INTRAVITAL MICROSCOPY IDENTIFIES ANTIBODY-DEPENDENT PHAGOCYTOSIS BY MACROPHAGES AS MAIN EFFECTOR MECHANISM TO PREVENT DEVELOPMENT OF LIVER METASTASES
Abstract

Monoclonal antibodies (mAbs) directed against tumor antigens are currently used as promising adjuvant therapies in addition to radio- and/or chemotherapy. Although clinical successes have been demonstrated, it is not completely understood how mAbs mediate tumor elimination. We previously demonstrated that mAb therapy is particularly successful in preventing surgery-induced liver metastases development in animal models. In the current study we used real-time intra-vital microscopy to elucidate the mechanisms that are involved in removal of tumor cells. We demonstrate that tumor cells are rapidly recognized and arrested by liver macrophages (Kupffer cells). Kupffer cells sampled tumor cells in the absence of mAb, but this did not lead to elimination. By contrast, mAb treatment resulted in fast phagocytosis of tumor cells by Kupffer cells, followed by degradation. As such, we propose that macrophages play a much more prominent role in mAb-mediated eradication of tumor cells in patients than is currently appreciated. With the use of intra-vital microscopy we show for the first time directly that antibody-dependent phagocytosis by macrophages is a prominent mechanism to induce therapeutic efficacy of mAbs. These findings may help to develop optimized mAb therapeutical strategies of many cancer patients, by aiming to enhancing macrophage recruitment and activity.
Introduction

Therapeutic monoclonal antibodies (mAb), which can be designed to specifically interact with tumor-associated antigens, represent a promising novel category of drugs for targeting malignancies in addition to chemo- or radiotherapy. The anti-CD20 mAb rituximab was one of the first that was approved for clinical use to treat B cell malignancies, and its unprecedented success prompted the development of a multitude of new anti-tumor mAb. The therapeutic mode of action of mAb is still incompletely understood and heavily debated, in spite of an overwhelming number of in vitro, in vivo and patient studies. Several direct and indirect mechanisms of mAb therapy have been proposed. Direct mechanisms include the induction of apoptosis, inhibition of proliferation or sensitization of tumor cells for chemotherapy. Furthermore, most mAb that are used in the clinic are of the IgG1 subclass, which activates the complement cascade through the classical pathway, leading to complement-dependent lysis. The Fc region of IgG additionally interacts with IgG Fc receptors (FcgR) that are expressed on immune cells. FcgR-mediated mechanisms proved essential for therapeutic efficacy in vivo, since mAb immunotherapy was ineffective in FcgR deficient mice. A strong association between certain FcR alleles and success of mAb therapy in patients was shown as well, as patients with the FcgRIIIa-158V allotype had a better clinical response compared to FcgRIIIa-158F allotype. This effect has mainly been attributed to FcgRIII-expressing natural killer (NK) cells, whose ability to lyse mAb-opsonized target cells is referred as antibody-dependent cellular cytotoxicity (ADCC). Recently, a role for macrophages was proposed as depletion with clodronate liposomes abrogated therapeutic efficacy of anti-murine CD20 mAb in a murine lymphoma model.

Notwithstanding the initial success of treating haematological malignancies with mAbs, therapeutic accomplishments in targeting solid tumors remain somewhat disappointing. This is partly explained by the lack of efficacy when direct effects of mAb are inhibited. For instance, the anti-HER-2 mAb trastuzumab is only effective when HER-2 is over-expressed, which is the case in ~20-25% of breast cancer patients, whereas mutations in the KRAS signaling pathway seriously hamper therapeutic success when colorectal patients are treated with anti-EGFR mAb. Understanding the precise mode of action of mAb therapy will allow the design of optimal mAb therapeutical strategies to improve efficacy in these patients.

We previously identified a novel mAb-based therapeutic strategy that may benefit a significant population of cancer patients. Approximately one million patients worldwide are diagnosed with colorectal cancer annually, and ~500,000 patients die from this disease each year. Resection of the primary tumor is the cornerstone of the treatment of colorectal cancer. However, disseminated circulating tumor cells can be detected in up to 70% of patients at the time of surgery. The presence of circulating tumor cells has furthermore been proposed as an independent prognostic factor for survival of patients with colorectal cancer.

