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Targeted microbeads for attraction and induction of specific innate immune response in the tumor microenvironment

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ABSTRACT

Antitumor activity of molecules and cells of the innate immune system has been reported. Here we propose a method for targeting preferred innate immune cells and magnifying their tumoricidal effect at the tumor microenvironment, by modular multiple-component complexes (termed TILTAN). As a model, micro-scale complexes were assembled carrying monoclonal anti-HER2 antibodies, lipopolysaccharide and/or mannose. The complexes showed high binding capacity to HER2-positive cancer cells *in vitro*, high induction of interleukin-1 RNA transcription by the activated monocytes and ability to mediate monocytes' attachment to HER2-positive cells. TILTAN treatment was found safe in *in vivo* testing and induced change in interleukin-1 RNA transcription in tumors xenografts. We thus present a new vision of targeting a desired innate immune response to the tumor microenvironment.

1. Introduction

Cancer immunotherapy is aimed at eliminating tumors by using components of the immune system. However, immunological treatments have had limited success in clinical trials. A major drawback is the presence of molecules and cells of the immune system in the tumor microenvironment that contribute to the tumor's development instead of its elimination. Various methods have been developed to overcome this obstacle, including nano- and microparticles carrying therapeutic agents, monoclonal antibodies, and innate immune cell activation at the tumor site [1].

Beads of various sizes and compositions have been tested as carriers of drugs to cancer cells [1]. The leakiness of blood vessels in the tumor microenvironment [2] allows extravasation of nano- or micro-sized particles to reach the targeted tumor cells: migration of particles as large as 2 μm through tumor vessels has been reported [3,4]. In some tumors, the blood vessel cell lining is separated by intracellular openings (mean diameter 1.7 μm, range 0.3–4.7 μm) and transcellular holes (mean diameter 0.6 μm) [5].

Monoclonal antibodies represent an attractive approach for the specific targeting of tumor cells, based on the detection of tumor-specific or overexpressed antigens on the surface of various cancer cells, e.g. HER2 [6] and specific MUC I on breast cancer cells [7]. In the last two decades, 12 antibodies have been approved for use for anticancer treatments, and dozens are in clinical trials [8]. One of the first such antibodies to be approved was herceptin, which is targeted to the HER2 receptor in breast cancer treatment [9]. While the expression of HER2 receptors in normal mammary tissues is low, it is overexpressed in approximately 30% of breast cancer patients. HER2 has also been reported to be overexpressed in other cancers, such as of the ovary, endometrium, bladder, prostate and lung [6], on which herceptin has no cytotoxic effect. For maximum effect on a solid tumor, the monoclonal antibodies must gain access at sufficient concentrations to all of its viable cells. Therefore, one of the major difficulties in developing monoclonal antibody treatments for solid tumors relates to their inability to reach and penetrate the tumor. An additional limitation in such treatments is the antigenic heterogenicity of tumor cells.

In cancer, a significant population of leukocytes is attracted to the tumor microenvironment, with the two major subpopulations being tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). Both suppress anticancer activities and are positively correlated with tumor-cell proliferation, increased angiogenesis and tumor metastases. TAMs may become cytotoxic to tumor cells and switch to a "classically" activated phenotype following "re-education" [10]. Polymorphonuclear neutrophils have been found to contribute to both tumor rejection and tumor promotion [11]. Pro-cancer activities of these cells may be switched to antitumor behavior by defined inducers [10], and these may alter the interactions between the tumor and the immune system in the

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microenvironment to enable the desired immunological responses [12].

Various molecules have been shown to elevate antitumor responses by induction via Toll-like receptors (TLRs) and Fc receptors (FcRs). TLRs, preferentially expressed on dendritic cells and macrophages, function as molecular sensors that detect pathogen-associated molecular patterns, triggering secretion of chemokines and cytokines to activate innate and adaptive immune cells [13]. The role of diverse TLRs in cancer immunotherapy has been recently described in many studies. In human breast cancer cells, for example, apoptosis was induced by double-stranded RNA in a TLR3-dependent manner [14]. In mouse models, antitumor activity of TLR9 induced by CpG was found [15,16]. Tumor regression and long-term antitumor immunity were achieved by treating mice with synthetic RNA, an agonist of TLR7 and TLR8 [12,17,18]. In another animal model, TLR5 activation following flagellin administration resulted in tumor-growth inhibition [19]. Those studies suggested that the antitumor activity of TLRs is mediated by several mechanisms, one possibly based on shifting the immune response toward CD8(+) T cells, decreasing the number of regulatory T cells [20], and reversing the suppressive function of those cells [21]. Another mechanism might be based on triggering the secretion of cytokines involved in the differentiation and induction of potent adaptive immunity [11].

FcR binds the Fc domain of antibodies that are attached to antigen, enhancing cellular uptake of the immune system. This receptor is expressed on most hematopoietic cells, e.g. monocytes, macrophages, interferon gamma-activated neutrophils, natural killer cells, mast cells and dendritic cells. The influence of Fc/FcR on antitumor responses has been summarized by Cassard et al. [22]. In some cancers, following IgG recognition of cancer cells, immune complexes are formed, activating Fc gamma receptor to induce the antitumor response [22].

Inducers may be administered intravenously or intra-tumorally as soluble molecules. In the first case, the substance spreads throughout the body and therefore, to have an antitumoral effect, it has to be administered at very high concentrations. The problem with the second method is that the inducer may not reach metastases, which are a major concern. The basic idea behind the approach presented in this article is to treat the tumor via immune mechanisms that are directed at rejecting foreign bodies (e.g., pathogens or transplants). The proposed treatment is aimed at attracting cells and molecules of the innate and adaptive arms of the immune system to the tumor microenvironment, whereas their effect is magnified while the target is the whole tumor mass. This may lead to tumor rejection through massive interruption of the tumor mass and microenvironment. The efficiency of the treatment should not be hampered by cell-antigenic diversity in the tumor. To achieve this effect, microparticles were constructed and tested as agents targeting the inducer to the tumor or its microenvironment where it should stimulate a desired immune response. The microcarriers mounted with both targeting and inducing agents were termed "TILTAN".

