Soluble β-glucan and heparin as modulators of the immune response elicited by vaccination against a tumor stromal antigen

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Abstract

In 2012, cancer was responsible for 8.2 million deaths worldwide. While incidence keeps rising, mortality however decreases. To keep the positive trend going, intensive research into possible cancer therapies is needed. One treatment option is therapeutic vaccination, where endogenous antibodies against tumor-associated proteins are generated through carefully developed immunization strategies used in combination with potent adjuvant mixtures. This putative cancer therapy has the benefits of being comparably inexpensive and potentially more efficient than existing treatment options, when targeting less transformed cancer-associated (stromal) tissue instead of tumor cells.

Alternatively spliced forms of the extracellular matrix (ECM) proteins fibronectin and tenascin-C have been identified as specific markers of the tumor stroma and vasculature. Here, the C domain of tenascin-C (TNCC) was selected as a target. A polyclonal antibody response against an ECM protein leads to local tissue destruction, including tumor blood vessels, hence blocking the supply of essential factors to the tumor. Soluble β-glucan is an immunostimulatory polysaccharide that can improve the efficacy of antibody-mediated anti-tumor effects by stimulation of neutrophil cytolytic capacities. Heparin, a heavily sulfated form of the ubiquitously expressed glycosaminoglycan heparan sulfate, is anti-inflammatory and may abrogate the immunostimulatory effects elicited by β-glucan. The aims of this study were to investigate the suitability of TNCC as a target for therapeutic cancer vaccination and to explore the possible immunomodulatory effects of β-glucan and heparin in conjunction with the immunization.

C57BL/6J mice were immunized with either TNCC or control substances. Subcutaneous grafting of Lewis lung carcinoma cells was undertaken when antibodies against TNCC had formed. When tumors were palpable, treatment with either β-glucan alone or β-glucan combined with heparin was initiated for a subset of TNCC-immunized mice. No significant differences with respect to tumor growth were found between the four groups. However, immunofluorescence staining with neutrophil marker Gr-1 revealed more infiltration of neutrophils in tumors from TNCC-immunized/β-glucan-treated mice compared with non-treated TNCC-immunized mice. The effect of β-glucan was reversed by treatment with heparin. Furthermore, tumors from TNCC-immunized/β-glucan-treated mice were more necrotic than those from TNCC-immunized mice.

These results show that soluble β-glucan, together with vaccination against TNCC, has an immunostimulatory effect which leads to an increased neutrophil infiltration and tumor necrosis. The effect on neutrophil recruitment can be abolished by addition of heparin. Whether β-glucan can potentiate the tumorstatic effects of a cancer vaccine must however be re-investigated in additional tumor models.
Contents

1 Introduction 5
  1.1 Cancer 5
  1.2 Vaccination against tumor stromal antigens 5
    1.2.1 The vaccination strategy 6
    1.2.2 Why target tumor stroma? 7
    1.2.3 Consequences of self-reactive antibodies 7
    1.2.4 Discrepancies between the human and mouse setting 8
  1.3 Tenascin-C and other ECM splice variants 8
  1.4 β-glucans 9
  1.5 Heparin 10
  1.6 Aims 10

2 Materials and methods 11
  2.1 Production of recombinant proteins 11
  2.2 Animals and immunization procedure 11
  2.3 Detection of anti-TNCC antibodies in mouse sera by ELISA 12
  2.4 Culture of Lewis lung carcinoma cells 12
  2.5 Lewis lung carcinoma tumor study 12
    2.5.1 Soluble β-glucan and heparin treatment 12
    2.5.2 Tumor study termination 13
    2.5.3 Tissue sampling 13
  2.6 Immunofluorescence staining for CD31 and Gr-1 13
  2.7 Necrosis scoring of immunofluorescence samples 14
  2.8 Statistical analysis 14

3 Results 15
  3.1 Lewis lung carcinoma tumors express TNCC 15
  3.2 Immunization against TNCC induced specific antibody formation 15
  3.3 Targeting of TNCC did not affect tumor growth or vessel density 15
  3.4 Neutrophil infiltration was affected by β-glucan and heparin treatments 17
  3.5 Large necrotic areas in tumors from β-glucan-treated mice 18

4 Discussion 20
  4.1 TNCC as a target for therapeutic vaccination 20
  4.2 Effect of β-glucan treatment in combination with the TNCC vaccine 21
  4.3 The effect of heparin addition on β-glucan treatment 23
  4.4 Future prospects 24
  4.5 Concluding remarks 24

5 Acknowledgements 25

6 References 26
**Abbreviations**

APC = antigen-presenting cell
BSA = bovine serum albumin
CR3 = complement receptor 3
CR3-DCC = CR3-dependent cellular cytotoxicity
ECM = extracellular matrix
ELISA = enzyme-linked immunosorbent assay
FCA = fetal calf serum
HRP = horse radish peroxidise
HS = heparan sulphate
iC3b = inactivated C3b
ICAM-1 = intercellular adhesion molecule 1
LFA-1 = leukocyte function-associated antigen 1
LLC = Lewis lung carcinoma
MHCII = major histocompatibility complex class II
NSG = neutral soluble glucan
PBS = phosphate-buffered saline
RT = room temperature
TNCC = extra domain C of tenascin-C
Trx = thioredoxin
uPAR = urokinase plasminogen activator
VEGF-A = vascular endothelial growth factor A
WGP = whole glucan particle
1 Introduction

1.1 Cancer

Cancer, the uncontrolled growth and spread of transformed cells which compromises the functionality of healthy tissues, was the cause of 8.2 million deaths worldwide in 2012 (World Health Organization, 2012). In Sweden, while total cancer incidence is rising, mortality decreases, an effect that can be attributed both to improved diagnostic methods and to the development of more efficient treatment methods (Cancerfonden, 2014). However, cancer is still the primary cause of death in Sweden for persons aged below 80 years (Cancerfonden, 2014; Socialstyrelsen, 2013).

Traditionally, cancer has been treated by surgical removal of the tumor tissue in combination with radio- and chemotherapy (Weinberg, 2014). As knowledge about the molecular underpinnings of tumor biology has grown, more refined methods have been developed. These include treatments with inhibitors of signaling molecules such as tyrosine kinases, monoclonal antibodies targeting various tumor-essential factors, and several different immunotherapies – methods that induce, promote or inhibit an endogenous immune reaction (Abbas et al., 2012; Weinberg, 2014).