We recently demonstrated that surgery paradoxically enhances the risk of liver metastases development, as it induces damage to the liver vasculature, which promotes adherence of circulating tumor cells. Importantly, anti-tumor mAb therapy prevented liver metastases outgrowth in mice and rats, which was depended on the presence of either FcgRI or FcgRIV. This supported the involvement of the tissue macrophages...
of the liver (Kupffer cells), because both receptors are only expressed by the myeloid mononuclear network in mice. Kupffer cells reside in the liver sinusoids and clear the hepatic blood circulation from bacteria, old blood cells or foreign substances. As such, they are the first line of defense against tumor cells entering the liver. In a previous study, we observed that ~80% of tumor cells were in contact with Kupffer cells in the absence of tumor-specific mAb, which was increased to approximately 90% after mAb treatment. Surprisingly, this modest difference completely prevented liver metastases development. To study the exact mechanisms of mAb therapy, we used real-time intravital microscopy of the liver of mice and in vitro live cell imaging to visualize the mode of action in more detail.

The B16F10 melanoma cell line was used as model system, since this is the only syngeneic solid tumor model for which an anti-tumor mAb is available (TA99; anti-gp75). Mice were either treated with an isotype control or with TA99. Real-time imaging in the livers of isotype control treated mice showed that Kupffer cells were able to sample small parts of tumor cells, which however did not result in elimination of tumor cells (Figure 1a, upper panels and supplemental movie 1). Still, these results explained why co-localization between Kupffer cells and tumor cells was observed in previous immunohistochemistry experiments. In contrast, tumor cells were rapidly recognized and phagocytosed in the livers of TA99 treated mice, (Figure 1a, lower panels and supplemental movie 2). Thus, although no difference was observed between the number of tumor cells that was in contact with Kupffer cells after either isotype or TA99 mAb therapy (Figure 1b), the number of tumor cells that had been phagocytosed was significantly increased after TA99 treatment (Figure 1c). Similar results were observed in in vitro live cell experiments. Even though cell-cell contact was observed for prolonged periods of time in the absence of tumor-specific mAb, macrophages were unable to prevent tumor growth (Figure 1d and supplementary movies). Addition of TA99 however resulted in rapid phagocytosis and elimination of tumor cells (Figure 1e, f, and supplementary movies).

Recently, it was suggested that macrophages eliminate tumor cells via ADCC, as synapse formation between tumor cells and macrophages was observed in peritoneal lavages of mAb treated mice. As uptake of tumor cells by macrophages requires a close interaction, synapse formation is likely involved. However, we now show that the main mechanism by which liver macrophages eliminate tumour cells is through antibody-dependent phagocytosis (ADPh). In addition, both murine bone marrow derived and human monocyte derived macrophages killed a wide variety of human tumor cells (colon carcinoma, vulvar carcinoma, B cell lymphoma) through ADPh in live cell imaging experiments (data not shown). Uptake of tumor cells was associated with phagosome-lysosome fusion, which led to rapid acidification, but slow degradation of tumor cells (Figure 2a and b, supplementary movies). This was comparable to intravital experiments. When livers of mice were studied 24 hours after injection of B16F10 cells and TA99, tumor cells had been degraded into smaller particles, but tumor material was still present (Figure 2c; right panel, 2d, 2e; lower panels and supplementary movie). Some small tumor particles were observed in Kupffer cells of isotype treated mice, but most tumor cells were present in larger clusters, indicative of tumor cell outgrowth (Figure 2c; left panel, 2d, 2e; upper panels and supplementary movie).
Depletion of Kupffer cells with clodronate liposomes abrogated therapeutic effects of TA99. Large tumor cell clusters were observed in either PBS or TA99 mAb treated mice 24 hours after injection of tumor cells in Kupffer cell-depleted mice (Figure 3a). There was no difference in size of tumor cells/particles between PBS/isotype control and TA99 treated mice (Figure 3b). Moreover, whereas treatment with TA99 almost completely prevented outgrowth of liver metastases in control mice, no therapeutic protection was observed in Kupffer cell-depleted mice (Figure 3c).