2. Materials and methods

2.1. Beads and particles

Carboxylated 10-μm polystyrene beads (Bangslabs, Fishers, IN) were covalently conjugated to avidin using a carbodiimide (EDC) commercial protocol. Yellow-green fluorescent polystyrene beads, 2 μm in diameter covalently coated with avidin, were purchased from Polysciences (Warrington, PA), and ~1.5-μm magnetic particles covalently coated with avidin (Biomag^{TM}) were also purchased from Bangslabs.

2.2. Cells and antibodies

SK-BR-3 and BT474 human breast cancer cells expressing HER2, G361 melanoma cells and THP-1 human monocytes were purchased from the American Type Culture Collection (ATCC, Rockville, MD). SK-BR-3 and G361 cells were grown in McCoy's 5A medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin streptomycin solution (Biological Industries, Beit Haemek, Israel). BT474 cells were grown in DMEM medium supplemented with 10% FCS, 1% L-glutamate, 1% sodium pyruvate and 1% penicillin streptomycin solution and 10 μg/ml insulin. THP-1 cells were grown in RPMI 1640 medium supplemented with 5% FCS, 2% L-glutamate, 1% sodium pyruvate and 1% penicillin streptomycin solution. Trastuzumab® (herceptin) anti-HER2 antibodies were from Roche Pharmaceutical (Basel, Switzerland).

2.3. Fluorescence-activated cell sorting (FACS)

FACS procedures were carried out by FACScalibur (Becton Dickinson, San Jose, CA). The FACS results were analyzed with WinMDI version 2.9 (Scripps Research Institute, San Diego, CA).

2.4. Preparation of microparticles carrying anti-HER2, mannose and lipopolysaccharide (LPS)

Antibodies were biotinylated using EZ-Link Biotin LC sulfo-NHS (Pierce, Appleton, WI), mixing a 1:10 molar weight ratio of antibodies to biotin. Samples were dialyzed and tested for biotin incorporation using a 2-(4-hydroxyphenylazo)benzoic acid (HABA) (Across Organics, Geel, Belgium) and avidin (Belovo, SA, Bastogne, Belgium) competitive assay, and DC protein assay (BioRad, Hercules, CA). Biotinylated anti-HER2 (BiH) antibodies were tested for antigen detection by FACS. The BiH antibodies were incubated for 1 h with SK-BR-3 cells, which were then washed and incubated with streptavidin-FITC (Jackson Immunoresearch, West Grove, PA) for 45 min.

LPS (L-2654, Sigma, St. Louis, MO) was biotinylated using a biotin hydrazide kit (Pierce). Biotinylation efficiency was tested by direct ELISA stained with streptavidin-peroxidase (HRP) (Jackson Immunoresearch) as described by Odeyale and Kang [23].

Biotinylated mannose (BiM) was synthesized as described by Vaya et al. [24].

To produce TILTAN complexes, BiH, BiM and biotinylated LPS (BiL) in various ratios were mixed with avidin-coated microparticles or microbeads and incubated overnight. The ratios of the components were assessed using biotin quantification following molecule biotinylation and based on the beads' or particles' binding capacity as stated by the manufacturer. Binding efficiency of BiH to carriers was examined by FACS. Samples were washed in FACS buffer: phosphate buffer (PBS) with 0.1% bovine serum albumin (BSA), and stained for 45 min with FITC-labeled goat anti-human Fc (Jackson Immunoresearch). Binding of BiL was measured indirectly by reduction of BiH fluorescence intensity.

2.5. TILTAN targeting to SK-BR-3 cells

Particles and beads were tested for their ability to bind to SK-BR-3 cells by FACS. Trypsinated cells were centrifuged, suspended in FACS buffer, then incubated for 1 h with 2-μm fluorescent beads or Biomag particles carrying BiH, BiL or BiM or their combinations in various ratios. Cell samples incubated with fluorescent beads were washed and tested by FACS. Samples incubated with Biomag particles were further washed and incubated with goat anti-human Fc-FITC.

2.6. Induction of TILTAN microbead uptake by THP-1 cells

THP-1 cells (10^6 per well) were grown overnight in 24-well plates. Fluorescent beads (2 μm), carrying BiH, BiL, BiM or their combinations in various ratios, were added and incubated for 3 h. Cells were then centrifuged, resuspended in PBS and analyzed by FACS.

2.7. TILTAN-mediated attachment of THP-1 monocytes to SK-BR-3 cells

SK-BR-3 cells (6×10^4 cells per well) were incubated overnight in 24-well plates. Biomag particles carrying BiH, BiL or their combinations were added and incubated for 1 h, followed by three washes with fresh medium. This was followed by the addition of THP-1 cells stained with carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye using the manufacturer's protocol (Invitrogen, Carlsbad, CA), and co-incubating for 3 h. Cells were washed and viewed by fluorescence microscopy.

2.8. Determination of TILTAN induction of IL-1β expression in THP-1 cells by semi-quantitive RT-PCR

THP-1 cells (10^6 per well) were incubated overnight in six-well plates. Biomag particles or 2-μm fluorescent beads were added and incubated for 3 h. Medium was discarded and cells were centrifuged.

Cellular RNA was extracted using TRISOL® reagent (Invitrogen). RT-PCR was performed on total mRNA using 20mer Oligo dT primers and the AMV kit protocol (Promega, Madison, WI). PCR was performed to propagate the IL-1β gene product and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control using a PCR kit (Bioline, London, UK). Primers used were human IL-1β (249-bp product), forward: 5′-GGCAGACTCAAATTCCAGCT-3′, reverse: 5′-GGACAGGATATGGAGCAACA-3′; GAPDH (158-bp product), forward: 5′-GAGTCAACGGATTTGGTCGT-3′, reverse: 5′-GGTGCCATGGAATTTGCCAT-3′.

The PCR product was electroporated on a 2.3% agarose gel and stained with ethidium bromide. IL-1β PCR product levels were analyzed relative to GAPDH using Fluor-S™ MultiImager by Quantity One software (BioRad).

2.9. TILTAN in vivo toxicity test and accumulation in tissues

To test systemic TILTAN toxicity, 30 BALB/c female mice 6–8 weeks old were divided into 10 groups and injected into the tail vain with 100 μl of TILTAN, at various concentrations. Behavior and weight loss were monitored for 3 weeks. Particles accumulation and clearance in liver, kidney and spleen were monitored daily by Magnetic resonance imaging (MRI) using T2* weighted scans. After 3 weeks animals were sacrificed and heart, lung, liver, spleen and kidney tissues were harvested and fixed in formaldehyde. Paraffin blocks were prepared, sliced and stained with hematoxylin and eosin (H&E) or Prussian blue iron staining. Stained slides were assessed for tissue damage and TILTAN remnants.