1.2 Vaccination against tumor stromal antigens

Monoclonal antibodies directed towards e.g. tumor-derived growth factors or tumor-associated antigens have proven to be efficient treatments of certain types of cancers (Weiner et al., 2010). Bevacizumab (Avastin), targeting vascular endothelial growth factor A (VEGF-A) which promotes tumor angiogenesis, is used in the clinic for treatment of some colorectal and lung cancer, and oncogene product HER2/Neu can be successfully targeted by trastuzumab (Herceptin) in certain breast cancers (Weiner et al., 2010). An alternative to monoclonal antibody therapy is therapeutic vaccination. The aim of this treatment strategy is to persuade the immune system to endogenously produce antibodies against tumor-associated antigens, rendering exogenous addition of these “magic bullets” superfluous. Therapeutic vaccination has the potential of being more cost-effective than treatments based on monoclonal antibodies, both in the production and administration process. It is however difficult to elicit the desired immune response, as tumors have endogenous origins. No self molecule should evoke an immune reaction – indeed, failures in the functions of this self-distinguishing machinery can lead to autoimmune disease (Abbas et al., 2012). Non-self peptides, on the other hand, should be readily recognized by the immune system and dealt with accordingly.

The mechanism that allows for self molecules to fly under the immune system’s radar is referred to as immunologic tolerance. T cells exhibit essentially perfect self tolerance, as autoreactive T cells commonly are thought to either be deleted in the thymus during maturation or to be put in a state of unresponsiveness, anergy, if they have reached the periphery (Abbas et al., 2012). B cell tolerance is less ubiquitous, but as B cells require T cell help to become activated, and autoreactive T cells are nigh non-existent, B cell autoreactivity is not in itself sufficient to develop an autoimmune response (Abbas et al., 2012).
1.2.1 The vaccination strategy

Cancers are, as already noted, endogenous tissues, but the transformation into tumor cells often entails differential expression of various molecules compared with normal organs, which opens up for specific tumor targeting. To overcome self tolerance, with the end goal of endogenous antibody production towards a self antigen, the vaccination strategy used must be carefully optimized. One method, described by Hellman (2008) and successfully used e.g. in Huijbers et al. (2010), is summarized in Figure 1. It is based on the fusion of the target self protein to an immunogenic non-self protein, in this case a bacterial thioredoxin. The fusion protein is taken up by antigen-presenting cells, such as dendritic cells, which cut it into smaller fragments and display these on surface-bound major histocompatibility complex class II (MHCII) molecules. Helper T cells, whose T cell receptor only recognizes proteins bound to MHCII molecules, identify the thioredoxin fragments as foreign and become activated. Simultaneously, autoreactive B cells with the right specificity binds to the self portion with their specific B cell receptor and internalizes the protein in its entirety. In the same way as the dendritic cells, the B cells process the fusion protein and display the fragments to T cells on MHCII. The thioredoxin-specific activated T cells will recognize the foreign thioredoxin part of the fusion protein on the B cell MHCII’s, and help activate the self-reactive B cells. The

![Figure 1: How to break self tolerance using a vaccination strategy based on the fusion of a self- and non-self protein.](image)

After vaccination, the fusion protein is taken up and processed by antigen-presenting cells (APCs), e.g. dendritic cells [1]. Self and non-self parts of the protein are shown to T cells on MHCII’s. The non-self parts are recognized by the T cells, which become activated and clonally expand [2]. Self-reactive B cells need assistance from helper T cells to become activated and produce antibodies. B cells also take up and process the fusion protein [3]. When B cells show the non-self parts on their MHCII, the activated helper T cells recognize these and activate the B cells, which expand and produce antibodies [4]. APC = antigen-presenting cell, MHC-II = major histocompatibility complex class II, TH = helper T cell, TCR = T cell receptor, B = B cell, BCR = B cell receptor. Figure from Huijbers et al. (2010), adapted from Hellman (2008).
physical linkage between the self and non-self protein is thus of utmost importance – if they were not covalently bound, the self-specific B cell would not take up the foreign molecule and therefore not receive help, essential for the B cell activation and self-antibody production, from the foreign-specific T cell. No activation of cytotoxic (CD8\(^+\)) T cells is expected, as such activation would necessitate the presence of self-specific CD8\(^+\) cells, thought to be extremely rare.

In addition to the fusion protein strategy used to overcome self-tolerance, the composition of the vaccination solution and the choice of target self protein must be carefully considered. To evoke an immune response against a self molecule, a potent adjuvant – a substance that improves the immune response towards an antigen (Abbas et al., 2012) – must be included in the immunization formula. Freund’s complete adjuvant (FCA) is an example of a very potent adjuvant that due to its high toxicity is not approved for human use. In fact, the most commonly used human adjuvant, aluminum hydroxide (alum), has a very weak immunostimulatory effect which is not sufficient to help create a robust immune response with antibody production towards a self antigen (Hellman, 2008). However, Montanide ISA 720, a non-toxic and biodegradable plant oil-based adjuvant, can in rats give as good antibody titers towards self antigen as FCA, if combined with unmethylated CpG oligonucleotides (Ringvall et al., 2009). Unmethylated CpG oligos mimic bacterial nucleic acids and stimulate Toll-like receptor 9, which leads to activation of several different immune cell populations (Steinhagen et al., 2011).

When choosing the self protein target of a vaccination scheme, it is wise not to opt for one that is ubiquitously expressed, as B cells autoreactive to such a protein are submitted to harsh suppressor mechanisms (Hellman, 2008). This criterion is met for the splice variants of extracellular matrix (ECM) molecules described below, as well as tumor-specificity (see section 1.3).

1.2.2 Why target tumor stroma?

A tumor consists both of dense, fast-growing tumor cell areas and of surrounding areas, the so-called tumor stroma. The tumor stroma comprises the ECM and all cells, e.g. endothelial cells, pericytes and fibroblasts, which in some way are associated with the tumor (Weinberg, 2014). There are several advantages to targeting the tumor stroma instead of, or in addition to, the tumor cells. Firstly, due to their proneness to mutational events, tumor cells are with time often able to escape treatments directed at them (Matejuk et al., 2011). The less transformed tumor stroma does not possess the same potential to evade attacks, but is nevertheless essential for the survival of the tumor. Secondly, tumor stroma and vasculature is more accessible for blood-borne treatments such as antibodies (Matejuk et al., 2011). Thirdly, gene expression signature can differ greatly between different tumor types and between patients and growth stages of the same tumor type, while ECM and vessel-associated molecules are more consistent. Targeting these can therefore potentially be a more general treatment option.