Thus, Kupffer cells are essential in mediating tumor cell elimination after mAb immunotherapy. Until now, modes of action of mAbs have been proposed based on in vitro results or experiments with mice that were either deficient for specific components of the immune system, or in which immune components/cells had been depleted.
Chapter 8 Intravital microscopy identifies antibody-dependent phagocytosis by macrophages as main effector mechanism to prevent development of liver metastases

Figure 2 Fast uptake, but slow degradation of tumour cells by macrophages

(a+b) Uptake of DiB-labeled (blue fluorescence) B16F10 tumour cell by (a) macrophage labeled with lysotracker (red fluorescence) or (b) Dil- labeled macrophage (red fluorescence) in the presence of TA99mAb. Asterisks in (a) indicate lysosome. Arrow heads in (b) show degraded particles of tumor cell within macrophage. Time points are indicated. Upper panels: overlay of bright field and fluorescence. Lower panels: fluorescence only. (c) Kupffer cells (blue) and B16F10 tumor cells (red) 24 hours after injection of tumor cells in PBS (left panel) or TA99 (right panel) treated mice. Arrow heads in show tumor cell particles within macrophages. Asterisks indicate whole tumor cells. (d) Quantification of size of tumor tumor cell particles in livers of PBS or TA99 treated mice (e) 3D reconstruction of Kupffer cells (blue) and B16F10 tumor cells (orange) in PBS (upper panels) or TA99 (lower panels) treated mice. Colors are artificial. Data are representative of three independent experiments.
With the use of intravital microscopy we now demonstrate for the first time directly that ADPh by macrophages is crucial for therapeutic efficacy of mAbs. As such, macrophages may play a much more prominent role in mAb-mediated eradication of tumor cells in patients than is currently appreciated. Because human macrophages express FcgRIII, we hypothesize that the difference in therapeutic efficacy of mAbs in patients with distinct Fc receptor allotype may be (partly) attributed to decreased/increased ADPh by macrophages in addition to NK cell- mediated ADCC, particularly because macrophages proved extremely efficient in phagocytosis of B cell lines in the presence of anti-CD20 mAb (data not shown).

Thus, our findings may significantly contribute to the design of optimized mAb therapeutical strategies of many cancer patients, by aiming to promote recruitment of macrophages as effector cells. In particular, we propose that patients undergoing resection for primary colorectal cancer may greatly benefit from peri-operative mAb immunotherapy, as this will lead to elimination of any remaining circulating tumor cells by Kupffer cells in the liver.
Chapter 8 Intravital microscopy identifies antibody-dependent phagocytosis by macrophages as main effector mechanism to prevent development of liver metastases
Material and methods

Animal models
Wild type C57BL/6 mice were purchased from Charles River. All mice had access to food and water ad libitum and were housed in specific pathogen–free, double-barrier units at the University of Calgary in Calgary or VU University Medical Center in Amsterdam. Protocols were in accordance with guidelines of University of Calgary Animal Care Committee and the Canadian Council on the Use of Laboratory Animals or the Committee for Animal Research of the VU University Medical Center. Mice were used between 8 and 12 weeks of age.

Cell culture
The mouse melanoma cell line B16F10, which expresses gp75, was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS, hereafter referred as complete growth medium. Previously, c-terminal melanosome retention signal in the human gp75, was identified as being NQPLLTD, of which the core dileucine motif (PLL) is highly conserved and was suggested to be responsible for the intracellular retention.\textsuperscript{21} In order to enable membrane expression of mouse gp75 we removed this motive from the full-length mGP75, by ordering a HindIII/EcoRI fragment containing the coding sequence of mGP75 (accession number NM_031202), but with only the first 10 membrane-proximal aminoacids of the c-terminus (RSRSTKNEAN), thus lacking the potential retention signal. This fragment was subcloned to pcDNA3.1 and transfected using Lipofectamine LTX with plus reagent (Invitrogen) according to the manufacturers’ instructions. After selection on 200 µg/ml of Zeocin (Sigma-Aldrich) for 7 days, the cells were sorted on cells staining positive for gp75 using mouse IgG2a TA99 mAb (VUMC, Amsterdam, The Netherlands) and goat-anti mouse IgG-FITC (Sanquin, Amsterdam, The Netherlands).In order to image tumor cells in vivo single cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution (Gibco, Irvine, UK). Viability was assessed by tryphan blue exclusion, which always exceeded 95%. For in vivo experiments, B16F10 cells ($5 \times 10^6$ cells/ml) were labeled fluorescently by incubating cells in complete growth medium containing 50 µg/ml 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbo cyanine perchlorate (DiI, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C and subsequently washed with Hanks’ balanced salt solution (HBSS; Gibco) according to the manufacturer’s instructions.