2.10. Human tumor xenografts

All experiments were carried out in accordance with guidelines of the Ethics Committee of the Hebrew University, Jerusalem. Twenty-five female, athymic (BALB/c nu) mice 6–8 weeks of age were kept under specific pathogene free conditions with free access to food and water. Two 60-day slow release 0.72 mg estradiol pellets (Innovative Research of America Sarasota, FL) were implanted 24 h prior to xenografts inoculation. BT474 breast cancer cells were trypsinated and washed twice with Hank's balanced solution (HBSS, Biological Industries) then 10^7 cells were suspended in 100 μL HBSS, mixed with 100 μL of ice cold Matrigel (BD Biosciences, Bedford, MA) and injected subcutaneous into the lateral flank.

2.11. TILTAN administration

The size of BT474 xenograft was monitored daily using caliper, until reaching 200–300 cm^3 [volume = (width^2 × length)/2]. Mice were divided into groups of 5 and injected with 100 μl of TILTAN, at various concentrations, into the tail vain. Forty-eight hours post injection animals were sacrificed, tumor, spleen and kidney tissues were harvested and taken for histology (H&E and Prussian blue). Tumor fresh samples were taken and checked by semi-quantitive RT-PCR for IL-1β expression.

3. Results

A specific composition of TILTAN was constructed to test the principle of targeting a desired immune response to the tumor site. Humanized monoclonal antibody anti-HER2 (herceptin) was used as the targeting agent, and LPS and mannose were used as inducers of innate immune cells.

Anti-HER2 antibodies, LPS and mannose molecules were biotinylated and conjugated to the same avidin-carrying microbead or microparticle through avidin-biotin interactions. Three carriers were tested as follows: 10-μm and 2-μm polystyrene beads and ~1.5-μm magnetic particles. Prior to loading onto the carrier, the activity of the biotinylated molecules was examined. BiH antibodies were found to attach to SK-BR-3 cells expressing high levels of HER2 (Fig. 1A). Activities of BiL and BiM were tested by induction of IL-1β in THP-1 monocytes. Biotinylation did not decrease IL-1β stimulation by mannose or LPS (data not shown).

Attachment of the biotinylated molecules to the avidin-carrying beads and particles was confirmed by FACS analysis. Fig. 1B describes attachment of BiH antibodies to the particles.

Specific targeting of beads carrying BiH was tested using two cancer cell lines: SK-BR-3 breast cancer cells that overexpress HER2, and G361 melanoma cells that do not express it (Fig. 2). Beads carrying BiH showed little or no binding to G361 cells (Fig. 2A), and high specific binding to the SK-BR-3 cells (Fig. 2C). Beads without BiH did not bind to the SK-BR-3 cells (Fig. 2B).

To confirm BiL binding to beads, various amounts of BiL were loaded on avidin-carrying beads, followed by incubation with an excess amount of BiH. The presence of BiH on the carrier was detected by FACS analysis, and was found to be dependent on the amount of BiL on the beads (Fig. 3A) so that as BiL amounts increased, the amount of BiH bound to the carrier decreased and the beads stained less intensively.

The ability of TILTAN complexes to attach to SK-BR-3 cells was tested. SK-BR-3 cells, monolayered or trypsinized, were incubated with Biomag particles carrying BiH and BiL, then incubated with goat anti-human Fc-FITC, and TILTAN attachment was detected by fluorescence microscopy and FACS (Fig. 3B). Binding of Biomag particles to SK-BR-3 cells was shown to be antibody-dependent (Fig. 3B): no attachment was observed following incubation with unloaded particles or particles loaded with BiL only (Fig. 3B1 and B6, respectively). The binding of Biomag particles was stable and was not affected by pre-FACS centrifugation or pipetting.

The attraction of monocytes by TILTAN loaded with various ratios of antibody and inducers was tested. As a first step, the binding ability of THP-1 cells to beads loaded with BiH following incubation was observed under a microscope. The presence of BiH was essential for THP-1 binding to the carriers (Fig. 4A). To test

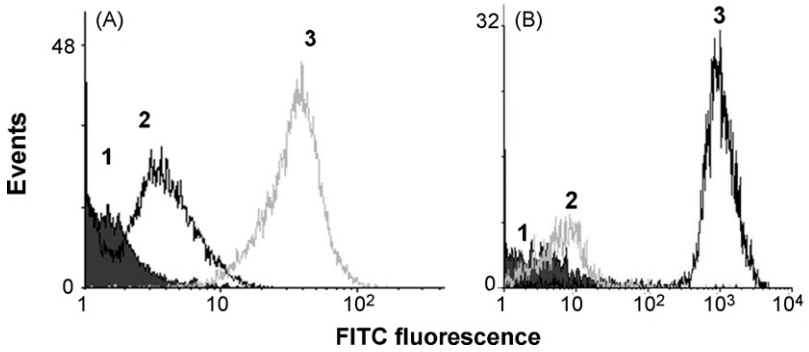

Fig. 1. Determination of biotinylated anti-HER2 antibody (BiH) attachment and assembly of the BiH carriers. (A) Attachment of BiH to SK-BR-3 cells. SK-BR-3 cells were incubated with: 1—biotinylated (Bi) non-relevant antibodies (negative control); 2—FACS buffer (negative control); 3—BiH, for 1 h. Samples were then fluorescently stained by incubation with strepavidin (SA)-FITC and detected by FACS. (B) Detection of BiH on Biomag magnetic particles. BiH was bound to avidin-carrying Biomag and then samples were stained with goat anti-human Fc-FITC antibodies. 1—Unbound Biomag (negative control); 2—Biomag bound to non-human Bi antibodies (negative control); 3—Biomag bound to BiH. FACS histograms are shown.