1.2.3 Consequences of self-reactive antibodies

Self-reactive antibodies produced by therapeutic vaccination towards tumor stromal antigens will selectively bind to their target molecules (described in section 1.3) present in the vicinity
of the tumor. Through Fc receptors, phagocytic neutrophils and macrophages will recognize the immune complexes (antibody-antigen complexes) formed and try to engulf them. Due to the tissue-bound nature of the target molecules, these attempts will result in so-called frustrated phagocytosis, where the phagocytes will release their granule contents of reactive oxygen species, proteases and peroxidases into the surroundings, with tissue destruction as a result. The same mechanism, albeit directly cytotoxic and stronger, is seen in hypersensitivity type II reactions, e.g. hyperacute transplant rejection (Abbas et al., 2012). If the antigen is vessel-associated, vessel deterioration will follow.

1.2.4 Discrepancies between the human and mouse setting

Unlike preventive vaccination, therapeutic vaccination is neither prophylactic, nor life-long (Hellman, 2008). However, when subcutaneous mouse tumor models are used, immunizations have to be done in advance. The reason for this is that it takes substantially longer for a robust antibody response to form, than for a subcutaneous tumor to reach its maximum allowed size. While subcutaneous tumor models thus are not fully reflective of the clinical situation, they are a good means for investigating basic mechanisms and for obtaining proofs of concept.

1.3 Tenascin-C and other ECM splice variants

Fibronectin and tenascin-C are examples of ECM molecules with splice variants that are differentially expressed in normal and tumor tissue. Extra domains A and B of fibronectin are good target candidates for therapeutic tumor vaccination, with reduced tumor progression as a result of immunization against these molecules (Femel et al., submitted; Huijbers et al., 2010).

Tenascin-C is an ECM glycoprotein that together with tenascins-R, -W and -X makes up the tenascin family (Midwood et al., 2011; Van Obberghen-Schilling et al., 2011). It is highly expressed during development but has a very restricted expression pattern in adult tissues (Midwood et al., 2011). However, upon tissue remodeling, e.g. in the event of injury or tumor formation, tenascin-C is re-expressed and deposited in the extracellular space (Midwood et al., 2011). It has both structural and signaling properties and can be found in the stroma of most solid cancers (Midwood and Orend, 2009). Tenascin-C promotes tumor progression partly through up-regulation of Wnt oncogenic signaling (Ruiz et al., 2004; Saupe et al., 2013) and supports breast cancer metastasis implicating Notch and, again, Wnt pathways (Oskarsson et al., 2011). Accordingly, high tenascin-C expression levels is a marker for bad prognosis of several different cancer types (Midwood et al., 2011; Orend and Chiquet-Ehrismann, 2006). Moreover, while expression of tenascin-C in ovarian carcinoma is restricted and vascular in benign tumors, malignant tumors exhibit more widespread and robust tenascin-C depositions, which also comprise stromal areas (Wilson et al., 1996).

Both human and mouse tenascin-C include several sequences that are subjected to alternative splicing (Joester and Faissner, 2001). The domains in question are similar to fibronectin type III-repeats and can be expressed in numerous combinations, producing a large amount of different tenascin-C isoforms (Joester and Faissner, 2001). The facultative domains, with a single exception comprising 91 amino acids, show a large species homology and are named accordingly in mouse and human (Joester and Faissner, 2001). Several of these extra domains are up-regulated in various cancer settings, e.g. the extra domain C of
tenascin-C (TNCC), which is over-expressed in high grade astrocytomas (Carnemolla et al., 1999) and which is a marker for angiogenesis in cavernoma (Viale et al., 2002). Moreover, TNCC expression in urothelial carcinoma shows the same patterning as overall tenascin-C in ovarian carcinoma – vascular in non-invasive tumors and extensive and stromal in invasive, malignant tumors (Richter et al., 2009). The amounts of TNCC shed in urine also increase with tumor progression (Richter et al., 2009). TNCC is expressed in most human lung cancers, in a stromal or vascular pattern, while no expression can be seen in normal adult tissues (Silacci et al., 2006). Also in glioblastoma, TNCC can be found in a stromal and vascular pattern (Carnemolla et al., 1999). However, as the expression of TNCC is highly dependent on the tumor type used, it must be verified for each tumor model independently (Berndt et al., 2010).

### 1.4 β-glucans

β-glucans, which can be found in the cell walls of certain plants, fungi and bacteria, have long been known to possess immunostimulatory capacity (Chan et al., 2009; Liu et al., 2009; Seljelid, 1986). They all consist of linear D-glucose chains with shorter D-glucose branches, however, different bindings of the glucose units to one another and of the branches to the backbone give β-glucans from different sources highly variable immunological properties (Chan et al., 2009). In β-glucan from Saccharomyces cerevisiae, both linear chains and branches are internally linked by 1→3 bonds, while the branches attach to the backbone chain by 1→6 linkages (Liu et al., 2009). There are three major forms of yeast-derived β-glucans used in medicine. Particulate β-glucan (whole glucan particles, WGP) are empty “skeletons” of fungal cell walls, soluble β-glucan (which is the focus of this study) has been purified into trimers with a molecular weight of approximately 150 kDa, and very small molecular mass β-glucan (neutral soluble glucan, NSG) has been processed into <20 kDa fragments and has a short half-life in vivo (Li et al., 2006; Liu et al., 2009). The different yeast-derived β-glucan formulations also have different anti-tumor properties. Particulate β-glucan has an intrinsic effect on the adaptive immune system, by activation of dendritic cells and both CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells (Qi et al., 2011). Both particulate and soluble β-glucan is eventually taken up by macrophages and processed into smaller fragments (Hong et al., 2004; Li et al., 2006). Cytotoxic functions of soluble β-glucan are only performed by these processed 25 kDa fragments, which act through a complement- and neutrophil-dependent mechanism (described in the next paragraph), in the same way as very small molecular mass β-glucan (Li et al., 2006). Both macrophage processing and complement activation are essential for the tumoricidal effects of soluble β-glucan (Allendorf et al., 2005; Hong et al., 2004; Qi et al., 2011).

The complement system is a collection of circulating proteins that once activated produce inflammatory chemoattractants and opsonizing agents (Abbas et al., 2012). Anti-tumor monoclonal antibodies complexed to tumor cell surfaces activate complement through the classical pathway, which leads to inactivated C3b (iC3b)-opsonization of the tumor cells and neutrophil recruitment through the release of chemoattractants such as C5a (Allendorf et al., 2005; Salvador et al., 2008). Neutrophils are essential effector cells of the processed β-glucan-
stimulated antibody-coupled anti-tumor response and their infiltration into the tissue is highly dependent on the release of said C5a (Allendorf et al., 2005).

