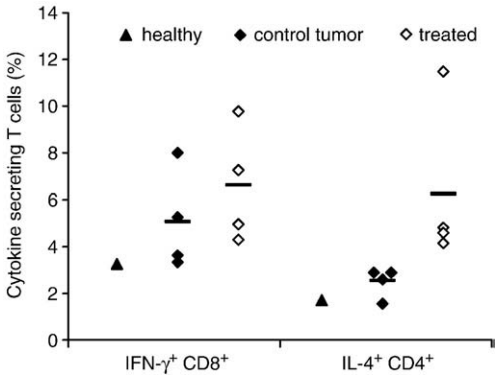

Fig. 4. Effect of the combined treatment on the percentage of IFN-γ and IL-4-secreting T cells. Splenocytes from a healthy mouse (▲), mice with control tumor (♦) and treated mice (◊) were stimulated *in vitro* with both peptides over four days and subsequently re-stimulated over 4 h followed by staining for CD8 and secreted IFN-γ or for CD4 and secreted IL-4. Cells were measured by flow cytometry and percentages of cytokine releasing T cells were calculated by quadrant analysis based upon gating on $CD4^+$ or $CD8^+$ cells. Data represent individual mice, and the horizontal bar indicates the mean for each group.

Therefore, the overall limited efficacy of our approach could rather be due to the late administration of the boost vaccination and by the route of administration of peptides and β-glucan. Firstly, the immunogenicity of peptide-pulsed mature DC may be superior to s.c. injected peptides and co-administered β-glucan, mainly for two reasons: 1) the peptides have to be taken up or bound by MHC class-I molecules on immature DC or macrophages in the subcutis which 2) have to migrate to adjacent lymph nodes to receive a further maturation signal by binding to β-glucan for optimal presentation of these antigens to naïve T cells [37].

Secondly, a low availability or even absence of β-glucan within the subcutis early after administration could have limited its stimulatory effect on skin-resident DC and macrophages. It was shown that orally administered soluble and particulate β-glucans are phagocytosed by gastrointestinal macrophages that transport them within three to four days to the spleen, lymphatic tissues and bone marrow [35]. In these tissues the macrophages partially degrade the polymeric sugar and release small biologically active fragments five to ten days after initiation of repeated administration. Thus, s.c. administration of β-glucan nearby to or mixed with the antigen could increase and accelerate its activating effect on dermal DC and induction of Th1 cells and CTL. This was recently shown by protein or peptide co-immunization with the β-(1–3)-D-glucan curdlan or unmethylated bacterial CpG-containing oligonucleotides, which protected mice against melanoma or breast carcinoma, respectively [3,21]. In addition, the s.c. injection of antigen plus curdlan reportedly induced a long-lasting CTL response, whereas in this study oral co-administration of β-glucan during peptide immunization failed to maintain the growth inhibitory effect over a similar period.

However, a different study reported that the translocation of orally administered soluble β-glucans into the circulation occurred within hours after their uptake by intestinal epithelial cells and gut-associated lymphoid tissue (GALT) and was correlated with elevated plasma levels of IL-12 [38]. This could have supported early systemic differentiation of Th1 cells and subsequent activation of naïve $CD8^+$ T cells, potentially also in mice treated with β-glucan alone. In contrast, the enhanced tumor growth after vaccination with peptides alone may have been caused by the presentation of survivin peptides by predominantly tolerogenic immature DC [39,40]. Immunosuppressive factors released by the tumor, such as TGF-β or IL-1β could evoke the expansion of immunosuppressive myeloid and T cells and impaired maturation of monocytes or iDC to functional APC inhibiting activation and effector function of lymphocytes especially in the tumor microenvironment [41–44]. However, the growth inhibitory effect of the combined treatment indicates incomplete suppression of CTL activity in these mice. Indeed, repeated immunizations were recently shown to be able to overcome the suppressive activity of Treg cells [3].

The presence of elevated numbers of $IL\text{-}4^+CD4^+$ T cells reflects the Th1/Th2 imbalance found in many mouse tumor models and cancer patients [20,45]. However, instead in untreated tumor-bearing mice a Th2 response was unexpectedly found in some of the mice undergoing prophylactic treatment. In these animals the β-glucan may have enhanced the differentiation of M2-type like tumor-associated macrophages being able to prime tumor-specific or unspecific Th2 responses [46,47].