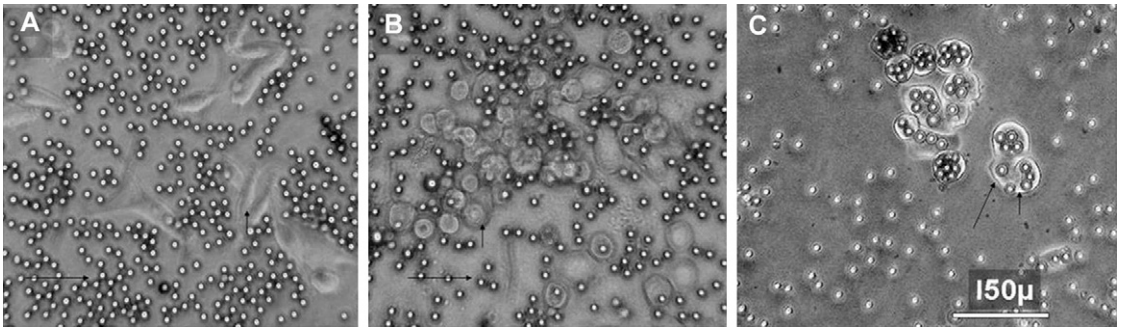

Fig. 2. Attachment of BiH beads (10 μm) to HER2-overexpressing cells. (A) BiH beads incubated with melanoma G361 cells (not expressing HER2). (B) Beads without BiH incubated with SK-BR-3 cells (negative control). (C) BiH beads incubated with SK-BR-3. Microscopic images are shown. Long arrows indicate beads; short arrows indicate cells.

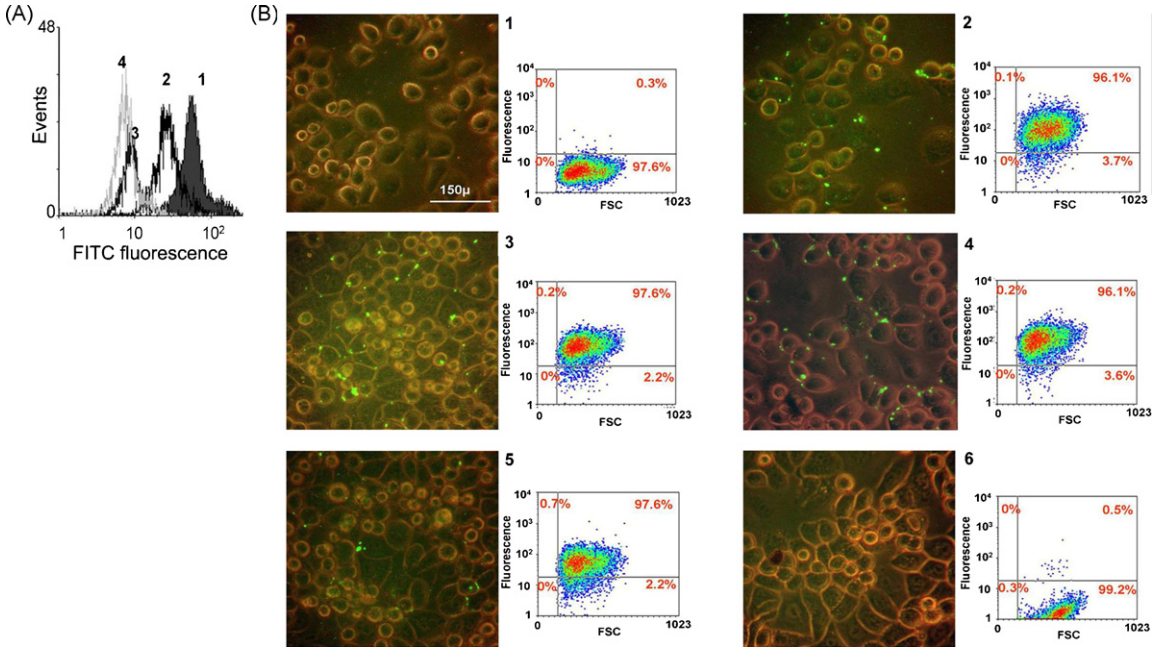

Fig. 3. Assembly of complexes with BiH and biotinylated LPS (BiL) at various ratios and their ability to bind to SK-BR-3 cells. (A) FACS histogram of BiH binding following loading of gradually increasing amounts of BiL on 10 μm avidin-coated beads. Samples were fluorescently stained with goat anti-human Fc-FITC: 1—no BiL added; 2—1 μl of BiL added; 3—5 μl of BiL added; 4—10 μl of BiL added. (B) Fluorescence microscopy images and FACS density plots demonstrating Biomag complexes' attachment to SK-BR-3 cells. Monolayered or trypsinized SK-BR-3 cells were incubated with Biomag particles for 1 h, followed by incubation with goat anti-human Fc-FITC. Biomag complexes were 1—non-loaded particles, or carried: 2—100% BiH; 3—75% BiH and 25% BiL; 4—50% BiH and 50% BiL; 5—25% BiH and 75% BiL; 6—100% BiL. FSC, forward scattering.