Complement receptor 3 (CR3, Mac-1, CD11b/CD18, αMβ2-integrin) is a leukocyte receptor with the ability to bind a large number of ligands and perform a multitude of different functions, e.g. adhesion to endothelial cells via intercellular adhesion molecule 1 (ICAM-1) and activation of cytotoxic functions in response to complement protein binding. It consists of two non-covalently linked subunits, CD11b or αM-integrin and CD18 or β2-integrin (Ross, 2002). The majority of ligands, e.g. iC3b, ICAM-1 and heparan sulfate, binds to overlapping but sometimes distinct sites of the I (inserted) domain, located distal from the transmembrane region of CR3 (Ross, 2002). A second, lectin-like site, situated close to the membrane, binds polysaccharides, e.g. β-glucan (Thornton et al., 1996). To enable high-affinity binding to the I site, a conformational change of CR3 must first take place (Ross, 2002). With the I site of CR3, neutrophils bind to iC3b, but co-ligation of β-glucan to the lectin-like site is necessary for eliciting cytotoxic functions mediated through CR3 (Li et al., 2006; Vetvicka et al., 1996; Xia et al., 1999). Processed β-glucan activation of CR3, an event which in the natural setting exclusively takes place when infectious agents introduce cell-wall β-glucan to the receptor, initiates CR3-dependent cellular cytotoxicity (CR3-DCC). This leads to the lysis of the iC3b-opsonized cell through an incompletely understood mechanism involving the tyrosine kinase Syk and phosphatidylinositol 3-kinase (Li et al., 2006; Liu et al., 2009).

Pilot studies indicate that while TNCC immunization alone had no effect on T241 fibrosarcoma growth, combining soluble β-glucan with the vaccination resulted in a decrease in tumor burden (Anna-Karin Olsson, personal communication).

1.5 Heparin

Heparin is a heavily sulfated form of the ubiquitously expressed glycosaminoglycan (polysaccharide) heparan sulfate (Nelson and Cox, 2008). Together with different core proteins, heparan sulfate make up several types of proteoglycans, which are abundant in the ECM and which play a part in many signaling events (Nelson and Cox, 2008; Parish, 2005). Heparin can be found in mast cell granules and its most common therapeutic use is as an anticoagulant (Nelson and Cox, 2008). It is also known to have anti-inflammatory effects (Parish, 2005). Unpublished findings suggest that heparin may inhibit the immunostimulatory effect of β-glucans (Ulf Lindahl, personal communication).

1.6 Aims

The overall aim of this study was to add more details to the present knowledge regarding therapeutic vaccination against tumor stromal antigens. Specifically, we wanted to investigate if TNCC is a suitable target molecule towards which self-antibodies can be raised, and whether such an immune activation can lead to halted tumor progression. We also wanted to examine whether soluble β-glucan can potentiate this effect. Lastly, to better understand the mechanisms behind soluble β-glucan immunostimulatory functions, we wanted to study the consequences of co-administration of heparin, a possible β-glucan effect inhibitor.
2 Materials and methods

2.1 Production of recombinant proteins

Recombinant Trx, Trx-TNCC and TNCC proteins for immunization and experimental purposes (Figure 2) were produced in *Escherichia coli* Rosetta-gami (DE3) (Novagen, EMD chemicals) as described for Trx-EDB in Huijbers et al. (2010). Recombinant proteins were produced by Falk Saupe and Elisabeth Huijbers.

2.2 Animals and immunization procedure

Animal work was approved by the Uppsala animal ethics committee (C114/13, C2/14). 7-week-old female C57BL/6J (Taconic) mice were immunized in the groin with fusion protein Trx-TNCC (100 μg/mouse, n = 30) or with Trx only (control, 50 μg/mouse, n = 10). The study time plan is presented in Figure 3. Immunization proteins were diluted in phosphate-buffered saline (PBS) together with 50 μg CpG oligo 1826 (Sigma-Aldrich) per mouse and mixed 50:50 with Montanide ISA 720 (SEPPIC). Booster immunizations were given two and five weeks after the initial immunization. All injections were given under isofluorane (KDG9623, Baxter) anaesthesia. Blood was drawn from the tail vein one week after the last booster, for measurements of antibody titers. Immunizations and blood sampling was done by Falk Saupe.

![Figure 2: Schematic overview of proteins used for immunizations and antibody detection. TRX = bacterial thioredoxin, mTNCC = mouse tenascin-C C domain. Proteins were produced as described in Huijbers et al. (2010).](image)

![Figure 3: Overview of the time scheme for the tenascin-C C domain (TNCC) vaccination and Lewis lung carcinoma (LLC) tumor study. Three immunizations were given followed by blood sampling and subcutaneous tumor cell grafting. For a subset of mice, treatment was started nine days after LLC grafting with intraperitoneal β-glucan injections every second day. Additionally, half of the β-glucan treated mice received daily subcutaneous injections of heparin. See also Table 1.](image)
2.3 Detection of anti-TNCC antibodies in mouse sera by ELISA

Blood for analysis of anti-TNCC antibody levels were coagulated at 4°C for a minimum of five hours. Samples were centrifuged 10 min at 2000 x g, the supernatant was collected and centrifugation at 13000 x g for 5 min followed. The serum obtained was frozen on dry ice and stored at -20°C.

ELISA plates (Multiwell Immuno Plate, Maxisorp, 96 well; M9410, Thermo Scientific) were coated with TNCC capture protein (8 μg/ml in PBS, 1 h), shortly rinsed with PBS and blocked with horse serum (Håtunalab, 1 h). Mouse sera were diluted 1:10 in horse serum and further 1:20 in E. coli Rosetta-gami whole cell extract (from a non-induced culture, to capture antibodies that may have been produced towards potential contamination from the immunization protein expression vector) to a final dilution of 1:200. Plates were incubated with duplicates of serum samples and controls (horse serum diluted 1:20 in Rosetta-gami extract) for 45 min. Wells were rinsed and washed. Biotinylated goat anti-mouse IgG (H+L) antibody (BA-9200, Vector Laboratories) diluted to 3 μg/ml in PBS with 0.1% Tween-20 (8.17072.1000, Merck) was added and incubated for 45 min. Plates were washed and subsequently incubated with streptavidin-coupled horse radish peroxidase (SA-HRP, SA-5004, Vector Laboratories) diluted to 2 μg/ml in PBS with 0.1% Tween-20 for 30 min, then washed again. All incubations were done at 37°C and all washings were done four times with PBS. Tetramethylbenzidine (TMB, T8665, Sigma-Aldrich) incubated at room temperature was used to measure HRP activity. Absorbance changes were continuously monitored during 15 min at 650 nm using a microplate reader. Incubation volumes were 50 μl per well.

2.4 Culture of Lewis lung carcinoma cells

Lewis lung carcinoma (LLC) cells (ATCC) were thawed from long-term storage (-150°C) and cultured in GlutaMAX Dulbecco’s Modified Eagle Medium (DMEM, 31966-021, Life technologies) supplemented with 10 % fetal calf serum (FCS, ECS 0180L, Euroclone), 100 U/ml penicillin and 100 μg/ml streptomycin (“1 % PeSt”, SVA). Cells were passaged twice before collection for tumor grafting.