Murine bone marrow macrophages
Bone marrow from wild type C57BL/6 mice was harvested from freshly isolated femur, tibia and humerus. After removal of connective tissues and muscles, bone marrow was flushed and single cell suspensions were made by passing bone marrow through a sterile 70 µm filter (BD Falcon, Bedford, MA). Macrophages were differentiated by incubating bone marrow cells for 7 days with complete DMEM, supplemented with 15 % L929 conditioned medium (containing macrophage–colony stimulating factor). Macrophages were harvested after 15 minute incubation with trypsin-EDTA and subsequent scraping using a cell scraper. For live cell imaging macrophages were seeded in 8 well ibiTreat µ-Slides ($2 \times 10^5$ cells/well, IBIDI, Munich, Germany).
Material and methods

In vivo experiments

**Intravital microscopy**
Twenty-four hours before imaging, mice were injected i.p. with 200 µg anti-gp75 TA99 mAb, or as a control with saline (250 µl). Animals were anesthetized by an i.p. injection of a mixture of 10 mg/kg xylazine (Bayer, Toronto, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Bimed-MTC, Cambridge, Ontario, Canada) prior to surgery. To maintain anesthesia or to inject antibody intravenously, tail vein of mice were canulated and anesthetics were injected every other 30 minutes. Alexa-647 labeled F4/80 (eBioscience, San Diego, CA) antibody was injected i.v. to stain Kupffer cells. A small incision was made in the left flank to reveal the spleen. Fluorescently labeled B16F10 cells (2x10^5 cells in 100 µl) were injected intra-splenically at a constant rate to allow flow of tumor cells toward the liver. After one minute, the spleen was removed to prevent bleeding. A midline and a left subcostal incision were made in order to exteriorize the liver and spleen. The hepatic ligaments were dissected and the intestine was covered with moist gauze. Animals were placed in a left supine position and the left liver lobe was positioned onto a Plexiglas microscopic stage. The liver surface was then covered with Saran Wrap to hold the organ in position. In additional experiments, Kupffer cells were depleted by i.v. injection of clodronate (Roche Diagnostics, Mannheim, Germany) encapsulated in liposomes or saline 3 days prior to tumor cells injection. Alternatively, to visualize tumor cells 24 hours after injection, fluorescently labeled tumor cells were injected in the spleen, after which splenectomy was performed and the incision sutured. Mice were allowed to recover for 24 hours after which animals were prepared for intravital microscopy of the liver as described above.

The liver microvasculature was visualized using a spinning-disk confocal microscopy and images were acquired with an Olympus IX81 inverted microscope with a 10/0.40 UplanFL N objective. The microscope was equipped with a confocal light path (WaveFx, Quorum, Guelph, ON) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Tokyo, Japan). Laser excitation wavelengths (Cobolt, Stockholm, Sweden) were used in rapid succession and visualized with the appropriate long-pass filters (Semrock, Rochester, NY). Typical exposure time for each excitation wavelength was 300 milli-seconds. A 512x512 pixel back-thinned electron-multiplying chargecoupled device camera (C9100-13, Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Volocity Acquisition software (Improvision) was used to drive the confocal microscope.

**Liver metastases development**
Effect of TA99 on tumor development in control and KCs depleted mice was studied. KCs were depleted as described and all animals were injected i.p. on days 0, 2, and 4, with 200 µg TA99 mAb, or as a control with saline (250 µl). Tumor cells were injected intrasplenically and splenectomy was performed as described here above. After 21 days, mice were sacrificed, and the number of liver metastases was scored in each mouse.
Material and methods

Quantification
Each contact between KCs and tumor cells was assigned as interaction between these two cell types and phagocytosis of tumor cells by KCs in the livers was determined as co-localization of complete red tumor cells within blue stained KCs. Differences between treatments were quantified in more than five independent experiments with AnalySIS software (Soft Imaging System GmbH).

In vitro cytotoxicity assays
Real-time cytotoxicity assays were performed with an Olympus CellR real-time live-imaging station (type IX81, UPLFLN 40 x O/1.3 lens, Münster, Germany). Macrophages were either labeled with DiO (green fluorescence), DiI (red fluorescence) or Lyso-ID Red. DiI, DiO, or DiB (blue fluorescence)-labeled B16F10 variant were added in an E:T ratio 15:1 with TA99 or MG-4 (irrelevant isotype control) antibodies. Pictures were taken every 5 minutes with an Olympus ColorView II camera for 3-6 h, followed by a 15 minutes interval for 18-21 hours. Additionally, random pictures were taken after 24h.

Statistical analysis
For comparisons between two groups student T-tests were used. Comparisons between multiple groups (>2) were performed with ANOVA. Statistical significance was accepted at p<0.05. Results are presented as mean +/- SEM.
Reference List