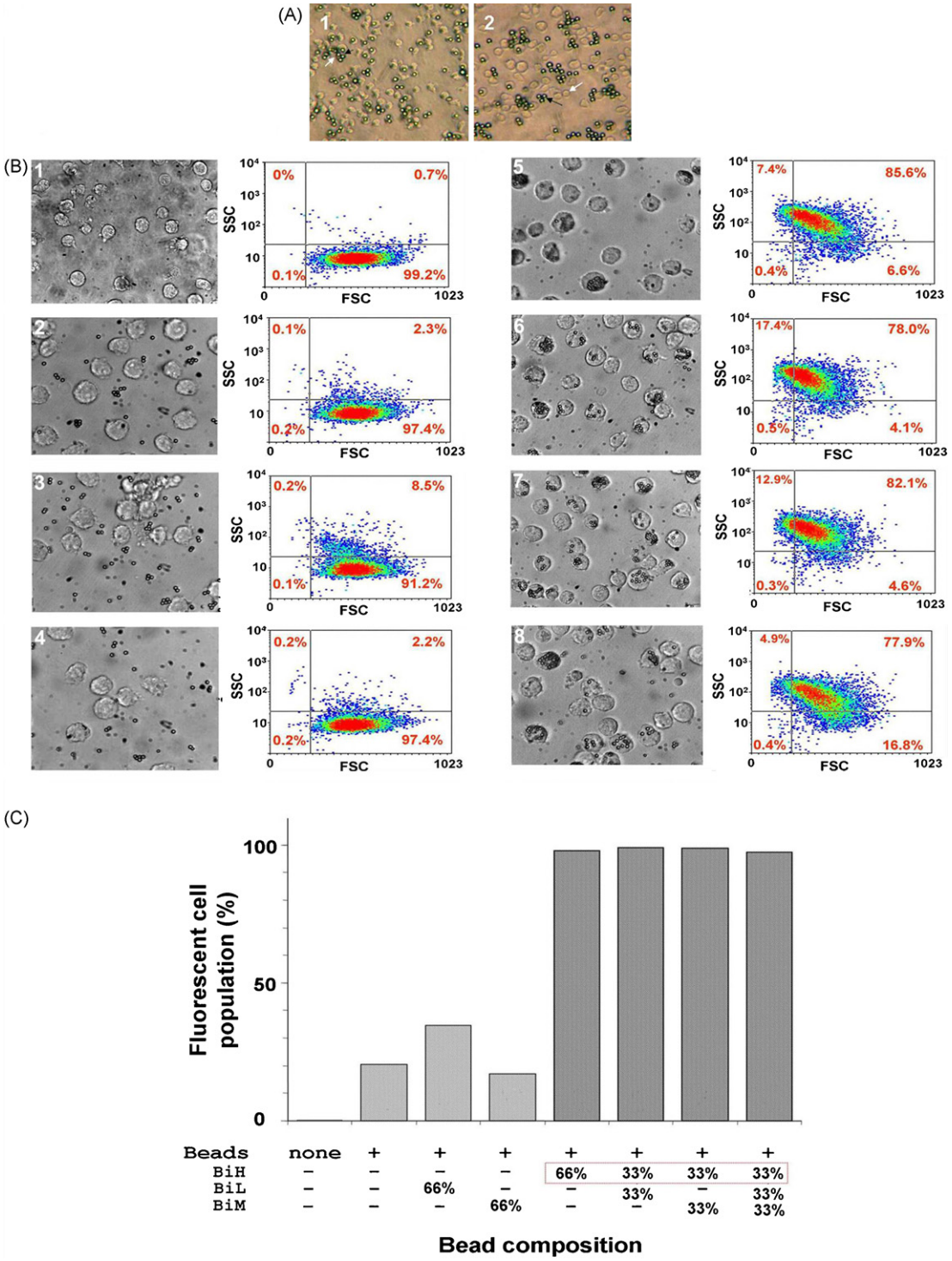

Fig. 4. Determination of THP-1 cell binding to beads and their uptake. (A) THP-1 cells were incubated with 10-μm beads: 1—BiH-loaded beads; 2—non-loaded beads. The cells' attachment to the beads was detected by microscopy. Black arrows show the beads, white arrows show THP-1 cells. (B and C) Complexes with various combinations of antibody/inducers on 2-μm fluorescent beads were incubated with THP-1 cells. The uptake of the complexes was detected by microscopy and FACS analysis. THP-1 cells were incubated with: 1—no beads, negative control; 2—non-coated beads, or with beads carrying: 3—66% BiL; 4—66% BiM; 5—66% BiH; 6—33% BiH and 33% BiL; 7—33% BiH and 33% BiM; 8—33% BiH, 33% BiL and 33% BiM. A—Microscopic image; B—microscopic image and FACS density plot, FSC, forward scattering, SSC, side scattering; C—graphic representation of fluorescent cell population.

the monocytes' ability to take up the complexes, microscopy and FACS analysis were performed following incubation of THP-1 cells with different TILTAN compositions. To determine complex uptake, the physical properties (forward and side scattering) of the THP-1 cells and their fluorescence were measured (Fig. 4B and C, respectively). FACS results, represented by density plots (Fig. 4B), showed changes in the physical properties of the THP-1 cells as a result of their incubation with the complexes. Bead attraction by THP-1 cells was significantly increased in the presence of BiH on the complexes, and the presence of LPS or mannose on the same carrier did not interfere with this. Over 96% of the cells were found to interact with the fluorescent beads carrying BiH (Fig. 4C).