2.5 Lewis lung carcinoma tumor study

53 days after the start of the immunization program (Figure 3), all mice were shaved at the upper left flank and received subcutaneous injections of 0.5 x 10^6 LLC cells in PBS. This day is referred to as day 0 of the tumor study. Tumor progression was carefully monitored and at day 9, when tumors were palpable, β-glucan or combined β-glucan and heparin treatment was started in 10 randomly chosen Trx-TNCC immunized mice per treatment regimen.

2.5.1 Soluble β-glucan and heparin treatment

Non-activated soluble β-glucan (Soluble yeast beta-glucan, SBG 13-BP-006, Biotec Pharmacon) was diluted in sterile PBS to a concentration of 4 mg/ml. Mice were intraperitoneally injected every second day with 125 μl of β-glucan solution, i.e. 500 μg per mouse (20 mg/kg for a 25 g mouse).

Heparin (585679, LEO Pharma) with an activity of 5000 IU/ml, approximately corresponding to a concentration of 25 mg/ml, was freshly diluted in sterile 0.9 % NaCl
solution to 0.25 mg/ml. Every day, mice were subcutaneously injected in the right flank with 125 μl of the heparin solution, i.e. 31.25 μg per mouse (1.25 mg/kg for a 25 g mouse). All injections were given under isofluorane anaesthesia.

2.5.2 Tumor study termination

Treatments were sustained until termination of the experiment, which occurred on day 22 after LLC grafting (Figure 3). Throughout the experiment, tumor dimensions were measured with a caliper and the volume was calculated using the formula \( V = \text{width}^2 \times \text{length} \times \pi/6 \).

2.5.3 Tissue sampling

Latest at day 22 after LLC grafting, mice were anaesthetized by intraperitoneal injection of 650 μl 3 % Avertin (T48402, Sigma-Aldrich) in PBS (w/v) and blood was drawn by heart puncture. Subsequently, mice were euthanized by heart removal and/or cervical dislocation. Lungs were perfused by injection through the trachea with 30 % sucrose (27480.360, D(+)-saccharose, VWR) in PBS and collected. Tumors were dissected out, measured by caliper and weighed. Both lungs and tumors were placed in 30 % sucrose in PBS overnight. The following day, tissue samples were embedded in OCT Cryomount (HistoLab 45830) tissue freezing medium and frozen in isopentane/dry ice.

2.6 Immunofluorescence staining for CD31 and Gr-1

Tumor samples were cut to 5 μm cryosections on a Cryotome FSE Cryostat (CryoStar NX70, ThermoScientific) at -20 °C (sample holder) and -22 °C (knife). The sections were fixed in ice-cold methanol for 10 min, rinsed in PBS and blocked for 45 min in 5 % bovine serum albumin (BSA, 10735094001, Fraction V, Roche) and 5 % horse serum (Håtunalab, Sweden) in PBS. Staining for blood vessels was done with 0.5 μg/ml primary antibody rat anti-mouse CD31 (PECAM-1, 553370, BD Pharmingen) in 5 % BSA in PBS for 1 h 45 min at room temperature (RT), washing, and secondary antibody Alexa 448-conjugated donkey anti-rat IgG (A21208, Invitrogen) diluted to 2 μg/ml in 5 % BSA in PBS for 45 min at RT. Slides were washed. Subsequently, staining for neutrophils was done with a 1.33 μg/ml dilution in 5 % BSA in PBS of APC-conjugated (allophycocyanin, IR emitting) rat anti-mouse Gr-1 antibody (Ly-6G and Ly-6C, 561083, BD Pharmingen) through over-night incubation at 4°C. After washing, nuclei were visualized through staining with Hoechst (33342, WVR) diluted to 1 μg/ml in PBS for 30 min at RT. Slides were washed and mounted with Fluoromount-G (0100-01, Southern Biotech). For negative controls, no primary antibodies were added. All washing steps were done 4 x 5 min in PBS. To visualize results, a Nikon eclipse 90i microscope (equipped with a DS-Qi1 Mc monochrome CCD camera and Nikon NIS Elements AR v3.2 software) and a SOLA SM light engine (Lumicor) was used. A minimum of eight images per sample were taken on random with a 20x objective from tumor areas with intact nuclear structures. Images were not taken in tissue proximal to wounds. Exposure times were kept constant at 150 milliseconds (ms) for CD31 (FITC) and 600 ms for Gr-1 (Cy5). The area of coverage in each colour channel was quantified with ImageJ version 1.48v (National Institutes of Health). Gr-1 coverage was normalized to nuclear staining for each image frame.
2.7 Necrosis scoring of immunofluorescence samples

Coverage of necrotic areas on samples stained for CD31 and Gr-1 was estimated by ocular inspection in a Nikon 90i microscope. Necrosis was defined as region containing no vessels, corrupt nuclear structures and large amounts of neutrophils. For each sample, this area compared to total tumor area was estimated on an ordinal scale comprising 0 = no, 1 = a little, 2 = some, 3 = quite some, 4 = much and 5 = very much.

2.8 Statistical analysis

Statistical analysis was done in GraphPad Prism version 5.0c. Planned comparisons between subsets of the four experimental groups were of interest (A vs. B, B vs. C, C vs. D and B vs. D, see Table 1). Therefore, no test for homogeneity across all groups was done. Multiple comparisons were not corrected for as individual hypothesis applied to different combinations of groups. For normally distributed data, Student’s t test was used. For non-normal data or data with small sample sizes, Mann-Whitney U test was performed. p values <0.05 were considered significant.

Table 1: Immunizations, treatments and sample sizes of the TNCC vaccination tumor study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization</th>
<th>Treatment</th>
<th>n (initial)</th>
<th>n (at termination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Trx</td>
<td>-</td>
<td>10</td>
<td>4</td>
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3 Results

3.1 Lewis lung carcinoma tumors express TNCC

To choose a tumor model system relevant to the vaccination strategy, TNCC expression in several different tumor types was investigated. Due to poor sample quality, only indicative results were obtained, why these experiments were omitted from the study. However, previous results from our laboratory have shown that TNCC is expressed in both T241 fibrosarcoma and Lewis lung carcinoma (LLC) (Figure 4) (Huijbers, 2012).