The induction of weakly cytotoxic tumor-specific CTL that failed to reject or diminish tumor growth was reported from untreated A20

implanted mice [48,49]. The expansion of IFNγ-secreting $CD8^+$ T cells in single untreated tumor-bearing mice could result from phagocytosis and presentation of antigenic tumor peptides by bystander DC or M1-type macrophages. Moreover, CTL could be induced by the presentation of immunogenic tumor-antigens such as survivin by the tumor itself due to its strong expression of MHC class-I and -II and low or moderate levels of costimulatory molecules [48–50].

Th2 cells are rather known as less or non-protective against tumor onset and progression, hence, the elevated titer of IL-4 secreting $CD4^+$ T cells in the mouse with the highest titer of tumor-specific $CD8^+$ T cells may have hampered CTL activity [20,51]. Otherwise, IL-4 and IL-12 were shown to act synergistically in the generation of CTL *in vitro* [41]. Moreover, significant antitumor immunity in mice implanted with a cerebral glioma and therapeutically vaccinated with a survivin peptide comprising MHC class-I and II-epitopes correlated with both the generation of strong Th1 and Th2 immune responses [29].

Induction of Th1 cells by the prophylactic treatment was not determined but might be feasible particularly in mice exhibiting a CTL response [17]. Accordingly, preferential priming of naïve T cells to a Th1- and Th17-type by β-glucan maturated DC and macrophages has been demonstrated in healthy or tumor-bearing mice [17,20,22,39]. Admittedly, the extent of CTL expansion and tumor growth inhibition in the present study argues against a strong induction of IFN-γ secreting $CD4^+$ T helper cells which is in accordance to the modest prolongation of survival of mice after therapeutic vaccination with MHC class-I restricted DC-loaded survivin peptides [29]. These mice completely lacked activation of $CD4^+$ T helper cells despite a twofold increase of peptide-specific CD8+ T cells as attained in our study. A recent study showed the induction of a potent CTL response and protective antitumor immunity completely independent from Th1 directing IL12 by a vaccine administered with the adjuvant curdlan [21].

Nevertheless, elevated levels of Th2 cells in single mice could have enhanced the activation of B cells and production of tumor-specific IgG1-type antibodies as demonstrated from oral co-administration of a synthetic β-(1–3), (1–6)-D-glucan to an HBsAg protein vaccine in tumor-free mice [46,52]. Accordingly, β-glucans used as co-immunizing agent to carbohydrate or protein vaccines induced the production of large amounts of antigen-specific IgM- and IgG-type antibodies or IgG1- and IgG2c-type antibodies, respectively, suggesting both Th1 and Th2 priming *in vivo* [17,23]. A production of C-activating IgM- and IgG-type antibodies could provide a beneficial effect due to their ability to induce coating of the surface of tumor cells with iC3b. Simultaneous binding of β-glucan and tumor cell deposited iC3b to distinct sites of CR3 expressed on granulocytes and NK cells is able to trigger cytotoxic activity of these cells directed against the tumor cells [25,26]. An activation of intestinal and peripheral blood innate immune effector cells by direct binding to the β-glucan, potentially enhanced by C-activating antibodies, could explain the slight tumor inhibitory effect of the sugar alone despite the lack of tumor-specific peptides [6–8,10,12,15,25,53].

In summary, we provide evidence that the combination of peptide vaccination and oral co-administration of β-glucan exerts a significant antitumor effect probably due to enhanced activation of macrophages and maturation of DC inducing tumor-specific CTL and sporadic expansion of Th2 cells. A reported ability of β-glucan to strongly activate $CR3^+$ innate immune effector cells in concert with C-activating antibodies may have contributed to the adjuvant effect.

Acknowledgements

We thank Prof. Rolf Seljelid, University of Tromso, Norway, for helpful discussions and Dr. Heiko Johnen for critical reading of the manuscript. We also thank B. Büttner and S. Gromova for excellent technical assistance in the *in vivo* experiments.

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