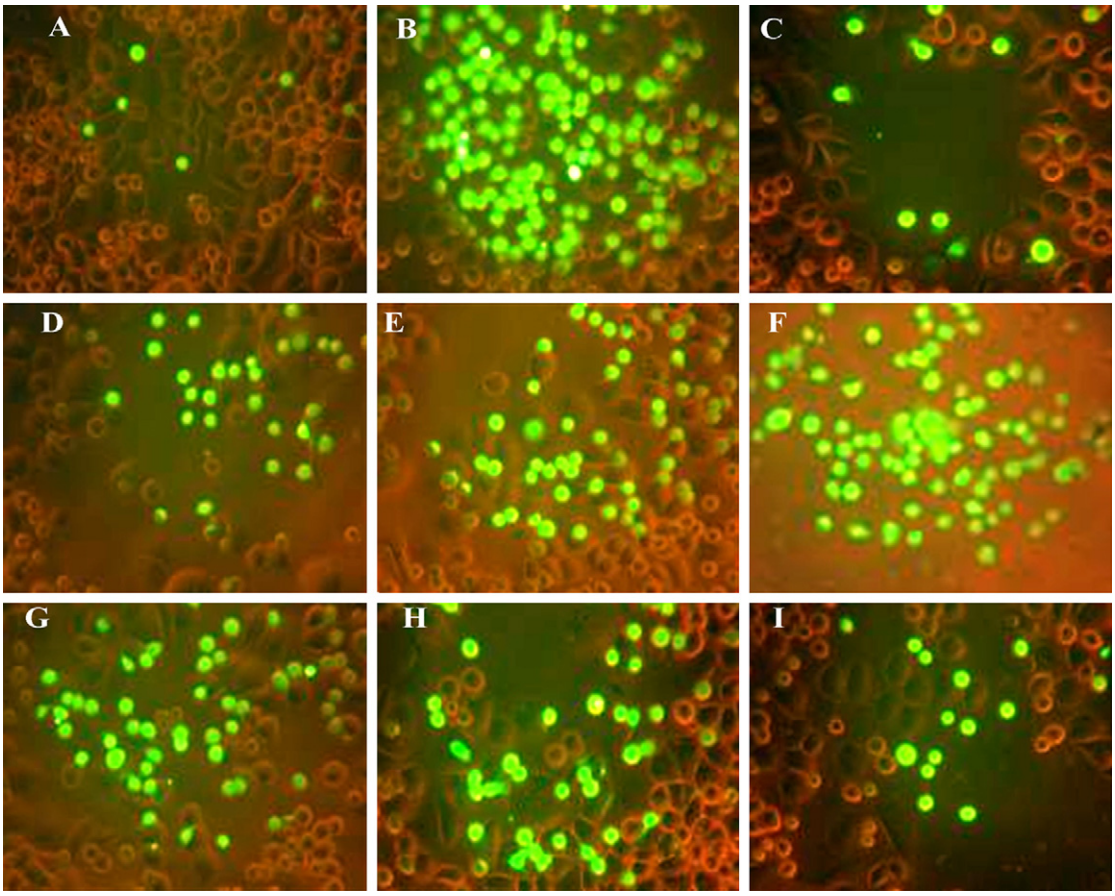

Fig. 5. TILTAN-mediated attachment of THP-1 to SK-BR-3 cells. SK-BR-3 cells were incubated with various complexes of antibody/inducers on Biomag particles, washed and then added to THP-1 cells, stained with CFSE fluorescent dye, and rewashed. Attachment of THP-1 cells to SK-BR-3 was visualized by fluorescence microscopy. Cells were incubated with: (A) soluble irrelevant antibodies (negative control); (B) soluble anti-HER2 (positive control); (C) soluble LPS, or Biomag particles carrying: (D) 100% BiL; (E) 100% BiH; (F) 25% BiH and 75% BiL; (G) 75% BiH and 25% BiL; (H) 50% BiH and 50% BiL; (I) non-coated Biomag particles.

To visualize TILTAN's ability to mediate attachment of target cells to monocytes, monolayered SK-BR-3 cells were incubated with different TILTAN complexes followed by washes and incubation with THP-1 cells pre-stained with CFSE fluorescent dye. The non-attached fluorescent THP-1 cells were then removed. The results presented in Fig. 5, confirmed TILTAN's ability to mediate attachment of monocytes to the targeted cancer cells (Fig. 5F–H).

The ability of the inducer, as a component of TILTAN, to stimulate IL-1β expression by monocytic cells was tested to evaluate possible induction of the innate immune response against the tumor. THP-1 cells were incubated with the complexes for 3 h, and then semi-quantitative RT-PCR for IL-1β and GAPDH genes was performed. RT-PCR products were visualized on an agarose gel (Figs. 6A and 7A), band density was quantified and IL-1β expression was normalized to that of GAPDH (Figs. 6B and 7B). Increased IL-1β expression was detected following incubation of THP-1 cells with Biomag particles carrying BiH and BiL at various ratios. A minor reduction in IL-1β expression was observed following a gradual decrease in the amount of BiL on the particle (Fig. 6, lanes 3–6). To test the induction of IL-1β expression by monocytes in response to a targeting agent and two inducers (BiL and BiM) on the same carrier, 2-μm fluorescent beads with various compositions were incubated with THP-1 cells. Beads carrying only BiH did not stimulate expression of IL-1β (Fig. 7, lane 6). However, the combination of BiH with BiM or BiL significantly increased the inductive effect. Indeed, beads carrying 33% BiM in combination with 33% BiH increased IL-1β expression level to over 2.5-fold higher than that of beads carrying 66% of a single BiM component (Fig. 7A and B, lanes 8 and 5, respec-

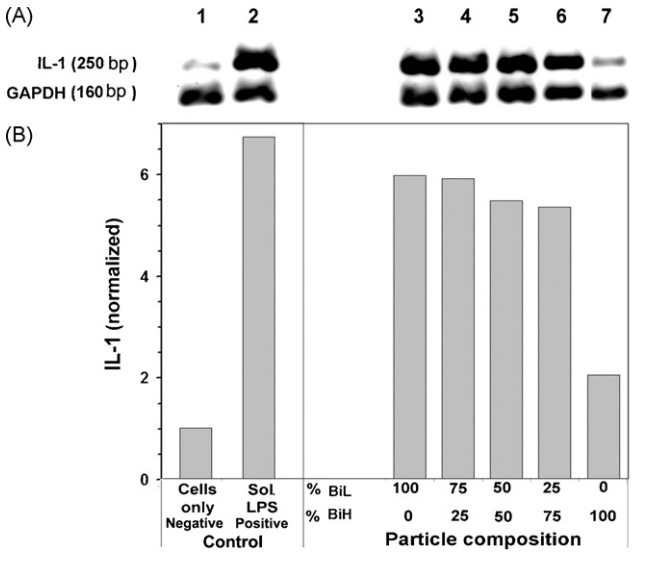

Fig. 6. Induction of IL-1β transcription in THP-1 monocytes by Biomag TILTAN. (A) Agarose gel of IL-1β (250 bp) and GAPDH (160 bp) RT-PCR products following THP-1 incubation with TILTANs. (B) Normalized IL-1β expression. A and B—Lanes and respective columns below represent PCR products of THP-1 cells incubated with: 1—cells only (negative control); 2—soluble LPS (positive control), or with Biomag particles carrying: 3—100% BiL; 4—75% BiL and 25% BiH; 5—50% BiL and 50% BiH; 6—25% BiL and 75% BiH; 7—100% BiH. Representative results of at least four independent experiments are shown.

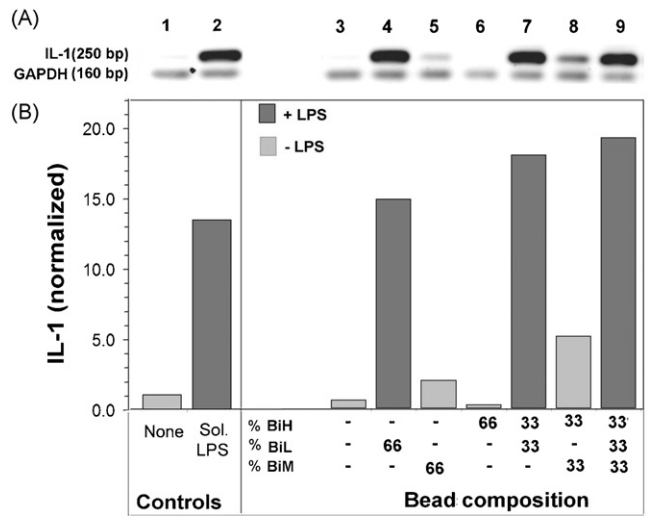

Fig. 7. Induction of IL-1β transcription in THP-1 monocytes by fluorescent 2-μm TILTAN beads. (A) Agarose gel of IL-1β (250 bp) and GAPDH (160 bp) RT-PCR products from THP-1 incubation with TILTANs. (B) Normalized IL-1β expression. A and B—lanes and respective column below represent PCR products of THP-1 cells incubated with: 1—cells only (negative control); 2—soluble LPS (positive control); 3—non-coated beads, or with beads carrying: 4—66% BiL; 5—66% BiM; 6—66% BiH; 7—33% BiH and 33% BiL; 8—33% BiH and 33% BiM; 9—33% BiH, 33% BiL and 33% BiM. Representative results of at least three independent experiments are shown.