![Figure 4: Tenascin-C C domain (TNCC) expression in healthy tissue and tumor tissue from mouse. Cryosections of healthy heart tissue (left panel) and tumor tissue (middle and right panel) were stained by immunohistochemistry with purified rabbit anti-mouse antibody against TNCC. Tumor tissue from T241 fibrosarcoma (T241) and Lewis lung carcinoma (LLC). Scale bar = 10 μm. Experiment performed by Elisabeth Huijbers, published in (E. J. Huijbers 2012, Manuscript IV).](image)

3.2 Immunization against TNCC induced specific antibody formation

To evoke an immune response against the tumor-associated antigen TNCC, 40 7-week-old female C57BL/6J mice were immunized either with fusion protein Trx-TNCC or with Trx only, as a control (Table 1). The immunization scheme is outlined in Figure 3.

One week after the last booster injection, antibody titers were measured by ELISA against recombinant TNCC protein (Figure 5). All Trx-TNCC vaccinated mice had detectable levels of anti-TNCC antibodies, while control (Trx) vaccinated mice did not, showing that the immunization was successful.

3.3 Targeting of TNCC did not affect tumor growth or vessel density

18 days after the last immunization, all mice were given subcutaneous injections of 0.5 x 10^6 LLC cells to the upper left flank. LLC was chosen as model tumor type as pilot studies had indicated no effect of anti-TNCC vaccination in T241 fibrosarcoma model.
When tumors were palpable, treatment with either β-glucan or β-glucan in combination with heparin was initiated in, for each group, ten randomly chosen mice (Table 1). Soluble β-glucan was intraperitoneally administered every second day, heparin was given daily by subcutaneous injections in the upper right flank. The treatment regimen was sustained until termination of the experiment, which occurred 22 days after LLC grafting for 21 of the 40 mice. Remaining 19 mice had to be prematurely euthanized due to wound formation in association with the tumors, which lead to a decline in group sizes and therefore a reduction in the strength of the study (Table 1). Tissue samples were collected from all animals.

The growth of the tumors was relatively uniform between the groups and increased as the study proceeded (Figure 6A). At the termination day, post-dissection tumor volumes and weights were distributed as depicted in Figure 6B and C. No statistically significant difference was seen between the groups of interest.

As TNCC vaccination is hypothesized to reduce tumor growth in part by disruption of the tumor vasculature, vessel density in proliferative tumor tissue was quantified. To increase the number of samples in each group and thereby increase the statistical power, tumors were taken from mice that participated in the study for a minimum of 19 days. Cryosections were stained by immunofluorescence with anti-CD31 antibody, an endothelial cell marker, and the area of CD31 coverage was quantified. Vessel appearance varied greatly between different tumor areas (Figure 7A). However, no differences in vessel density could be found between the groups (Figure 7B).
Results

To address soluble β-glucan activation of immunological anti-tumor responses, neutrophil infiltration in the tumor tissue was assessed. Cryosections were stained by immunofluorescence with an anti-Gr-1 antibody, a marker which predominantly binds to neutrophils, and the fraction of neutrophils compared to total cell coverage was quantified (Figure 7C). The neutrophil infiltration was higher in the β-glucan treated group (C) than in both the immunization-only group (B) and the β-glucan/heparin combination treated group (D). No difference was seen between the two later groups. Neutrophil infiltration did neither correlate with tumor size nor with tumor wound status (data not shown). Examples of the characteristic neutrophil spot like staining can be seen in Figure 7A.

3.4 Neutrophil infiltration was affected by β-glucan and heparin treatments

Figure 6: Growth of subcutaneous LLC tumors in mice subjected to different TNCC vaccination-coupled treatments. C57BL/6J mice were immunized with fusion protein Trx-TNCC or Trx only and inoculated with LLC cells. A subset of mice was from day 9 after grafting treated with either β-glucan or β-glucan in combination with heparin. Throughout the experiment, tumor dimensions were measured using a caliper and the volume was calculated using the formula $V = \frac{\text{width}^2 \times \text{length}}{6}$. 

- **A.** Tumor growth over time. Data are visualized as medians with interquartile range.
- **B, C.** Volume and weight of dissected tumors at termination of the study, day 22. One dot corresponds to one animal. No significant differences between the indicated treatment groups were found with the Mann-Whitney U-test. Medians are depicted as horizontal bars. LLC = Lewis lung carcinoma, TNCC = tenascin-C C domain, Trx = bacterial thioredoxin, β-g = β-glucan, Hep = heparin.
RESULTS

Large necrotic areas in tumors from β-glucan-treated mice

To approximate tumor viability, necrosis was estimated from cryosections stained by immunofluorescence. On a scale from 0 (no) to 5 (very much), the relative area of each tumor interpreted as necrotic was estimated. These areas were identified as containing no vessels, corrupt nuclear structures and a very high neutrophil presence. According to this classifica-
tion, tumors from the β-glucan treated group (C) were more necrotic than those from the immunization-only group (B) (Figure 8).

**Figure 8: Necrosis score of LLC tumors from mice subjected to different TNCC vaccination-coupled treatments.** Immunization, tumor grafting and treatments were performed as described in Figure 6. The analysis was done on tumor samples collected between day 19 and day 22 after tumor cell grafting. Tumor necrosis was investigated on cryosections which were immunofluorescently stained for neutrophils, blood vessels and cell nuclei. Necrosis was scored relative to total tumor area on an ordinal scale from 0 (no) to 5 (very much). Mann-Whitney U test, B-C p = 0.0347. Medians are depicted as horizontal bars. LLC = Lewis lung carcinoma, TNCC = tenascin-C C domain, Trx = bacterial thioredoxin, β-g = β-glucan, Hep = heparin.
4 Discussion

The overall aim of this study was to add more details to the present knowledge regarding therapeutic vaccination against tumor stromal antigens. We wanted to investigate if TNCC is a suitable tumor stroma target molecule towards which endogenously produced self-reactive antibodies can be raised and whether the potential antibody-mediated anti-tumor effect can be modulated by treatment with soluble β-glucan and heparin. While no effects of TNCC vaccination were seen on tumor progression, immune activation by soluble β-glucan was observed.

4.1 TNCC as a target for therapeutic vaccination

To see if an immune response could be elicited towards the self molecule TNCC, antibody titers were measured after the end of the vaccination scheme. All vaccinated mice produced antibodies against TNCC (Figure 5), but the variability in antibody titers between animals was substantial. The measurement of antibody levels was however done only one week after the last booster immunization, which may not have been long enough for the antibody production to peak. Indeed, immunization studies on other target proteins (extra domains A and B of fibronectin) have shown that antibody titers rise substantially between one and four weeks following the last booster immunization (Femel et al., submitted). Measurements at the time of tumor grafting or treatment initiation would most likely have shown a higher and more even antibody distribution.

In a previous pilot experiment, TNCC vaccination did not have any effect on growth of T241 fibrosarcoma tumors (Anna-Karin Olsson, personal communication). The primary reason to choose LLC as the tumor type in this study was to see if this would be a suitable model for studies of effects of immunization against TNCC on tumor growth. Also, most human lung cancers robustly express TNCC (Silacci et al., 2006).