tively). Similarly, IL-1β expression level induced by a combination of 33% BiL and 33% BiH was 1.2-fold higher than that induced by beads carrying 66% BiL alone (Fig. 7A and B, lanes 7 and 4, respectively). A combination of the two inducers, BiL and BiM with BiH in 1:1:1 ratio, did not interfere with IL-1β induction (Fig. 7, lane 9), indicating the feasibility of constructing an active complex that carries more than one inducer.

The complexes' ability to bind target cells and to induce IL-1β expression when loaded on different carriers is described in Figs. 2, 3B, 6 and 7 and summarized in Table 1. The proposed TILTAN complexes, consisting of the microcarrier, BiH and BiL/BiM, attached preferentially to the target cells (Fig. 2), mediated monocyte attachment to the cancer cells (Fig. 5) and increased monocyte susceptibility to the inducer, triggering an immune response that is determined by the inducer on the carrier, demonstrated in this case by IL-1β expression (Figs. 6 and 7).

Mice injected with high dose of TILTAN-LPS (10^8 particles) showed initially slight weight loss, which completely recovered after two days. Mice injected with lower TILTAN-LPS doses or with high doses of BiH or uncoated Biomag did not show any sign of stress or weight loss.

MRI results show particles cleared from liver and kidney after 6 days (Fig. 8A and B). Pathologic analysis of lung, liver, kidney and spleen tissues showed transient accumulation of iron particles in tissues after 1.5 h which cleared within 3 weeks (Fig. 8C). No particle accumulation was shown in the heart.

Semi-quntitative analysis of induction of RNA transcription in the tumor did not show increase in IL-1 expression, rather a slight and still a nonsignificant reduction in this gene expression after injection of TILTAN-LPS was recorded (data not shown).

4. Discussion

A number of factors may interfere with the development of an effective antitumoral immune response, including antigen loss [25,26], downregulation of MHC class I presentation [25,27,28], secretion of downregulatory cytokines [29,30], and recruitment of immune-system cells and molecules that support tumor growth and suppress the immune response. In addition, the tumor is composed of a variety of heterogeneous cells, of which only some present the cancer-specific antigen that is detected by a targeting antibody. Therefore, treatments with specific antibodies are limited to fractions of cells within tumors. Moreover, the cells of the tumor may change over time and reduce antigen presentation. To enable a more efficient immune response against tumors, the equilibrium in the microenvironment between tumor and immune system needs to be destabilized. Various studies using molecules such as TLR ligands for the activation of a desired antitumor response have demonstrated the feasibility of this approach [12,14–19].

The basic idea of this study was to develop a tool that would treat the tumor mass as a foreign body, stimulating a local targeted immune response at the tumor site. The modular particle TILTAN was constructed from a microcarrier loaded with a targeting agent and inducer, in order to direct selected innate immune cells to the tumor following stimulation of a desired response in the tumor microenvironment.

Micro- and nanocarriers have been previously used to deliver compounds for various purposes [31]. In this work, the size of the carrier was dictated by the need for systemic administration, lim-

Table 1
Beads and particles tested for binding SK-BR-3 target cells and inducing IL-1β in THP-1 cells. (–) No response; (+–) inconclusive results; (+) weak response; (++) moderate response; (+++) strong response; (++++) very strong response.

Carrier	Composition (%)			Activity	
	Ab	LPS	Mannose	Binding target cells	Inducing IL-1
10 mm p/s	100	0	0	+	+–
	0	0	0	–	–
1.5 mm Biomag™	100	0	0	++++	+–
	90	10	0	++++	+++
	75	25	0	++++	+++
	50	50	0	++++	+++
	25	75	0	++++	+++
	0	100	0	–	+++
	0	10	0	–	+++
	0	0	0	–	–
2 mm YG	66	0	0	+++	–
	0	66	0	–	+++
	0	0	66	–	+
	33	33	0	++	+++
	33	0	33	+++	++
	33	33	33	+	+++
	0	0	0	–	–

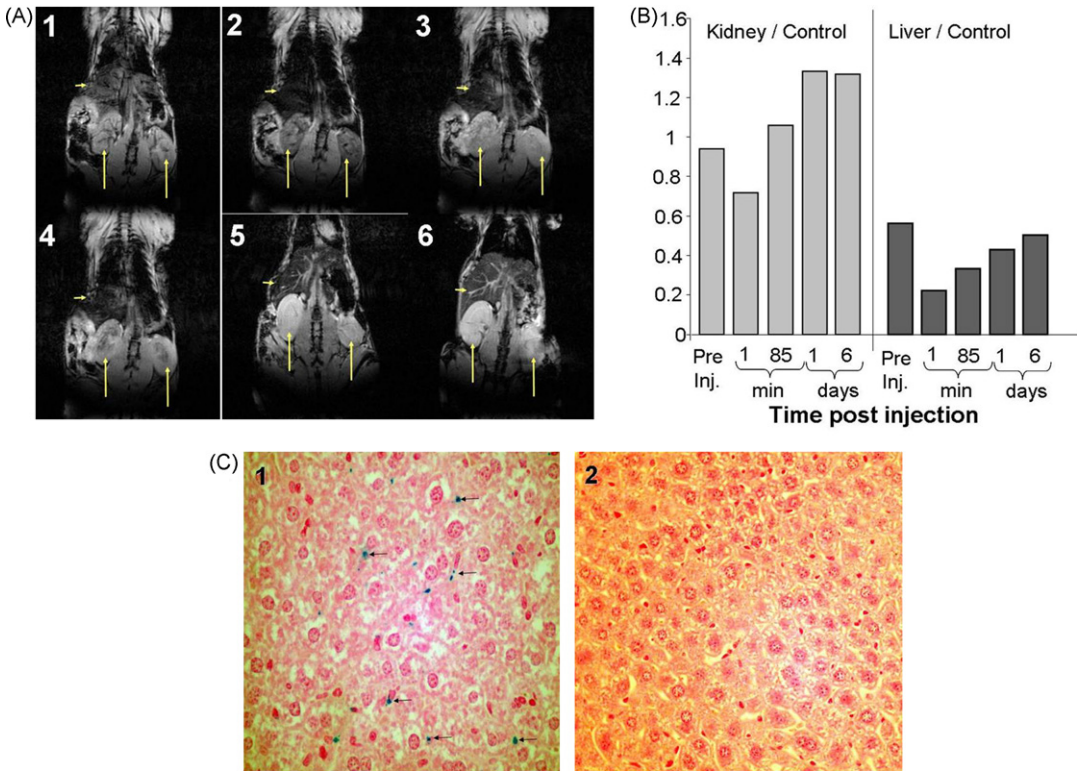

Fig. 8. In vivo testing of Biomag TILTAN. (A) Micro-MRI (T2* weighted scans) for surveillance of a mouse injected with 10^8 particles of Biomag TILTAN into tail vain 1—pre injection; 2—1 min post injection; 3—20 min post injection, 4—85 min post injection; 5—1 day post injection; 6—6 days post injection. Long vertical arrows indicate kidney; short horizontal arrows indicate liver. (B) Calculated presence of Biomag TILTAN in liver and kidney over 6 days normalized with a control muscle tissue. (C) Microscope images (×40) of liver tissues, iron stained with Prussian blue 1—1.5 h (C1) and 2—3 weeks (C2), post injection of 10^8 particles of Biomag TILTAN. Arrows indicate TILTAN blue particles identified by iron staining.

iting it to that of blood cells, while allowing for extravasation at the tumor site. Based on these limitations, TILTAN carriers of 1–2 μm were chosen. The presence of a targeting molecule (e.g. antibody) and inducer molecule (e.g. LPS) on the carrier allowed binding of TILTAN to the cancer cells and stimulation of the desired response. Microcarriers with multiple components have the potential to improve existing antibody-based antitumor therapy by inducing amplified reactions in solid cancers. In this study, we determined the ability to bind anti-HER2 antibody and the inducers LPS and mannose to the same carrier while preserving their activities.

Three types of carriers differing in size and surface properties were tested. All carriers were adequate for binding the molecules of interest at the examined ratios. However, due to their higher surface area, Biomag particles could bind more targeting and inducer molecules, thereby creating better attachments to the cancer cells and better induction of immune cells.

Because of its modularity, TILTAN can be designed for diverse types of cancers. A variety of antibodies to known cancer antigens may be used to target specific cancers, e.g. anti-insulin-like growth factor receptor, anti-epidermal growth factor receptor, vascular endothelial growth factor, anti-mucin 1, and anti-HER2 [8].

HER2 is overexpressed in various types of cancers [32]. However, humanized monoclonal antibodies targeting this antigen have been found to be effective only for the treatment of breast cancer [33,34]. The TILTAN approach may expand the possible applications of herceptin for additional types of cancer that overexpress HER2. Herein, herceptin was used only as a targeting element of the TILTAN complex and was not expected by itself to modulate a cytotoxic response. The presence of the antibody on the carrier was required for its binding to HER2-expressing cells and for its uptake by monocytes. This systemic approach with TILTAN complexes may enable treatment of primary tumors as well as metastases overexpressing HER2. Furthermore, TILTAN complexes showed the ability to bridge between cancer cells expressing a specific antigen and innate immune cells, probably mediated by the Fc fragment of the antibodies. Part of the regression of a tumor may be attributed to the Fc fragment's induction of various immunological responses [22], thus the immunoglobulin Fc on the bead may also serve as an inducer. Attachment of Fc gamma to TILTAN targeted to the tumor may direct a desired immunological cascade, by attracting neutrophils, macrophages or natural killer cells to the tumor and initiating a massive local response.

The type and activity of immune cells attracted to tumors treated with TILTAN are proposed to be determined by the inducer carried by the complex. In previous cancer immunotherapy studies, inducers were given as soluble molecules that were expected to be distributed throughout the body with only a small portion reaching the tumor. Therefore, the treatment was given at high doses, or injected into the tumor while ignoring the metastases.

Using inducers such as CpG, flagellin or double-stranded RNA, the tumor is expected to be marked by the immune system as a pathogen, followed by the desired anticancer response [14–16,19]. In the current study, LPS and mannose were used as model inducers to test monocyte activation by the TILTAN construct. The expression of IL-1β induced by LPS or mannose loaded alone on the carrier was intensified when anti-HER2 was included. In vivo, no over-expression of IL-1 in the tumor following TILTAN treatment was recorded. Rather, some decrease in this gene expression was monitored by semi-quantitative PCR. This change in IL-1 transcription, points to the fact that TILTAN affected the tumor. The relatively slight response may be due to the short-term and low-concentration treatment, and will be tested in the future.

Combining specific targeting with the innate branch of the immune system may enable the production of inflammation or a

rejection response at the tumor site. Tumor initiation, progression and metastasis have been shown in many studies to be influenced by inflammation mediated by the innate immune cells [35,36]. However, peripheral naive cells of the innate immune system, which are not recruited by the tumor, may be activated to induce tumor destruction [37]. In addition, the pro-cancerous properties of innate immune cells that have already been recruited by the tumor have been shown to be reversible via the re-education of TAMs into antitumor-activated macrophages [10]. In another study, TLR-8 ligand was found to mediate the reversal of immune tolerance induced by regulatory T cells [18,21]. The modularity of TILTAN, with its ability to carry multiple inducer combinations, may allow treatment of diverse tumors via adjustment of the type and intensity of the immune response.

In summary, the current study shows the feasibility of constructing carriers mounted with targeting and inducer agents to treat tumors. These constructs show the ability to bind to the target cells, as well as to attract monocytes and stimulate them for cytokine expression. Based on the fact that TILTAN was found safe to use, the next stage will focus on testing modifications and optimizing the doses in *in vivo* tests, for the design of a TILTAN based future clinical applications.

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