Due to wound formation at the tumor site, near half of the animals originally included had to be prematurely removed from study. The mice remaining at the end of the experiment were too few to produce solid data, both from a biological and a statistical point of view. Thus, no differences in tumor progression could be corroborated (Figure 6). Future studies with adequate sample sizes will be needed to examine the present issues further. It is also crucial to develop a robust system for detection of the target molecule, in this case TNCC, in the available tumor model systems, as low levels of antigen expression may not be sufficient to elicit tumoricidal effects, despite successful antibody generation.

The cause of the wound development is not known. Hypothetically, LLC cells used for grafting were not fully syngenic to the host mice, even though both were C57BL/6J. Minor immunological differences between the host and the tumor cells could have lead to an adverse reaction to the forming tumor, resulting in tissue destruction. Such a global immune response directed at the tumor might mask or compromise effects on immune functions elicited by the vaccination or by the treatments. However, effects are always seen in comparison to a control group, and wound formation appeared in all groups, somehow normalizing this feature, why experimentally induced effects nevertheless could be discernible. To confirm the results
however, the study should be undertaken once more with a validated LLC cell line that is known to match the host mouse strain.

As no effects were seen on tumor growth, it is not surprising that vessel coverage was uniform between groups. In similar vaccination studies (directed at extra domains A or B of fibronectin) where tumor burden was significantly diminished, no effect on vascular distribution was seen, instead, vascular function was affected (Femel et al., submitted; Huijbers et al., 2010). Assessing the functionality of the vasculature could thus be of interest for upcoming studies.

4.2 Effect of β-glucan treatment in combination with the TNCC vaccine

To assess immune activation of treatment with soluble β-glucan, neutrophil recruitment to the tumors was quantified by staining for the neutrophil marker Gr-1. No significant difference was seen between Trx- (A, control) and Trx-TNCC-immunized (B) groups, suggesting that in this experiment the vaccination did not affect immune cell infiltration. Earlier studies however suggest that in well-functioning vaccination setups, more leukocytes are recruited to the tumor tissue (Femel et al., submitted; Huijbers et al., 2010)

More neutrophils were found in tumors from β-glucan treated mice (C) compared to the immunized-only (B) mice (Figure 7). It could be hypothesized that activation of CR3 through β-glucan ligation could result in increased neutrophil binding to endothelial ICAM-1 and subsequent extravasation. However, the conformational change elicited by β-glucan binding does not entail exposure of the adhesion-associated ICAM-1 high-affinity binding site (Vetvicka et al., 1996). Additionally, leukocyte function-associated antigen 1 (LFA-1) is the primary player in integrin-mediated diapedesis adhesion, whereas CR3 is responsible for neutrophil crawling on the endothelial surface to the optimal site for extravasation (Phillipson et al., 2006). Negative effects of β-glucan ligation on neutrophil endothelial adhesion has however been reported, as urokinase plasminogen activator (uPAR) needs to bind the same CR3 lectin-like site as β-glucan in order to induce the high-affinity ICAM-1 binding state (Xia et al., 2002). In vitro, this effect can result in reduced transmigration of human neutrophils (Tsikitis et al., 2004a). In contrast, in vivo experiments in rats show that soluble β-glucan treatment causes an increased infiltration of neutrophils to both sterile inflamed and Escherichia coli pneumonia-infected sites (LeBlanc et al., 2006). The enhanced human neutrophil chemotaxis towards C5a elicited by soluble β-glucan ligation possibly overrides the reduced transmigration effect (Tsikitis et al., 2004b). The observed increase in neutrophil numbers could thus be due to an increased chemotactic capacity elicited by non-processed soluble β-glucan.

Reports in literature on neutrophil residence in tumor tissue as a response to β-glucan treatment are inconsistent. Allendorf et al. (2005) mention that they do not see any difference in neutrophil counts between tumors from control and mAb/β-glucan-treated mice in a subcutaneous lymphoma model, while Salvador et al. (2008) and Zhong et al. (2009) state that in subcutaneous xenograft models, neutrophils are more common in mAb/β-glucan-treated mice than in controls and only β-glucan-treated. However, while Allendorf et al. used particulate β-glucan, in the studies done by Salvador et al. and Zhong et al. soluble β-glucan was the variant administered. This underscores the importance of carefully examining which
specific β-glucan has which effect, and the notion that these effects are not automatically transferrable between β-glucan types. The increase in chemotaxis in response to C5a has only been validated for soluble, and not particulate, β-glucan (Tsikitis et al., 2004b).

Combining soluble β-glucan with anti-VEGF-A monoclonal antibody (bevacizumab) gives an increased tumoricidal effect only if tumor cells express VEGF-A on the cell surface (Salvador et al., 2008). Antibodies binding to soluble VEGF-A does not induce the same augmented anti-tumor effect as a response to β-glucan addition, suggesting that the complexing of antibodies is crucial to the complement activation needed for the induction of CR3-DCC (Salvador et al., 2008). TNCC is not membrane-bound and no cell-surface opsonization of iC3b is thought to occur, however, complement is still thought to be activated through antibody complex formation in the ECM (Abbas et al., 2012). Antibodies elicited by vaccination are polyclonal, thus binding to the target molecule at multiple sites, which abrogates the necessity for several closely located identical binding sites, e.g. multiple adjacent receptors on a cell surface, to enable immune complexing. In addition, iC3b can not only be deposited on cell surfaces, but also on the complement-activating antibody-antigen complexes (Abbas et al., 2012). Candida albicans hyphae, β-glucan-containing structures which are too large to be ingested by phagocytes, provoke CR3-dependent frustrated phagocytosis and a release of reactive oxygen species from neutrophils (Lavigne et al., 2006). Hypothetically, ECM bound iC3b in conjunction with exogenously added β-glucan could elicit this same response, which is potentially similar to CR3-DCC, but directed at ECM rather than at cells. The CR3 activation would add to the tissue-destroying effect of antibody opsonization, which would be beneficial from a therapeutic point of view.

As mentioned, anti-tumor effects of soluble β-glucan are dependent upon complement activation elicited by antibody complexing (Allendorf et al., 2005; Salvador et al., 2008). Effects of treatment with soluble β-glucan alone, which however sometimes can be detected, may arise from slight complement activation mediated by naturally occurring antibodies against tumor proteins (Harnack et al., 2009; Hong et al., 2004). However, when directed at the extra domain B of fibronectin, the vaccination strategy used in our lab predominantly induces antibodies of the mouse subclass IgG1, which is not supposed to activate complement (Huibjers et al., 2012; Neuberger and Rajewsky, 1981). IgM, a less specialized antibody type with the ability to activate complement, may however also be present. Antibody subtype signature as a response to TNCC vaccination should be investigated in the present experimental setup. To verify complement activation, staining for e.g. C5a and iC3b could be performed.

It takes five to ten days for macrophages to process orally administered particulate β-glucan into bioactive 25 kDa moieties (Hong et al., 2004). When treating by intravenous injection of soluble β-glucan, the numbers of 25 kDa-fragment β-glucan-primed neutrophils rise from none to around 10 %, which seem to be a maximum amount possible, between the first and the seventh days after administration (Li et al., 2006). The delay time between the start of the treatment with soluble β-glucan and the start of the effects of this treatment must be taken into consideration when planning future experiments. The time that soluble β-glucan can have a biological effect should not be too short, or offset with respect to heparin treatment. If full effect is not reached until seven days after the first injection, the mice in this study were only subjected to full β-glucan-induced cytotoxicity for a total of six days. In that
case, it is not surprising that effects were seen only on non-processed soluble β-glucan-mediated augmentation of neutrophil recruitment (Figure 7C), while the processed β-glucan-dependent activation necessary for CR3-dependent frustrated phagocytosis and effects on tumor growth were undetectable (Figure 6).

The occurrence of more necrosis in the β-glucan treated group (C) than the immunized-only group (B, Figure 8), together with the higher neutrophil infiltration (B, C, Figure 7C), suggest that there is indeed an augmented immune response with increased tissue destruction ongoing in the β-glucan treated tumors. Zhong et al. (2009) report more necrosis in mAb/β-glucan co-treated samples than only mAb treated in human lung cancer orthotopic xenografts in mice, where survival was significantly increased in the former group. It is possible that the effects on tumor necrosis seen here also would have affected tumor growth, if treatment had been initiated at an earlier stage or if a more slow-growing tumor model had been used. However, the accuracy of the necrosis assessment procedure used here must be confirmed by re-evaluation with a validated method.

4.3 The effect of heparin addition on β-glucan treatment

Heparin, a potential inhibitor of β-glucan-mediated anti-tumor effects, was included in the study to possibly shed light on the mechanism of action of soluble β-glucan in the present system. The increase in neutrophil infiltration that was seen in the β-glucan treated group (C) compared to the immunized-only group (B) was indeed extinguished when heparin was added to the treatment regimen (D) (Figure 7C). The inhibition of neutrophil recruitment could however have several explanations. Endogenous heparan sulfate (HS) binds pro-inflammatory chemoattractants, chemokines, and transports them to the luminal side of endothelial cell layers, where they present them to leukocytes (Parish, 2005). Most chemokines possess a positively charged C terminus which can bind to the negatively charged HS (Parish, 2005) – and possibly even better to the even more negative heparin. The reason for the decrease in neutrophil infiltration caused by heparin treatment may therefore be that the soluble heparin out-competes the HS, bound at the site of inflammation, for chemokine binding, thereby inhibiting leukocytes recognition of the site for extravasation. Indeed, in a model of neuroinflammation, mice overexpressing heparanase, an HS-cleaving enzyme, exhibit impaired leukocyte infiltration and transmigration in response to chemokine release (Zhang et al., 2012). HS and heparin are also ligands for L-selectin, which leukocytes, including neutrophils, use for the initial adherence to vessel walls at sites of inflammation (Göttte, 2003). L-selectin ligand binding is necessary to slow down the rolling in preparation for diapedesis. Leukocytes adhering to the circulating heparin instead of the HS might thus also play a part in the effect. Furthermore, heparin is a ligand to the I site of CR3 (Diamond et al., 1995). Neutrophil migration is significantly diminished in heparin-treated cells, an effect that can be attributed to heparin binding to the CD11b part of CR3 (Salas et al., 2000). In addition to possible effects on leukocyte infiltration, heparin also competes with iC3b for binding to CR3 (Peter et al., 1999), thereby possibly blocking not only the recruitment but also the effector functions of the neutrophils. This remains to be investigated.
4.4 Future prospects

In this study, no effects of anti-TNCC vaccination and treatments were seen on tumor growth. Whether this is a genuine reflection of biological circumstances or an effect of the premature loss of experimental animals can however at present not be known. The questions posed are still of great interest to the development of anti-cancer immunization schemes, and deserve to be re-examined under more optimal conditions. The suitability of TNCC as a cancer vasculature target can be studied in a well-known tumor model with a validated TNCC expression.

Anti-tumor effects of soluble β-glucan and heparin can be further investigated in a robust vaccination/tumor model, such as extra domain B of fibronectin combined with T241 fibrosarcoma (Huijbers et al., 2010) or extra domain A of fibronectin combined with MMTV-PyMT, a model of spontaneous formation of breast cancer and lung metastasis (Femel et al., submitted; Huijbers et al., 2010). The mechanisms of action of soluble β-glucan and heparin in conjunction with therapeutic vaccination against an ECM protein should also be elucidated. Neutrophils are essential as effector cells for anti-tumor responses in mAb and soluble β-glucan co-treated mice (Allendorf et al., 2005). To assess whether neutrophils are the effectors also in our experimental system, a setup where these cells are absent (e.g. transiently depleted using an anti-Gr-1 antibody (Allendorf et al., 2005) or non-functional (as in CR3−/− mice (Li et al., 2006) can be used. To investigate if putative β-glucan effects are iC3b/CR3-dependent, CR3 conformation can be assessed with an antibody targeting the neoepitope which appears only after CR3 activation, as described in (Li et al., 2006), or in C3−/− mice, as in (Hong et al., 2004). Also, the potentially separate effects of non-processed soluble β-glucan on neutrophil recruitment and of processed β-glucan fragments on cytotoxic functions must be confirmed.

As tenascin-C promotes metastasis in human breast cancers (Oskarsson et al., 2011), effects of TNCC vaccination on metastasizing capacities could also be investigated. We collected tissue samples (lung) which allows for future investigations of that sort even in the scope of this study. Also, if TNCC is expressed in MMTV-PyMT tumors, this could be a suitable model for investigation of effects of TNCC immunization on metastasis formation.

4.5 Concluding remarks

Therapeutic vaccination against tumor stromal targets is a promising new avenue in the cancer therapy field of research. This study contributes to the body of knowledge on the immune functions of therapeutic anti-self antibodies and the interplay between the adaptive and innate parts of the immune system. When future studies further clarify the intricate web of immune reactions and the involvement and roles of immune cells and factors, therapeutic vaccination strategies and adjuvant formulations can be increasingly well adapted in order to optimize the exciting possibility of endogenous suppression of cancer progression.
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